THE SYNTHESIS AND STUDY OF STREPTOMYCIN DERIVATIVES

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T H E S I S

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by

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C O N T E N T S

HISTORICAL

DISCUSSION

EXPERIMENTAL

HISTORICAL

INTRODUCTION

Tuberculosis has plagued mankind for many thousands of years. Human skeletons have been unearthed which show evidence of tuberculous lesions and establish that the disease was known as early as 5000 B.C.[1] Hippocrates, " the father of medicine" , described its semiology and pathology more than 2000 years ago, mentioning phymata or nodules in the lungs, and stressed the importance of personal hygiene with proper diet in the control of the disease. Tuberculosis became commonly known as consumption or phthisis because of its most conspicuous external feature, wasting, and in the past was considered to be of many varieties and origins. The unity of the disease, however, was established experimentally by Robert Koch when he identified the causative organism in 1882 and knowledge of the disease has progressed rapidly from that time. Even today, despite the magnitude of achievements in the field of chemotherapy, tuberculosis still remains a serious threat to life in many parts of the world. In recent years, however, mortality from tuberculosis has declined[2] rapidly and recent statistical surveys[3,4] have shown this to have been particularly marked since 1947. Statistics for the period 1947 to 1953 show that the fall in death rate in western countries due to

respiratory tuberculosis has ranged from 53 per cent in France to 83 per cent in Ireland.[5] Chronologically this corresponds more or less with the introduction of streptomycin, p-amino-salicylic acid and isoniazid (isonicotinic acid hydrazide). The decline in mortality from tuberculosis, however, has not been accompanied by a parallel decrease in the morbidity.[5,6] This may be attributed to better methods of investigation and, in particular, to mass-radiography which has revealed many new cases which otherwise would have remained undetected until some later date.

The Nature of the Disease.

Tuberculosis is characterised pathologically by inflammatory infiltrations, tubercles, caseous necrosis, abcesses, fibrosis and calcification. This communicable disease mostly affects the lungs due to inhalation of tubercle bacilli, though the gastro-intestinal and genito-urinary tracts, bones and joints may occasionally be infested.

The causatine organism, the tubercle bacillus, was first described by Robert Koch in 1882[1], and various types, human, bovine and avian are known.[7] Infection in man is mainly by human or bovine types but seldom by avian strain. The tubercle bacillus belongs to the genus "Mycobacteria"

which is characterised by its acid fastness, a property attributed mainly to mycolic acid[8] which is present in combination with polysaccharides. This characteristic is most pronounced in the lipoids of the cytoplasmic membrane and in the internal granules of the cell.[9]

The concept of a protective lipoid capsule[10] which possibly arose from the hydrophobic and lipophilic properties of the tubercle bacillus has been proved to be erroneous.[11] The minute architecture of tubercle bacillus has been revealed by electron microscopy,[12] and the central cytoplasmic mass shown to be surrounded by thin cytoplasmic membranes enclosed by a thin cell wall. Both extracellular and intracellular constituents have been investigated extensively in the past because of the unique immunological and bacteriological properties of Mycobacterium tuberculosis. Possibly no other bacterial group has received quite the same attention as the Mycobacteria or yielded in return so rich a harvest of chemical knowledge.

CHEMISTRY OF MYCOBACTERIUM TUBERCULOSIS

Like all other microbial cells, the tubercle bacillus synthesises a wide variety of giant molecules or macromolecules from the simplest of carbon and nitrogen sources, such as asparagine, glycerol, and salts of sodium, potassium and magnesium[13a]. The number and complexity of

its macromolecular constituents is bewildering,[14] though their nature (polysaccharide, fat, protein and nucleic acid) and proportion are dependent upon the type of medium[15] and the strain[16] of the organism. Macromolecules obtained from tubercle bacillus have important biological functions in the animal body. The proteins and polysaccharides manifest specific biological activities such as the "Tuberculin" reaction, whilst the lipoid complexes cause tubercle and giant cell formation.[17]

Today, isolation and study of constituents of the tubercle bacillus have not succeeded in opening any new approach to chemotherapeutic investigation. Such information, however, is essential to provide insight into the mechanism of the cell reactions. Some of the biologically important constituents which have been isolated from the tubercle bacillus are, therefore, briefly considered here.

CARBOHYDRATES

The complexity of the polysaccharides in tubercle bacillus is beyond doubt since more than eight structurally different polysaccharides have been isolated. Some of these have been found to be present in combination with lipoids while others form complexes with nucleic acids or with proteins. These complex polysaccharides are distributed throughout the cellular material and some

diffuse from the outer surface into the culture medium. Accordingly, tubercle polysaccharides have been considered in three groups:

(a) those of somatic origin,

(b) those combined with the lipoids, and

(c) those associated with the "tuberculin" from the culture medium.

(a) Polysaccharides Associated with Somatic Components of Defatted Cells.

Earlier investigations have been reviewed by Stacey and Kent[18] and Andrejew.[19] In the past attempts have been made to establish the presence of cellulose, hemicellulose and chitin but these have not been confirmed by more recent studies. The presence of glucosamine, the essential component of chitin, however, has been demonstrated in the carbohydrate fractions of the tubercle bacillus.[20] Besides this, many investigators have established the presence of glycogen by its reaction with iodine and by enzymatic hydrolysis.

In 1925, Laidlaw and Dudley[21] pioneered the study of carbohydrates in the bacillus by isolating a substance from defatted dead bacilli having the characteristics of glycogen. They also characterised another polysaccharide

having $[\alpha]_D^{20}$ + 67° which on hydrolysis with dilute acid yielded a mixture of reducing sugars containing 30 per cent pentoses. The polysaccharide was found to be serologically active in animals which had been immunised with dead tubercle bacilli and this substance was regarded as a hapten. Maxim[22] isolated two polysaccharides having $[\alpha]_D$ + 64.5° and + 80.2° respectively by extracting fat-free bacilli with dilute acid, and showed them to be serologically active. Heidelberger and Menzel[23,24] extracted defatted cells by percolation with dilute acetic acid and also obtained two dextrorotary specific polysaccharides. The one with high rotation($[\alpha]_D$ + 85°) yielded arabinose and D-mannose on hydrolysis, while the other ($[\alpha]_D$ + 34.7°) gave only D-arabinose. They also isolated identical polysaccharides from Mycobacterium tuberculosis (bovine strain). Heidelberger and Menzel established that serological activity was related to the pentose fragments of the substances, and suggested that sugar acids were not responsible for immunological specificity in the tubercle bacillus. They also suggested that the constituents isolated from culture filtrates by Masucci, McAlpine and Glenn[25] were identical with those in polysaccharides of somatic origin.

Gough[26] isolated a serologically active polysaccharide by the action of 4 per cent sodium hydroxide on the moist bacilli. Ludewig and Anderson,[27] and du Mont and Anderson[28] also isolated from Mycobacterium tuberculosis (human strain) a purified polysaccharide fraction which yielded inositol, D-arabinose and D-mannose on hydrolysis. The latter authors also obtained the same results from avian strains of bacilli.

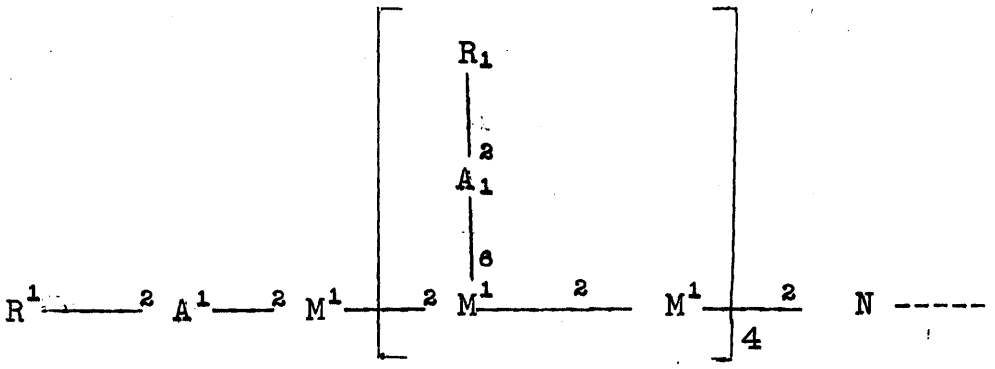
Chargaff and Schaefer[29] using methods similar to those of Heidelberger and Menzel[23] isolated polysaccharide constituents from Bacillus Calmette Guerin (B.C.G.). Besides glycogen, two specific polysaccharides were fractionated, and one having $[\alpha]_D$ + 77°, yielded D-mannose, D-arabinose and small quantities of inositol and D-glucosamine on hydrolysis. Karjala and Heidelberger[30] isolated polysaccharides from an avian strain of tubercle bacilli with 3.5 per cent acetic acid. The active polysaccharide containing the greatest amount of pentoses showed the same immunological properties as those derived from human and bovine strains. Chargaff and Moore[31] demonstrated that purified glycogen fractions were inactive biologically.

It is evident that the various investigators have between them isolated a large number of polysaccharides by

adopting different procedures, but without embarking upon structural studies. Haworth, Kent and Stacey,[32] however, have not only confirmed the existence of two stable serologically active polysaccharides, one having $[\alpha]_D^{18}$ + 85° from the somatic part of the cell and the other having $[\alpha]_D^{18}$ + 25° bound to the cellular lipoids, but also have studied the details of their structure.

The polysaccharide of high dextrorotation was isolated[32] by defatting moist steam-killed cells with organic solvents and treating with N sodium hydroxide for a few days. The crude polysaccharide was precipitated by the addition of ethanol. After acidification, it was dialysed and again fractionated with ethanol. Methylation of the polysaccharide with dimethyl sulphate and sodium hydroxide yielded a brown gum which on hydrolysis with methanolic hydrogen chloride gave a mixture of methyl glycosides. These were fractionated using high vacuum and were characterised as 3,5-dimethyl-methyl-D-arabofuranoside, 2,3,4-trimethyl-methyl-L-rhamnopyranoside, 3,4,6-trimethyl--methyl-D-mannopyranoside, 3,4-dimethyl-methyl-D-mannopyranoside and an unidentified dimethyl-methyl-2-acetamidohexoside in the molecular proportion of 5,5,5,4,1 respectively.
On the basis of these conclusions, the structure (1) has been

assigned[32] to the somatic polysaccharide (the numerals represent points of mutual attachment).



R = L-rhamnopyranose, M = D-mannopyranose

A = D-arbofuranose, N = 2-amino-hexose

(I)

In this representation, all the linkages engaging the glycosidic groups have been depicted in the α-form. The somatic polysaccharide molecule may be any multiple of the above suggested structure linked glycosidically through its amino sugar residue. Osmotic pressure determinations indicated a minimum molecular weight of 12000, which suggests that at lease three such structures are joined together in the intact polysaccharide molecule.

(b) Polysaccharides combined with the Lipoids.

Certain earlier workers[18] in this field observed the unusual complexity of the lipoid constituents of Mycobacterium

tuberculosis and attributed the property of acid fastness to the "fat" or lipoids present in the organisms. Long and Campbell[33] obtained a waxy substance which was found to be a difficultly hydrolysable ester of a high molecular weight alcohol. Anderson and his collaborators[34] undertook a systematic study of the lipoids of the tubercle bacilli, as a result of which carbohydrates have been shown to be associated by direct chemical combination with four types of lipoid substances:

(a) Acetone-soluble fats,

(b) Phosphatides,

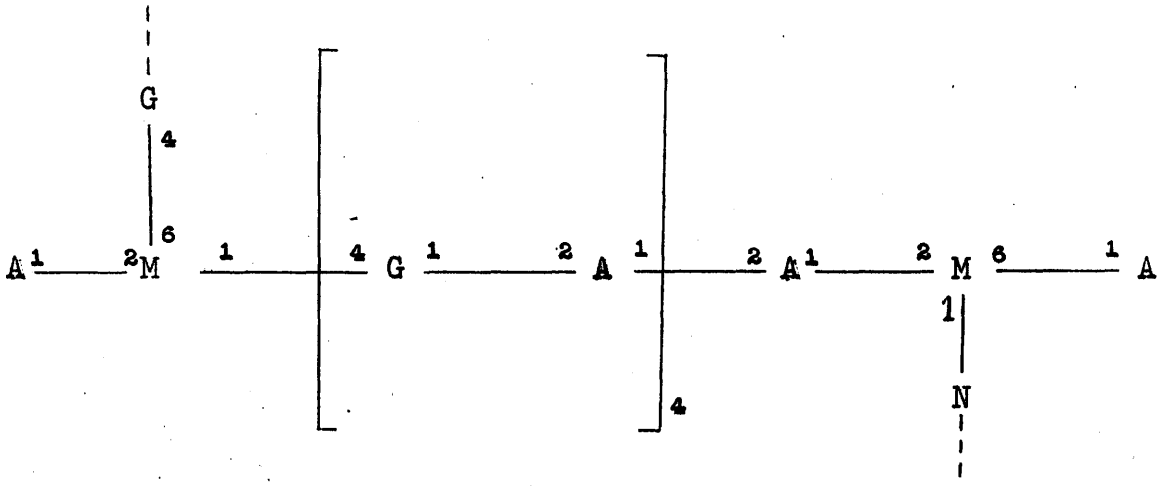
(c) Waxes and

(d) Firmly bound lipoids.

The acetone-soluble fats were largely composed of free fatty acids in addition to fatty esters but were devoid of glycerides. Alkaline hydrolysis[35] of fats derived from human strain, but not those obtained from the bovine and avian strains of tubercle bacilli, yielded a water-soluble disaccharide which was identified as trehalose.[36] The phosphatides[37] or nitrogen- and phosphorus- containing fractions, when hydrolysed with 5 per cent sulphuric acid gave a mixture of fats (65 per cent) and water-soluble components (33.6 per cent) consisting of glycerophosphoric

acid, inositol, D-mannose and an unidentified hexose. A chloroform-soluble wax[38] yielded D-mannose, D-galactose and D-arabinose, together with small amounts of D-glucosamine and inositol. Similar products were obtained from bovine[39] and avian[40] strains.

Saponification of firmly-bound lipoids[41] afforded polysaccharides, having properties similar to those obtained from purified wax and were shown to contain D-arabinose, D-mannose and D-galactose. Haworth, Kent and Stacey[42] isolated a serologically active lipoid-bound polysaccharide by saponification of a urea extracted complex of heat-killed bacilli. This had $[\alpha]_D^{18}$ + 25° in water. Structural studies of the polysaccharide were undertaken after purification by fractionation and repeated methylation which afforded a brown gum. Hydrolysis of the fully methylated polysaccharide with methanolic hydrochloric acid yielded 2,3,5--trimethyl methyl-D-arabofuranoside, 3,5-dimethyl methyl-D-arabofuranoside, 2,3,6-trimethyl methyl-D-galactopyranoside, 3,4-dimethyl methyl-D-mannopyranoside and a dimethyl methyl--D-glucosaminide in the molecular proportions of 2:5:5:2:1 respectively. Structure (II) has been proposed for the lipoid-bound polysaccharide (the numerals represent points of mutual attachment).



A = D-arabofuranose M = D-mannopyranose,

G = D-galactopyranose N = D-glucosamine

(II)

(c) Polysaccharides Associated with Tuberculin and the Culture Medium.

Culture filtrates obtained from the media after the growth of tubercle bacilli contain complex polysaccharides as do the bacillary bodies themselves. Mueller[43] isolated a polysaccharide from the six week old culture filtrate of tubercle bacilli in the form of a yellow gum free from proteins and having $[\alpha]_D$ + 24.2°. This product though containing less than one per cent of nitrogen, reacted with immune serum at a dilution of one in a million. After acid hydrolysis, the product contained 54.8 per cent of pentoses and the specific rotation had changed to -22.9°. It was

suggested that the polysaccharide was similar in nature to that isolated by Laidlaw and Dudley[21] from the somatic portion of the cell. Enders[44] studies its biological properties and concluded that the polysaccharide induced lethal anaphylactic shock in tuberculous guinea pigs. Masucci, McAlpine and Glenn,[25] and Renfrew[45] isolated a serologically active polysaccharide which on acid hydrolysis, liberated mannose, D-arabinose and an unidentified sugar acid. McAlpine and Masucci[46] isolated two fractions, one of low and the other of high dextrorotation by fractional hydrolysis and attributed their biological activity to pentose constituents.

Dorset and Henley[47] obtained a polysaccharide using Long's synthetic medium which yielded D-arabinose and D-mannose on hydrolysis. They claimed that the biological activity of this product was due to proteins (tuberculin) with which it was associated and these results were subsequently confirmed by Seibert and Munday.[48] Spiegel-Adolf and Seibert[49] investigated various tuberculins spectrographically and found that the tuberculin protein purified by trichloracetic acid (Purified protein derivatives; P.P.D.) exhibited an absorption band at 265-267 mμ which was identical with that displayed by deoxyribonucleic acid.

Seibert and her associates[50] separated nucleic acid, polysaccharide and protein fractions of tuberculin by electrophoresis, a technique which was developed later by Seibert and Watson[51] for large scale work in this field. The relative immobility of the polysaccharide permitted its separation from the protein and nucleic acid, which move independently at high pH.[52] The nucleic acid did not show any antigenic properties.[53,54]

A distinction has now been made between the polysaccharides of the culture filtrate on the basis of electrophoretic analysis and ethyl alcohol fractionation. The first of these, designated as "Polysaccharide 1" was separated by Seibert and Watson.[51] It was shown to contain units of mannose, galactose and arabinose and was found to be highly active serologically. It did not elicit antibodies and had a relatively low molecular weight of about 7000-9000. The "Polysaccharide II" was isolated and examined by Seibert, Stacey and Kent[55] and shown to be similar in structure to dextran. It gave a high precipitin titre with horse antituberculous serum and lower titre with anti-B.C.G. serum and with rabbit and human antituberculous sera. It was able to induce formation of homologous antibodies when injected into rabbits but did not produce

any immunity. Kent[56] has claimed that it contains about 8 per cent of bound lipoid component and that the glucose units are joined by (1⟶2) linkages.

A third polysaccharide isolated by Seibert from the supernatent remaining after precipitation of P.P.D. with half saturated ammonium sulphate, was purified by ultra-filtration. More recently it has been shown by Stacey[57a] to contain 80 per cent of arabinose with small amounts of mannose and galactose and a trace of an amino sugar.

It is clear, therefore, that components of polysaccharides in the culture filtrates resemble closely those present in the cells. Stacey[57a] has drawn attention to similarities in the structure of the somatic polysaccharides and more especially of the "lipoid bound" polysaccharide, to that of streptomycin; the streptose fragment of the latter corresponding to D-arabofuranose, the L-glucosamine of streptomycin to D-hexosamine and the inositol (streptidine) of streptomycin to the hexoses. There are grounds, therefore, for considering the possibility that streptomycin might act as a competitive metabolite by inhibiting the biosynthesis of an essential tuberculosis polysaccharide.

Biological Properties of Polysaccharides.

Various polysaccharide fractions isolated from the bacillus and culture filtrates react strongly in precipitin test with the serum of animals immunized against tubercle bacilli[21,42,50] but none stimulate antibody formation. These carbohydrates are, therefore, not true antigens but rather haptens.[46] Furthermore, they are non-toxic for the normal body and do not induce hypersensitivity of the tissues.[17]

LIPOIDS

The pioneering investigations of Anderson and his collaborators into the extraction and fractionation of lipoid components of Mycobacterium tuberculosis and other acid-fast bacilli with organic solvents have been extensively reviewed elsewhere[34, 58, 59]. Four main groups of lipoids have been recognised on the basis of solubility relations, viz: acetone-soluble fats, acetone-insoluble phosphatides, chloroform-soluble waxes and the firmly-bound lipoids. A general scheme for the fractionation of lipoids from tubercle bacilli has been cited by Seibert[60] and Long[13b]. Biological studies of various fractions isolated by Anderson have been recorded by Sabin[61,62], and lipoids seem to play an important role in the process of tuberculous infections.

Lipoids constitute about 20 to 40 per cent of the tubercle bacillus, depending upon the composition of the medium[15] and the strain of the organism used[16]. Acetone--soluble fats and phosphatides have yielded on saponification mostly the normal fatty acids such as palmitic, stearic, n-hexacosanoic ($C_{26}H_{52}O_2$) and oleic acids besides several specific branched-chain fatty acids, of which the most important are the methyl-branched fatty acids, tuberculostearic acid and phthioic acids. On the other hand the chloroform-soluble waxes and firmly-bound lipoids gave mycoceranic acid, mycolic acid and a toxic fraction, 'cord factor.'

Tuberculostearic acid ($C_{19}H_{38}O_2$) which was isolated by Anderson and Chargaff[64], is biologically[62] and optically[65] inactive. Its structure assigned as (±)-10-methylstearic acid (III) by Spielman[65], is supported by the X-ray diffraction

$$CH_3.(CH_2)_7.\underset{\displaystyle CH_3}{\underset{|}{CH}}.(CH_2)_8.COOH$$

(III)

studies of Velick[66]. Prout, Cason and Ingersoll[67] have since established its structure as the optically active (-)-10--methyloctadecanoic acid ($[\alpha]_D$ ca. -0.05°) by step-wise synthesis. A new and improved method of synthesis of (±)-10--methyloctadecanoic acid has been reported by Schmidt and Shirley[68].

Spielman and Anderson[69] isolated phthioic acid, $C_{26}H_{52}O_2$, as a white solid, m.p. 20-21°, $[\alpha]_D$ + 12.6°, from the tubercle bacillus, and considered it to be a saturated branched-chain acid. Its name is derived from the fact that it causes typical tubercular lesions on intraperitoneal injection into test animals[62]. Preliminary investigation led to the erroneous assumption that crude phthioic acid was saturated. This arose through a misinterpretation of the zero iodine value of phthioic acid and the use of hydrogenation in its isolation. Chanley and Polgar[70], however, drew attention to the non-reactivity of α,β-unsaturated acids to halogens and showed that these dextrorotatory phthioic acids were in fact α,β-unsaturated. Efficient fractionation by various workers[71-76], has shown that phthioic acid is a complex mixture of fatty acids containing at least thirteen saturated and unsaturated acids.

αβ-Unsaturated acids consisting of at least six dextrorotatory α-alkyl-αβ-unsaturated acids have been identified[75] as C_{25}-, C_{26}-, C_{27}- and C_{29}- phthienoic acids. The latter acid has been shown on the basis of physical data and the Kuhn-Roth determination of C-methyl, to possess one more branch in the chain than do the other phthienoic acids. The structure, 3,13,19-trimethyltricosanoic acid, proposed[77] for C_{27}-phthienoic acid, was shown to be untenable[78]. Cason and his associates,[79,80] assigned it the partial structure,

L(+)-2,4-dimethyl-2-alkenoic acid and degradative studies[73] of mycolipenic acid, a dextrorotatory α,β-unsaturated acid, believed to be identical[81] with C_{27}-phthienoic acid, have shown this to be (+)-2,4,6-trimethyl tetracos-2-enoic acid (IV). The physical constants cited by Polgar[70,72,] and by Cason[74] for the two acids are, however different.

$$CH_3.(CH_2)_{17}.CHMe.CH_2.CHMe.CH{:}CMe.CO_2H.$$

(IV)

Of the several laevorotatory saturated acids present in the lipoids of tubercle bacillus, mycocerosic acid, a C_{31}-laevorotatory saturated acid, discovered by Ginger and Anderson[82] has been assigned the structure (V) by Polgar[73], who has named it "mycoceranic acid". More recently Cason and Fonken[75] have proposed the name "C_{31}-mycosanoic" acid

$$CH_3.(CH_2)_n.CHMe.CH_2.CHMe.CH_2.CHMe.CO_2H.$$

(n is probably 21)

(V)

($C_{31}H_{62}O_2$) for an identical acid.

Mycolic acid, a long chain branched, saturated, fatty acid, $C_{88}H_{176}O_4$, isolated by Stodola, Lesuk and Anderson[83] from a human strain of tubercle bacillus, is at least partly responsible for the property of acid fastness in Mycobacteria[8]. It has been shown to contain carboxyl, hydroxyl and methoxyl groups in the ratio 1:1:1. Degradation[84] by pyrolysis at 300° in vacuo, and chromic acid oxidation yielded n-hexacosanoic

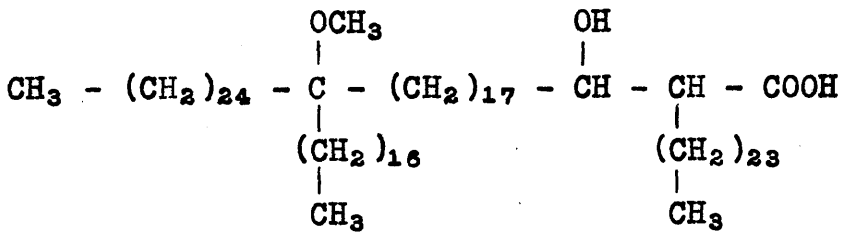
acid ($C_{26}H_{52}O_2$), stearic acid and hexadecane-1,16-dicarboxylic acid. Chromatography of mycolic acid on alumina by Asselineau and Lederer[85] gave two isomers, α- and β- mycolic acids. Analytical data confirmed Anderson's molecular formula for mycolic acid as $C_{88}H_{176}O_4$ but still with an uncertainty of about $\pm 5CH_2$. Pyrolytic decomposition of the α- and β- mycolic acids is characteristic of α- substituted β- hydroxy acids and the following mechanism has been proposed[86], though the reaction seems to be more

$$R - CHOH - \underset{\displaystyle C_{24}H_{49}}{\overset{\displaystyle |}{CH}} - COOH \longrightarrow R - CHO + \underset{\displaystyle C_{24}H_{49}}{\overset{\displaystyle |}{CH_2}}COOH$$

(VI)

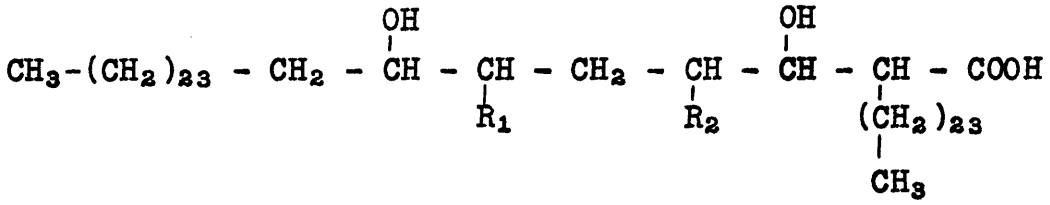
complicated than this would indicate since no aldehyde was isolated. However the partial structure (VI) has been confirmed by dehydration to the corresponding anhydro-mycolic acid and subsequent ozonolysis. About forty different types of mycolic acid having identical molecular weights have been isolated[57b] from various strains of Mycobacteria and tentative structures for some have been proposed as follows:-

(i) α-mycolic acid[87a] (VII), 3-hydroxy-x-methoxy-mycolanoic acid ($C_{88}H_{176}O_4$),



(VII)

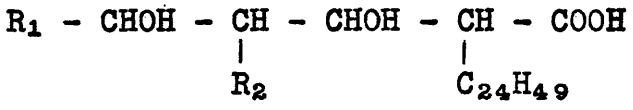
(ii) α_2-mycolic acid[87b] (VIII), 3,7(?)-dihydroxy-mycolanoic acid, ($C_{88}H_{174}O_4$ $\pm 5CH_2$),



$R_1 + R_2 \simeq C_{31}H_{62}$ $\pm 5CH_2$

(VIII)

(iii) α-mycolic acid Brevannes[88] (IX), 3,5(?)-dihydroxy-mycolanoic acid (1-Brevannes).



$R_1 + R_2 = C_{58}H_{118}$ $\pm 5CH_2$

(IX)

The chemical nature of the toxic substance, designated "cord factor" and isolated from the wax fraction of tubercle bacillus, has been revealed by Noll, Bloch, Asselineau and Lederer[89]. The purified sample[89,90] of cord factor having m.p. 37-38°, $[\alpha]_D$ + 31°, molecular formula $C_{186}H_{366}O_{17}$ $\pm$ 10(CH_2), was identified[89] as trehalose - 6,6'-dimycolate

and this was confirmed by synthesis[91,92].

The chemistry of other natural constituents of the purified wax[93] and the acetone-soluble fat[93,94] has also been investigated. Such chemical studies have permitted both qualitative and quantitative distinctions to be made with accuracy in the classification of various strains of tubercle bacillus by infrared spectroscopy[95].

Biological Properties of Lipoids.

Investigations carried out by Sabin and her co-workers[17,96] have revealed that formation of typical giant cells and epitheloid cells could be provoked not only by dead tubercle bacilli but also by their lipoid constituents. Whilst confirming these results various workers[97,98] have shown that the methylated branched-chain acids of the phthioic acids type cause the formation of typical epithelioid cells, and that laevorotatory acids produce more granulomatous lesions than dextrorotatory acids in guinea pigs.

Typical tuberculin hypersensitivity has been demonstrated by injecting wax fraction from Myco. tuberculosis (human strain) together with tuberculoprotein[99] into guinea pigs. After testing the various crude and purified components of wax, it has been concluded[100] that the so-called "cord factor" is responsible for hypersensitivity. The

relationship of virulence to cord factor in Mycobacteria, however, has not been substantiated.[101]

PROTEINS.

The study of protein constituents has been found to be far more complicated than that of the other constituents of the tubercle bacillus, due to the complexity of protein macromolecules. The proteins are usually isolated from the liquid culture filtrates which in addition to the constituents of the medium contain proteins, nucleic acids, carbohydrates and bacillary metabolites which have diffused out from the bacterial cells. Robert Koch isolated the substance, tuberculin, which gave characteristic protein reactions, from culture filtrates of tubercle bacilli by precipitation with ethanol.

Though the extensive study of various protein fractions has not provided any new approach to the chemotherapy of tuberculosis, their immunological properties have been of considerable value as a diagnostic aid in clinical assessment of the disease. The tuberculin reaction of Koch[13c], or the specific allergic reaction, involves the injection of a minute amount of tuberculin into the skin of a tuberculous animal and is manifested by a zone of inflammation. Numerous investigations have been carried out in the past for the

isolation of the pure fractions responsible for the tuberculin reaction.

Fractionation and purification of various protein components has been achieved as a result of the brilliant researches of Seibert and these have been summarised in a number of reviews[53,60,102]. The specific protein fractions, tuberculin, in its crude form is a water-soluble mixture of protein, nucleic acid and polysaccharide. The specific potent protein known as Old Tuberculin (O.T.) was precipitated from heated culture filtrates with trichloracetic acid[103], the latter being washed off subsequently with ether[104]. The product which was designated as purified protein derivative (PPD), was found to be stable, uniform and effective[105].

PPD was contaminated substantially with nucleic acid and polysaccharide, though each of these impurities was inactive in tuberculin reaction. The nucleic acid was separated by precipitation of the protein at pH7. A standard purified protein derivative[106] (PPD-S) was prepared by ultra filtration of the culture filtrate and by precipitating the protein repeatedly with neutral saturated ammonium sulphate at pH7. This purified product contained 1.2 per cent of nucleic acid (diphenylamine reaction) and 5.9 per cent of polysaccharide (carbazole reaction) and had a potency twice that of the former purified protein derivative. Subsequently this

preparation was adopted as the international standard tuberculin[107]. Seibert[54] has since indicated that this purified product contains at least two proteins which show differences in their solubility, coagulability, diffusibility, sedimentability, electrophoretic mobility, antigenicity and potency. Thus the native or undenatured proteins in raw tuberculin have been designated as proteins A and B. These proteins are soluble at all hydrogen-ion concentrations but are differenciated by the fact that protein A has a lower electrophoretic mobility than the B. The dissociation constants of the more polar groups in protein B suggest that they may be imidazole structures.

The denatured proteins A' and B' corresponding to the native proteins A and B have been identified by precipitation at pH4 to 5 and show lower potency for eliciting the tuberculin skin reaction than the native proteins. The two proteins isolated by Bevilacqua and McCarter[108], have been shown to be separate entities and one is not the degradative product of the other. Such studies of different protein components and their denaturation has led to the use of unheated culture filtrates[109].

More recently Seibert has affected still further purification of PPD-S by precipitation of unheated culture filtrates with ammonium sulphate[110] and also with ethanol[109]

at low temperatures and at controlled pH and salt concentration. The three groups of proteins designated as A, B and C in the order of their electrophoretic mobility were fractionated[111] in this way. The least potent fraction which was obtained by simple precipitation at pH4, had the greatest mobility. The fraction B having mobility similar to that of fraction C, was precipitated with 30 per cent ethanol at pH 4.6. The fraction A which was the least mobile, required 70 per cent of ethanol at pH 4.6 for its precipitation. This fraction was the least homogeneous, being appreciably contaminated with carbohydrate. Proteins A and C had similar ultraviolet absorption spectra[109] at 2800 Å., but at 2500 Å., protein C had nearly twice the optical density of protein A.

The amino acid constituents of various protein fractions have been studied by Seibert[112]. The A proteins showed a higher content of proline, glutamic acid-alanine and the basic amino acids, arginine and lysine than that of the C proteins while the latter contained more leucine - isoleucine, phenylalanine and valine. These differences particularly in basic amino acids for which nucleic acid has special affinity may explain the difference in absorption spectra of these protein fractions.

Biological properties of these fractions were studied by Seibert[110,112]. The fraction A was found to be most active in tuberculous reaction and particularly in eliciting antibodies in normal animals. The greater tuberculin activity of protein A was attributed to the presence of more phenolic groups than in protein C.

Besides the isolation of protein fractions from culture filtrates, many investigators have attempted to extract the biologically active proteins from the bacillary bodies. Heidelburger and Menzel[113] obtained three antigenically different protein fractions by treating the acetone-extracted cells with dilute acetate buffer under increasingly alkaline conditions. Heckly and Watson[114] extracted the mechanically ruptured bacilli with phosphate and borate buffers and isolated various protein fractions. More recently steam-killed cells were treated with urea[112,115] and the purified product[116] so obtained, found to be homogeneous in its electrophoretic behaviour. It resembled Seibert's C proteins in its ultraviolet and electrophoretic characteristics but was similar to proteins A in eliciting skin reactions in tuberculous animals.

It is evident that certain protein fractions which are antigenic, produce a specific reaction in infected animals. It has been demonstrated that proteins from

different strains of tubercle bacilli elicit a certain amount of immunological specificity. Aronson[117] has confirmed Koch's observation that animals previously infected become resistant to reinfection. This principle has been applied from time to time in an effort to produce specific immunizing agents from modified and heat-killed tubercle bacilli. Tubercle bacilli modified with radium bromide, and with ultraviolet light[118], protected guinea pigs to a certain degree against reinfection. Heat-killed tubercle bacilli[119] increased resistance to reinfection with virulent strains. The B.C.G. vaccine, a living attenuated bovine strain, on the other hand, has been shown to confer greater resistance in both experimental and clinical studies[117]. Recently M-vaccine[120] prepared from an attenuated murine type strain has been claimed to possess manifold advantages over B.C.G. vaccine.

THE CHEMOTHERAPY OF TUBERCULOSIS

Synthetic Tuberculostatics

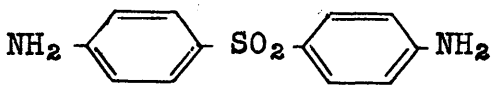
Little progress was achieved in the chemotherapy of tuberculosis in the first three decades of the present century. Until then the older remedies such as creosote,[121] salts of heavy metals particularly of gold[122] and dyes such as methylene blue and its derivatives,[123] were frequently used

in combating tuberculosis, though without marked success. Their administration, too, was often attended by a marked risk of toxic side reactions. Cod liver oil[124] and vitamin preparations were also used extensively as adjuncts mainly because of their beneficial contribution to the general health of the patient.

Sulphonamides and Sulphones.

The advent of sulphonamides in 1935 brought the opportunity to explore their potentiality in experimental tuberculosis. Sulphanilamide,[125] sulphapyridine,[126] sulphathiazole,[127] and sulphadiazine[128] showed some activity against Mycobacterium tuberculosis both in vitro and in vivo. While these investigations did not warrant the clinical use of sulphonamides, they did lead to a new class of compounds, namely the sulphones.

4,4'-Diaminodiphenyl sulphone[129] (X) (Dapsone), the prototype of this group, was first tested in 1937[130] and was shown to be active both in vitro and in vivo.[131]

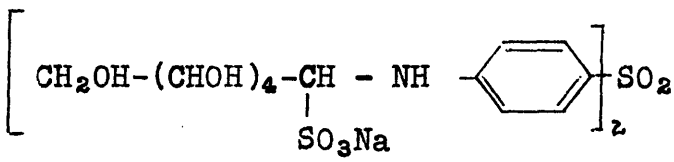


(X)

Its toxicity, however, arrested its clinical use in tuberculosis, but Lowe[132] found it to be a valuable agent

in the treatment of leprosy.

A large number of less toxic derivatives of Dapsone were prepared by substitution in the amino group and drugs of particular interest have been designated as Promin[133] (XI), (disodium 4,4'-diaminodiphenyl sulphone-N,N'-diglucose sulphonate), Promizole[134] (XII) (4,2'-diaminodiphenyl--5-thiazolyl sulphone), Solapsone or Sulphetrone[135] (XIII) (tetrasodium 4,4'-bis-√ -phenyl-n-propylaminodiphenyl sulphone tetrasulphonate) and Diasone[136] (XIV) (4,4'--diaminodiphenyl sulphone disodium formaldehyde sulphoxylate). All these compounds were of limited clinical use.



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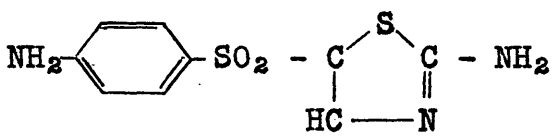

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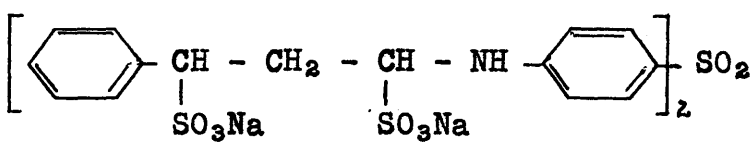

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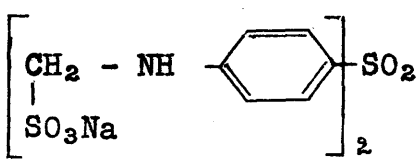

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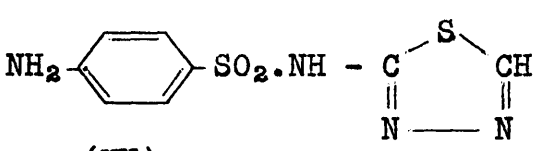
Thiosemicarbazones.

Domagk[137] inferred from comparative studies of the tuberculostatic activity of sulphathiazole, sulphathiadiazole (XV) and promizole that much of the activity was contributed by the thiazole ring and as a result of this discovery, commenced the examination of structurally analogous compounds. He showed that the open chain 4-acetylaminobenzaldehyde thiosemicarbazone (Thiacetazone XVI, Conteben, Tb 1/698) was the most active of the



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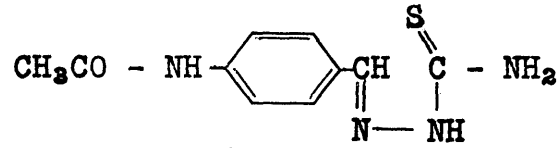


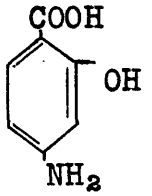
(XVI)

substances examined. Its efficiency has been confirmed in experimental animals[138] and in clinical trials[139] but due to its high toxicity, it could not compete with other equally effective tuberculostatics which were to be introduced later on.

Para-Aminosalicylic Acid (PAS)

The work of Bernheim[140] which demonstrated the inhibitory effects of benzoates and salicylates on the respiration of tubercle bacilli, prompted Lehmann[141] in 1946 to test a series of benzoic and salicylic acid derivatives against Mycobacterium tuberculosis. Of these,

p-aminosalicylic acid (PAS) (XVII) was found to be the most active in both experimental[141,142] and clinical studies[141,143].

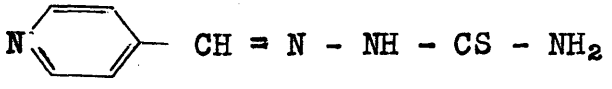


(XVII)

Comparative trials have since shown it to be much less active than either isoniazid or streptomycin but in combination with either of the latter, drug action is reinforced and the emergence of resistant strains delayed[144,145].

Isonicotinic Acid Hydrazide (Isoniazid).

The discovery of p-acetamidobenzaldehyde thiosemicarbazone (XV) by Domagk[137] as an effective tuberculostatic, prompted Fox[146] to synthesise isonicotinaldehyde thiosemicarbazone (XVIII) through the intermediate isonicotinic acid hydrazide, (XIX). The latter was proved to be a potent chemotherapeutic agent in tuberculosis, being much more effective than the semicarbazone.



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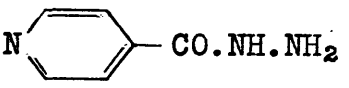
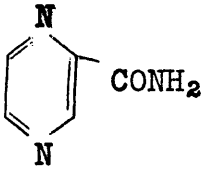


(XIX)

After encouraging reports based on in vitro[147] experiments, isoniazid was found to exhibit a high level of activity in experimental tuberculosis[148] and in man[149] and rapidly became established as the most potent anti-tuberculous agent. Co-administration with pyridoxine has been found to prevent undue toxicity when higher dose levels are required[150]. Like PAS, isoniazid is immediately absorbed after oral administration[151]. In the last few years a large number of isoniazid derivatives have been examined for tuberculostatic activity but without success.

The antituberculous activity exhibited by nicotinamide and some of its derivatives[152] led to the synthesis of pyrazine analogues[153]. Of these, pyrazinamide (XX) was regarded as promising[154] but has been shown to be unsafe for routine clinical use due to its high toxicity.[155]



(XX)

Surface-Active Agents.

A commercial non-ionic surface-active agent "Triton A20" and other similar macromolecular polyoxyethylene ethers were found by Cornforth and others[156] to suppress the development of experimental tuberculosis in the mouse and

this was confirmed by Solotorovsky and Gregory[157]. Increase in the number of ethylene oxide units from between 10 and 20 (with highest activity) to 25 to 30 abolished the therapeutic activity and with yet further increase to between 45 and 90 units gave substances which exhibited protuberculous effect[158]. The opinion has been expressed that these surface-active agents possibly influence tuberculous infections by modifying the surface lipoids of the tuberculous bacillus within the monocytes in experimental animals.

Hinconstarch

Hinconstarch is a polymer which is prepared from periodate oxidised potato starch by condensation with equimolar proportions of isoniazid and p-aminobenzalthiosemicarbazone[159]. It is a yellow amorphous powder, insoluble in water and accordingly its antituberculosis activity has not been investigated *in vitro*[160]. It was shown, however, to be effective in experimental tuberculosis[160] and in clinical trials[161] but haemolytic reactions and hepatic toxicity limit its usefulness. A further disadvantage is the instability of the polymer in the presence of dilute acid or alkali.

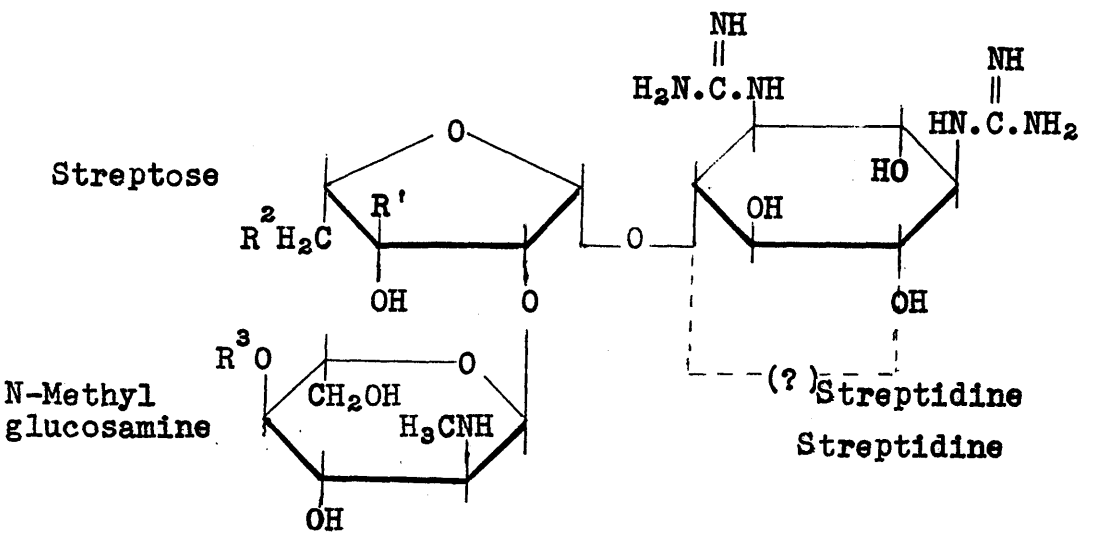
Antibiotics

Out of the several antibiotics so far studied for antitubercular activity, only a few have passed beyond the stage of laboratory experiment due either to high toxicity or to diminished activity. Streptomycin one of the first antibiotics found to be effective in the control of tuberculosis is still used more extensively than any other for this purpose.

Streptomycin and its Derivatives

Streptomycin (XXIa) was isolated from *Streptomyces griseus* in 1944 by Schatz, Bugie and Waksman[162] as a result of a long and painstaking search among actinomycetes for antibacterially active metabolic products[163]. Schatz and Waksman[164] demonstrated its activity against *Mycobacterium tuberculosis in vitro* and this was confirmed by Youman and Feldman[165]. Tests on experimental animals,[166] and clinical trials[167] soon followed establishing its effectiveness as a bacteriostatic agent[168] in the treatment of a wide variety of tuberculous lesions. Response to streptomycin is most favourable in acute miliary[169], pulmonary[170] and meningeal[171] tuberculosis, but chronic fibrotic lesions are little affected. A serious disadvantage is the need to administer the drug by intramuscular injection due to its poor

absorption from the intestine. A further limiting factor to prolonged therapy is the rapidity with which the tubercle bacillus acquires resistance to streptomycin[172]. This may be obviated, however, by administering p-amino-salicylic acid[173] and isoniazid[174] in conjunction with streptomycin, a form of treatment which significantly delays the emergence of streptomycin resistance



(a) $R' = CHO$; $R^2 = R^3 = H$

(b) $R' = CH_2OH$, $R^2 = R^3 = H$

(c) $R' = CHO$, $R^2 = H$, R^3 = D-mannose.

(d) $R' = CHO$, $R^2 = OH$, $R^3 = H$

(e) $R' = COOH$, $R^2 = R^3 = H$

(XXI)

Some toxic effects are produced by prolonged treatment with streptomycin. These include impairment of renal function, giddiness and allergic manifestations though by far the most serious are disturbances of auditory and vestibular function[175]. This neurotoxicity was once attributed to the presence of the aldehydic group since it is partly eliminated in dihydrostreptomycin[176]. Other guanidino-substituted antibiotics also cause vestibular disfunction and it is now thought that in streptomycin, this is probably associated with the streptidine part of the molecule.[177]

Dihydrostreptomycin (XXIb) which is formed by catalytic reduction[178,179] of the aldehydic group to the corresponding primary alcohol was found in both *in vitro*[180] and *in vivo*[181] tests, and also in clinical trials[182] to be almost as effective as streptomycin against *Myco.tuberculosis*. Despite early reports to the contrary, impairment of hearing mechanism is even greater than with streptomycin. In order to minimise the appearance of toxic symptoms, the use of both streptomycin and dihydrostreptomycin in admixture, has been proposed, so that equilibratory toxicity of streptomycin and auditory nerve toxicity of dihydrostreptomycin are lessened.[183]

Mannosidostreptomycin (Streptomycin B) (XXIc) was isolated by Fried and Titus[184] from the crude streptomycin

concentrates and purified[185] by conversion into the reineckate. Structural analysis[186] revealed that the molecule consists of streptomycin joined glycosidically to D-mannose. Mannosidostreptomycin can be converted to streptomycin by the enzyme mannosidostreptomycinase obtained from the cell-free preparations of Streptomyces griseus[187], and the antibiotic is thought to be the precursor of streptomycin. The smaller yield of mannosidostreptomycin compared with streptomycin from the culture filtrates lends further support to this argument. Because it is comparatively less active than streptomycin[188], it has not been used clinically.

Hydroxystreptomycin (XXId) has also been isolated from various species of Streptomyces[189,190]. Its biological activity is comparable to that of streptomycin but it exhibits rather high ototoxicity[191] and for this reason it has not been developed as a practical agent for the treatment of tuberculosis.

Recently Málek and his coworkers[192] have suggested a new and interesting form of streptomycin therapy. They have combined streptomycin with polyacrylic and natural polycarboxylic acids and with sulphonic and phosphorylated polysaccharides to form a series of so-called lymphotropic antibiotics. These macromolecular salts act as pure colloids which are absorbed by the lymphatic system and become

concentrated in lymphnodes. On the other hand, much lower blood levels are achieved than with the parent antibiotic but these are maintained for longer periods, and consequently excretion is delayed. Acute toxicity also is much less than that of streptomycin in experimental animals. Similar results have been obtained with neomycin, viomycin and other antibiotics.

The Problem of Streptomycin - Resistance

An antitubercular agent should preferably be highly bactericidal for the causative organisms in concentrations that do not produce toxic symptoms in the host. But even the most potent tuberculostatics such as streptomycin, isoniazid and p-aminosalicylic acid are bacteriostatic rather than bactericidal agents when administered in therapeutic doses. This necessitates the prolonged therapy which induces the emergence of bacterial resistance.

Very soon after streptomycin became available for laboratory experiment and clinical trials, reports of the development of streptomycin-resistance began to appear[193,194]. The resistance was also observed[195] during serial subculturing on media containing increasing concentrations of streptomycin. A careful *in vitro* study[196] revealed that streptomycin-resistant variants appeared in the cultures

of susceptible strains as a result of random mutations in bacterial population. Resistance is, therefore, considered to be a heritable character arising possibly by gene mutation[197] and this has been confirmed[196] by prolonged sub-cultivation on streptomycin - free media. One hypothesis suggests that streptomycin-resistance is somehow associated with desoxyribonucleic acid but no satisfactory answer has as yet been found[198].

Miller and Bohnhoff[199] observed the development of streptomycin - dependent organisms. Colonies of E. Coli grew larger on media containing high concentrations of streptomycin. These dependent variants were known to be genetically alike and the larger size was due to direct stimulation by streptomycin.

Streptomycin-resistant and dependent-strains have been utilized in the identification of streptomycin-producing moulds;[200] they have also been used as a tool in bacterial genetics as well as to explore the mode of action of antimicrobial agents.[163a] The growth of streptomycin-dependent meningococci is not stimulated[163a] by streptomycin degradation products such as streptamine, streptidine and streptobiosamine, by lipositol and inositol or by streptomycin inactivated with hydroxylamine hydrochloride and cysteine hydrochloride. Rake[201], however, demonstrated that

preparations such as lipositol, N-acetyl-mannosidostreptomycin and streptobiosamine possessed some activity. This facet of the problem, therefore, still requires further investigation.

The efficacy of streptomycin would be naturally enhanced if the obstacle of streptomycin-resistance could be eradicated though in preliminary investigations, intermittent administration or combination of streptomycin with other tuberculostatics was suggested to delay the onset of streptomycin resistance.

The Mode of Action of Streptomycin.

Henry and Hobby[163b] have cited several ways in which streptomycin might interfere with bacterial metabolism but little is known with certainty about its mode of action against the tubercle bacillus[202,203].

The action of streptomycin on the bacterial cell is influenced by several factors, namely, concentration of the antibiotic[204,205], the size of the inoculum[204], the nature of the medium, its pH[206] and the length of time for which the cells are exposed to the antibiotic. Thus the efficiency of streptomycin decreases with increase in the size of the inoculum which is correlated with the presence of greater numbers of resistant organisms[204]. Further, the activity is

pronounced when the culture consists of young actively growing cells[205] but is diminished against resting cells or older cultures[207]. This seems to suggest that streptomycin blocks the synthesis of bacterial metabolites or the process of cell division.

The activity of streptomycin is reduced by inclusion in the culture medium, of peptone and amino acids such as methionine, cysteine, proline, tyrosine and aspartic acids[208]. Cysteine, however, does not inactivate dihydrostreptomycin[209] and this has been interpreted to imply that the aldehyde group may not be necessary for the action of streptomycin. On the other hand, Bailey and Cavallito[210] have suggested that dihydrostreptomycin is first oxidised to streptomycin by the organism and only then is it inactivated by cysteine. It has been concluded that streptomycin possibly acts by interference with amino-acid synthesis at enzyme level. Nevertheless, Henry and others[211] failed to observe inhibition of purified enzymes with streptomycin.

Streptomycin probably exerts certain growth-inhibitory effects by virtue of its action on cellular metabolism. Certain cell constituents such as proteins[212], desoxyribonucleic acid[213] and lipoids[212] form complex precipitates with streptomycin is possibly brought about by the diguanide part of the antibiotic, but as a basis of the mode of action

this is still controversial[203] since the streptidine fragment does not show any activity. On the other hand, the surface adsorptive capacity of polysaccharides in the tubercle bacillus has been suggested by Stacey and Kent[18] to help in the fixation of streptomycin and thereby modify the permeability of the cell.

The inhibition of terminal respiration has been interpreted as an important mechanism of the mode of action of streptomycin. Umbreit[214] observed that the inhibition of fumarate and pyruvate oxidation by streptomycin in E.coli results in a block of the terminal respiration system which involves a pyruvate-oxalacetate condensation. The nature of this reaction has not been fully elucidated but a possible intermediate involved is 2-phospho-4-hydroxy-4-hydroxycarbonyladipic acid[215]. Oginsky, Smith and Solotorovsky[216] have suggested that streptomycin interferes in the oxidation of higher fatty acids synthesised by tubercle bacillus and may, thus, block the utilization of such materials.

Most of the observations which have bearing on the mode of action of streptomycin, have been limited to either resting cells, growing cells or isolated enzymes and other constituents of the parasite but the superiority of anyone over the others has not been demonstrated. Possibly,

each factor is responsible for a number of toxic reactions that contribute to the therapeutic effectiveness of the antibiotic.

The Chemistry and Biological Activity of Streptomycin and its Degradative Products.

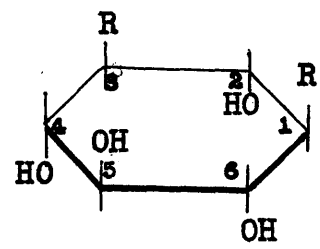
Streptomycin is a laevorotatory, triacidic base with the molecular composition, $C_{21}H_{39}O_{12}N_7$, which does not crystallise, but which forms a crystalline calcium chloride complex, $2(C_{21}H_{39}O_{12}N_7.3HCl).CaCl_2$. Other salts of commercial importance are, the sulphate $2(C_{21}H_{39}O_{12}N_7).3H_2SO_4$ and the trihydrochloride. The crystalline complex has proved to be of great value in eliminating impurities in crude streptomycin concentrates,[217] though chromatographic techniques have also been exploited commercially[218] for the purpose. Other crystalline salts have also been recorded for characterisation and purification of the antibiotic.[218]

Streptomycin is soluble in water and insoluble in almost all organic solvents. The hydrochloride is quite soluble in methyl alcohol in contrast to the sulphate but both are soluble in water. Streptomycin is relatively stable at pH 1-10 but quite stable at pH 3-7 at temperatures below 28°[219].

The chemical investigations which led to the elucidation of the structure of streptomycin were carried out principally

by Folkers, Wintersteiner, Carter, Wolfrom and their respective collaborators. These studies have been reviewed elsewhere[220,221]. Streptomycin is composed of three moieties, streptidine, streptose and N-methyl-L--glucosamine joined together by glycosidic linkages.

Wolfrom and his coworkers[222] have pointed out that positions 4 and 6 of the streptidine moiety (XXI) are not configurationally equivalent and that substitution of the same optically active streptobiosamine fragment for the hydroxyl hydrogens at these points will lead to diastereo-isomers. This configurational point in the streptomycin molecule remains to be elucidated though hydroxyl hydrogen at position 4 is generally accepted to be linked glycosidically to streptobiosamine. However, the same authors have established that the linkages between streptidine and streptose is β- and that between streptose and N-methyl-L-glucosamine is α- . The latter linkage is comparatively stable and requires drastic acidic conditions for its cleavage while the former weaker glycosidic bond between streptidine and streptose is readily cleaved by acid hydrolysis to give streptobiosamine[223] (XXII) and streptidine[224] (XXIIIa). The latter has been shown by degradation to be the mesoform of 1,3-diguanidino-2,4,5,6-tetrahydroxycyclohexane and this structure has been confirmed



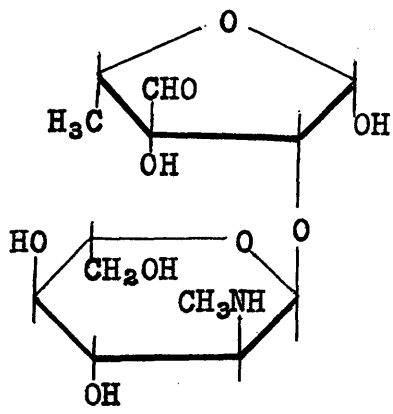
(a) R = -NH-C(=NH)-NH$_2$

(b) R = -NH.C$^{\oplus}$(=NH$_2$)

(c) R = -NH$_2$

(XXIII)

Streptomycin (XXIa)



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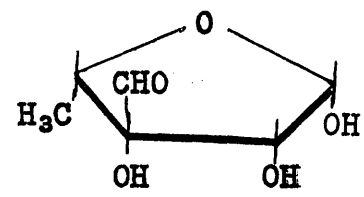
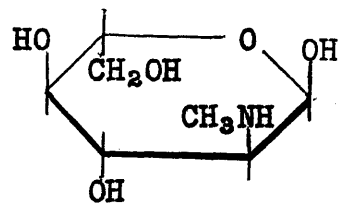


(XXV)



(XXIV)

by synthesis.[225] Neither of these hydrolytic products either individually or mixed together exhibit any antitubercular activity.

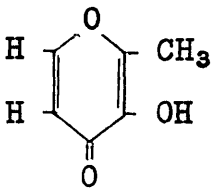
The optically inactive, diacidic base, streptidine

was converted by stepwise alkaline degradation[224] with barium hydroxide solution, first into strepturea (XXIIIb) and then to streptamine (XXIIIc). The basic disaccharide streptobiosamine (XXII) was further degraded by acid hydrolysis to N-methyl-L-glucosamine (XXIV) whose structure was confirmed by synthesis.[226] The instability of streptose moiety under drastic hydrolytic conditions presented considerable difficulties in the elucidation of its structure but was finally shown to be 3-C-formyl-5--deoxy-L-lyxose (XXV), a pentose containing a reactive aldehyde group[227].

The presence of the aldehydic group in streptomycin was established by oxidation with bromine to streptomycinic acid[228] (XXIe) and by the formation of an oxime, semicarbazone and phenylhydrazone[229], all of which were biologically inactive. On the other hand, a low antitubercular activity has been demonstrated with streptomycylidene isonicotinyl hydrazine which was obtained by condensation of streptomycin and isoniazid[230]. The desirable attributes of both drugs are thus combined in a single preparation which has the added advantage of being devoid of serious toxic effects attributable to streptomycin.

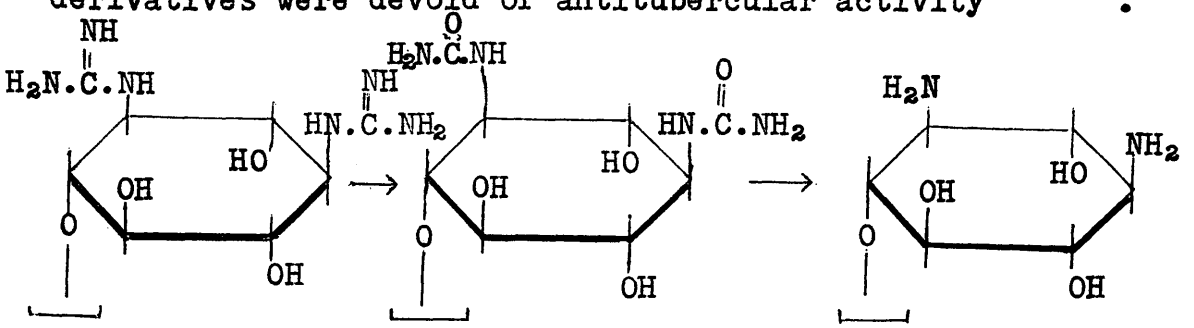
Reduction of the aldehydic group to the corresponding primary alcohol by hydrogenation with platinum[178] or nickel[231]

gave dihydrosterptomycin which showed biological activity comparable to streptomycin. Dihydrostreptomycin base unlike streptomycin has been crystallised[232], though it does not form calcium chloride double salt and is not decomposed by alkali to yield maltol[178] (XXVI).



(XXVI)

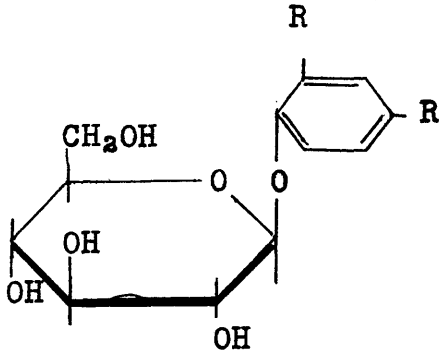
Owing to the relative stability of the dihydrostreptose fragment to alkali, it was possible to degrade dihydrostreptomycin (XXIb) with barium hydroxide solution first to strepturea dihydrostreptobiosaminide (XXVII) and then to streptamine dihydrostreptobiosaminide (XXVIII). These derivatives were devoid of antitubercular activity[233,234].



(XXIb) (XXVII) (XXVIII)

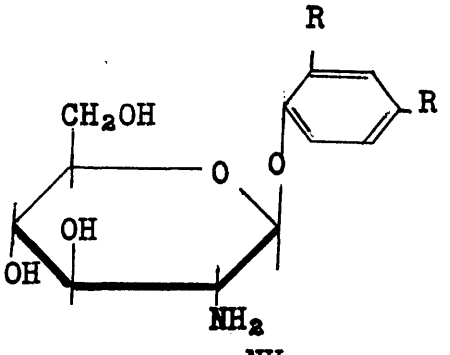
Since the substitution of guanidino groups in dihydrostreptomycin abolishes its activity, the significance of these groups has been tested by Latham, May and Mosettig[235]

by synthesising diamino- and diguanidino- phenyl β-D-glycosides (XXIX, XXX). But none of these compounds were



R = $-NH_2$ or $-NH-\overset{\overset{NH}{\|}}{C}-NH_2$

(XXIX)

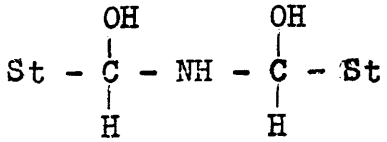


R = $-NH_2$ or $-NH-\overset{\overset{NH}{\|}}{C}-NH_2$

(XXX)

found to be active in vitro against Myco. tuberculosis.

During the preliminary investigations of streptomycin, it was observed that crude concentrates were more toxic to experimental animals than streptomycin itself. Further purification of streptomycin resulted in the isolation of an interesting substance which proved lethal to mice. Characterisation, structural determination and synthesis of this substance led to the discovery that under certain conditions ammonia unites with two molecules of streptomycin to form bis-(α-hydroxystreptomycyl)-amine (XXXI) which is much more toxic than pure streptomycin[236].

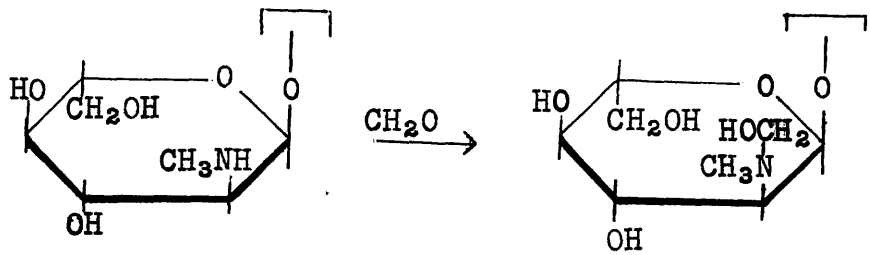


St is streptomycin less - CHO.

(XXXI)

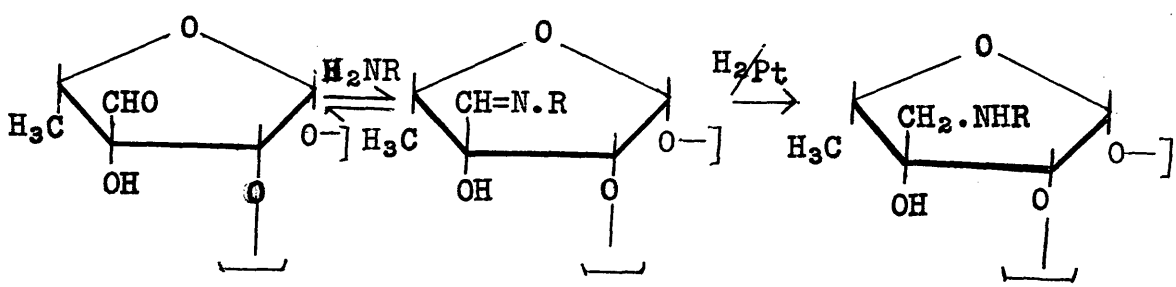
Mannosidostereptomycin (XXIc) is comparatively less active than streptomycin[188] while hydroxystreptomycin (XXId) possesses biological activity comparable with that of streptomycin but it exhibits much higher ototoxicity[191].

In the past, various attempts have been made to modify the structure of streptomycin in order to enhance the antitubercular activity or to reduce its toxicity. Jarowski and Murphy[237] have demonstrated that hydroxymethyl streptomycin (XXXII) prepared by reaction with formaldehyde is comparable in biological activity to streptomycin but its



(XXIa) (XXXII)

neurotoxicity is significantly lower than that of the latter. Redmond and Cummings[238] have observed that conversion to



(XXIa) (XXXIII)

N-substituted streptomycylamines (XXXIII)[239] by condensation with primary amine and subsequent reduction modifies the biological activity of streptomycin in three ways. Thus not only is tuberculostatic activity decreased but it varies with sensitive, resistant and dependent strains of tubercle bacilli and antistreptomycin activity also appears.

Tuberculostatic activity is completely lost with decyl- or dodecyl-streptomycylamine but is only reduced when streptomycin is condensed with a shorter chain base such as propylamine. Streptomycylamine and some of its derivatives such as N'-p-sulphonamidobenzyl-, N'-n-octadecyl-, N'-2-butoxy-5-pyridyl-streptomycylamine and methylolstreptomycin inhibited the resistant strain at 10 μg per ml. while dimethylol N'-n-dodecyl-streptomycylamine antagonised the action of streptomycin against streptomycin-sensitive and dependent strain[238].

It would appear from structures of the three naturally occurring streptomycins and from the biologically active derivatives of streptomycin such as dihydrostreptomycin, methylolstreptomycin and streptomycylamines that some structural variation is permissible without the destruction of the antibacterial properties.

Other Antibiotics

Neomycin

Neomycin,[240] a complex metabolic product obtained from *Streptomyces fradiae*, has been shown by chromatography[241] to be a mixture of two closely related substances, neomycins B and C (neomycin A is probably neamine,[242] a degradation product of neomycins B and C). They resemble streptomycin in that a disaccharide unit, termed neobiosamine is linked glycosidically to a basic component, neamine (deoxystreptamine) analogous to streptidine. The formula (XXXIV, R = H) for neobiosamines B and C from neomycins B and C (XXXIV, R = $C_{12}H_{25}N_4O_5$), have recently been proposed[243] on the basis of physico-chemical study.

$$R - O - C_5H_6(OH)_2 - O - C_6H_7O(OH)_2(NH_2)_2$$

D-Ribose Diaminohexose

(XXXIV)

Neomycin is active against tubercle bacilli *in vitro*[244] but in clinical tuberculosis[245] its effectiveness is associated with serious toxic effects such as renal damage and deafness. It is chemically more stable than streptomycin; it also appears to induce bacterial resistance at a slower rate and is active against streptomycin-sensitive and -resistant strains[246]. Its toxic symptoms have limited its use to topical application and pre-operative sterilization of the gut.

Viomycin.

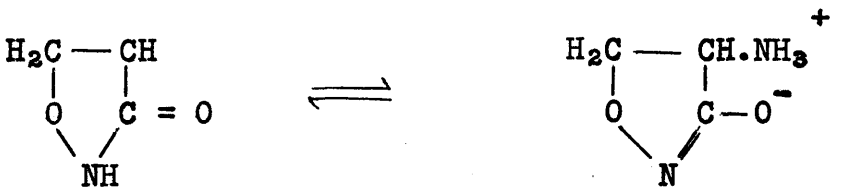
Viomycin,[247] an antimicrobial agent produced by Streptomyces puniceus and Streptomyces floridae, is a polyacidic base with empirical formula $C_{18}H_{31-33}N_9O_8$. It is slightly less potent than streptomycin in tuberculous infections[248] but is also active against streptomycin-resistant tubercle bacilli.[249] Its use in clinical tuberculosis has been restricted by its toxicity to those cases when neither streptomycin nor isoniazid can be used[250]. Toxic symptoms such as skin irritation, renal damage and vestibular or auditory disfunction can be lessened or obviated by suitable selection of dose regimens. As with streptomycin, the neurotoxicity is possibly a function of the guanidine moiety of the molecule. Solutions of viomycin sulphate are moderately stable at room temperature and pH 5-6 and are administered intramuscularly.

Cycloserine (Syn: Oxamycin, seromycin)

Cycloserine,[251] which is produced by Streptomyces orchidaceus, is effective against the tubercle bacillus both in vitro and in vivo. It has been proved to be of value in experimental and clinical tuberculosis. Due to its relative non-toxicity, it has been found valuable as an alternative to streptomycin in acute and chronic tuberculosis.[252]

Recent reports,[253] following its more widespread use, indicate that it is not entirely free from toxic side effects.

It is excreted in the urine after intramuscular administration in greater amounts than when the drug is administered orally possibly because cycloserine is unstable in strongly acidic or strongly alkaline media[254]. It is very soluble in water and is comparatively stable in alkaline solutions. Kuehl and his associates[255] have identified it to be D-4-amino-3-isoxazolidone (XXXV).



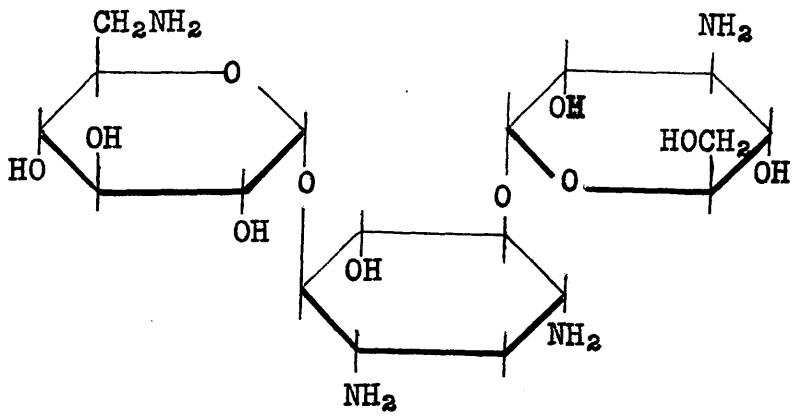
(XXXV)

Kanamycin

Kanamycin, an antimicrobial agent from Streptomyces kanamyceticus was isolated by Umezawa and associates[256] in 1957. It has been reported to be effective both in vitro and in vivo against Myco.tuberculosis and proved to be reasonably non-toxic in experimental animals. Preliminary clinical trials indicate that kanamycin exhibited a pattern of toxicity similar to that of streptomycin and its antituberculous activity upon streptomycin-susceptible and

-resistant tubercle bacilli has been demonstrated by Steenken and others[257]. On the other hand, the emergence of kanamycin-resistant strains is relatively much faster than with streptomycin.

Kanamycin is soluble in water but insoluble in the common alcohols. Its aqueous solution is stable at 100° for thirty minutes at pH 6 to 8. It is poorly absorbed following oral administration but is well absorbed by the intramuscular route[258]. On the basis of physico-chemical evidence, Cron and others[259] have assigned, to kanamycin, the structure (XXXVI) as that of a deoxystreptamine-bis--hexosaminide, thereby establishing a formal relationship with streptomycin.



(XXXVI)

D I S C U S S I O N

INTRODUCTION

Although the action of streptomycin has been studied with many different organisms, it is clear from the preceding introduction that the mechanism of its action against Mycobacterium tuberculosis is only incompletely understood. Little, too, is known of the relationship between the chemical structure of streptomycin and its biological activity, though the examination of modified structures against specified micro-organisms can provide useful points to their mechanism of action.

Considerable interest attaches to the function of the streptose aldehydic group since reaction with carbonyl reagents, such as hydroxylamine, phenylhydrazine and semicarbazide,[229] with cysteine, or oxidation to corresponding acid[228] leads to products which are inactive against Myco. tuberculosis. Streptomycin isonicotinylhydrazone[230] is an exception and has a significant level of tuberculostatic activity, but this is attributed to the isonicotinylhydrazine moiety of the molecule. On the other hand, the retention of full tuberculostatic activity by dihydrostreptomycin, in which the aldehyde group of streptomycin has been reduced to the corresponding primary alcohol conflicts with the evidence of the aldehyde group's importance for activity. However, despite reports to the contrary[209], Bailey and Cavallito[210], have shown that like streptomycin, dihydrostreptomycin is also inactivated by cysteine. In their view, dihydrostreptomycin is oxidised by

the micro-organisms to streptomycin and it would not be unreasonable, therefore, to suppose that dihydrostreptomycin depends upon such an oxidation for its activity. The related streptomycylamines[239] (XXXIII) also show a wide range of antibacterial activity, though, in general, it is lower than that of streptomycin, but nothing is known either of their mode of action or of their possible inactivation by such substances as cysteine.

Part of the present work has been concerned with the preparation of dihydrostreptomycin derivatives in which the primary alcoholic group (XXIb) is blocked to prevent its oxidation. Comparison of the tuberculostatic activity of such derivatives should provide a means of substantiating the conclusion of Bailey and Cavallito[210] and establishing unequivocally the importance of the streptose-aldehyde function as a factor contributing to the activity of the molecule as a whole. The choice of a suitable blocking group posed a number of problems. Ideally, this group should be, at least, moderately stable, and conversion of the particular hydroxyl group to an ether link appeared to offer the best possibility of success. Both streptomycin and dihydrostreptomycin are, however, polyhydroxy compounds and protection of the remaining hydroxyls by groups which could be readily inserted and equally readily removed was a prime requirement of any synthetic route. This, coupled with the known

instability to acid of the streptose-streptidine link in streptomycin and of the dihydrostreptose-streptidine link in dihydrostreptomycin, placed a severe limitation on the choice of test compound. A further limitation was also imposed by the known instability of streptomycin to alkali which readily degrades it to maltol[260] (XXVI). Dihydrostreptomycin is stable in this respect[178] but the guanidine groups are unstable to strong alkali, when strepturea dihydrostreptobiosaminide (XXVII) and ultimately streptamine dihydrostreptobiosaminide (XXVIII) are formed.

The preparation of N-acetyltrityldihydrostreptomycin (XXXVIIIa), therefore, represents a compromise which is recognised as departing from ideality in at least two respects. The trityl ether link is cleaved by aqueous acid[261] and probably has no greater stability than that of the dihydrostreptose--streptidine link while its stability to alkali permits the easy removal of all acetyl substituents except one (N-methyl--N-acetyl). The retention of this group in the final product follows from the restriction to mild de-acetylation conditions (ammonia-methanol at 0°C) imposed by the presence of guanidino groups.

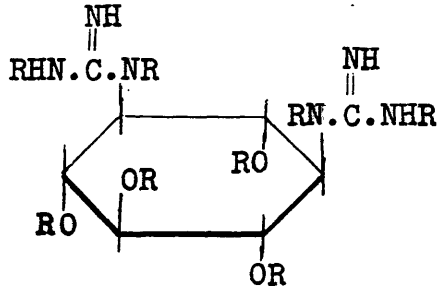
The disadvantage of a test compound in which the N-acetyl group is retained on the N-methyl-L-glucosamine fragment, is perhaps not so serious as would first appear. The addition of formaldehyde to streptomycin gives the N-hydroxymethyl derivative[237] (XXXII) which is claimed to

exhibit biological activity comparable with that of the parent compound. Furthermore, it is less liable to induce neurotoxicity. The choice of N-acetyltrityldihydrostreptomycin as the test compound, however, required that it should be compared with N-acetyl-dihydrostreptomycin and N-acetylstreptomycin.

N-Acetyltrityldihydrostreptomycin was prepared from streptomycin as outlined in the scheme on next page.

Acetylation of Dihydrostreptomycin and Streptomycin Sulphates.

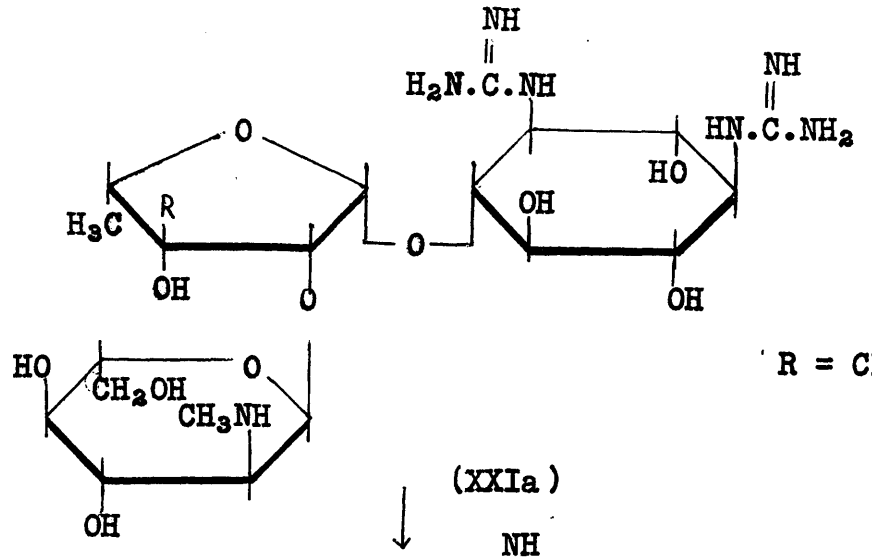
Acetylation was used to protect the hydroxyl groups in streptomycin prior to tritylation rather than benzoylation[262] since octabenzoylstreptidine (XXXIXa) yields a monobenzoylstreptidine when treated with sodium methoxide in pyridine--methanol solution and complete debenzoylation can only be accomplished by refluxing with 6N hydrochloric acid. The latter condition would cleave the glycosidic linkages and this method was, therefore, considered unsuitable. Octaacetylstreptidine (XXXIXb), on the other hand, had been



(XXXIX)

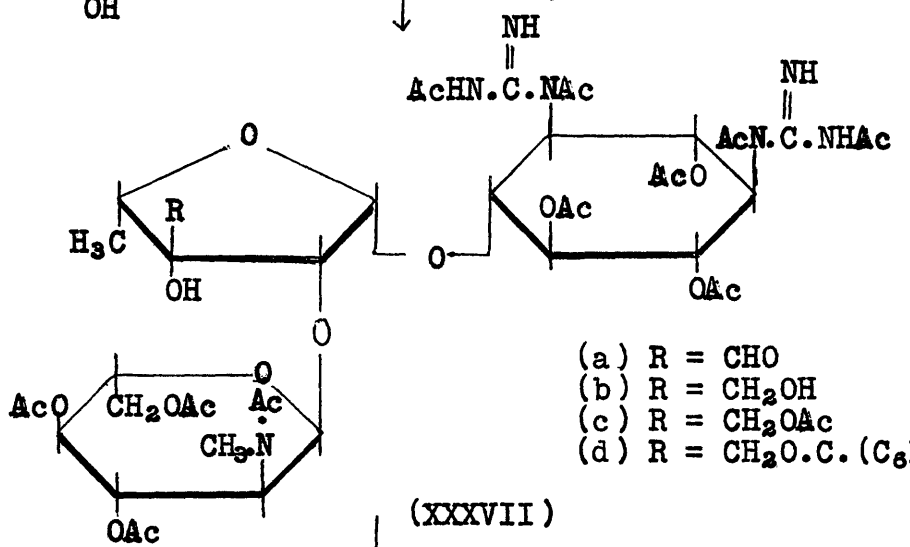
(a) R = $CO.C_6H_5$

(b) R = $CO.CH_3$



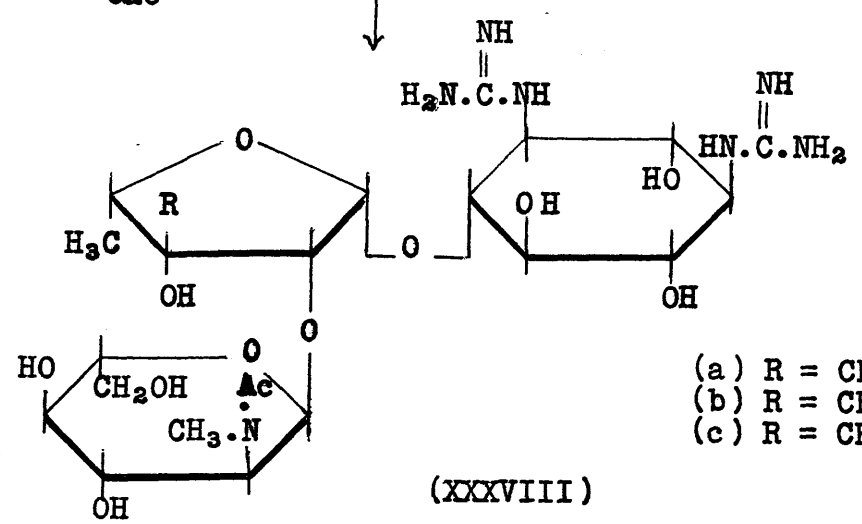
R = CHO

(XXIa)



(a) R = CHO
(b) R = CH_2OH
(c) R = CH_2OAc
(d) R = $CH_2O.C.(C_6H_5)_3$

(XXXVII)



(a) R = $CH_2O.C.(C_6H_5)_3$
(b) R = CH_2OH
(c) R = CHO

(XXXVIII)

deacetylated[262] completely at room temperature in methanolic ammonia. So, on these grounds, acetylation offered a more suitable alternative route.

Streptomycin and dihydrostreptomycin were available as sulphates and a preliminary investigation was carried out to see whether these salts could be acetylated directly to the corresponding undeca-acetyl and dodeca-acetyl derivatives. Dodeca-acetyldihydrostreptomycin (XXXVIIc) had previously been prepared only from the corresponding free base by two stage acetylation[222] with acetic anhydride-pyridine. Attempts to acetylate dihydrostreptomycin sulphate by this method were unsuccessful.

The free base was, therefore, prepared by deionization (de-acidite F.F.) of aqueous dihydrostreptomycin sulphate solution, isolated by freeze-drying and acetylated by Wolfrom's two stage procedure.[222] Later, the tedious deionization and freeze-drying procedure was found to be unnecessary and dihydrostreptomycin sulphate was acetylated directly in the presence of fused sodium acetate which in addition to being a base catalyst, helped in the removal of sulphate ions as sodium sulphate. A much better yield (75-80 per cent) of analytically pure dodeca-acetyl dihydrostreptomycin was obtained by repeated precipitation from benzene-petroleum ether (b.p. 80-100°) than the chromatographic purification[222] which gave a very low yield.

Streptomycin sulphate was also acetylated similarly to undeca-acetylstreptomycin (XXXVIIa) m.p. 168-170° (decomp.), $[\alpha]_D^{21}$ -64.5° (c,1.1, chloroform).

Hydrogenation of Undeca-acetylstreptomycin.

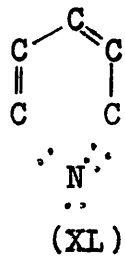
Attempts to hydrogenate undeca-acetylstreptomycin to undeca-acetyldihydrostreptomycin (XXXVIIb) at a platinum catalyst in methanol or ethanol failed completely, starting material being recovered in each case. However, the aldehyde group was slowly reduced when glacial acetic acid was used as the solvent, hydrogenation being complete in about eighteen hours at room temperature. Completion of the reaction was confirmed by testing the product with Fehling's solution which was not reduced on boiling.

The influence of solvent in the hydrogenation of bases has been explained by Maxted and Walker[263] and others[264].

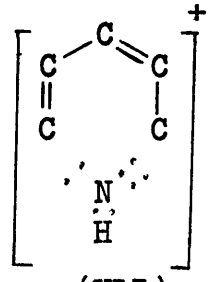
Nitrogenous aromatic bases undergo catalytic hydrogenation in solution over platinic oxide at a considerably lower rate than, for instance, benzene under similar conditions. Hydrogenation of these bases is facilitated by using either an acidic solvent or the hydrochloride instead of the free base.

Maxted and Walker[263] attributed this slow hydrogenation to a self-poisoning effect of pyridine (XL) and piperidine (XLII). They suggested on the basis of their experimental

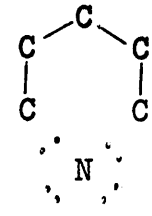
data that the free electron pair on the nitrogen atom of pyridine and piperidine leads to a strong adsorption by the metallic catalyst. This self-poisoning effect vanishes



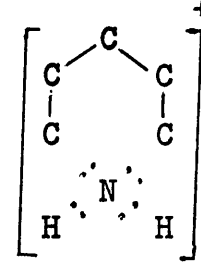
(XL)



(XLI)

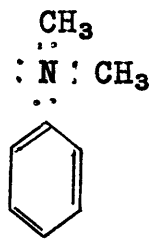


(XLII)

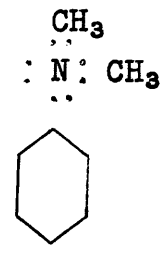


(XLIII)

with the addition of mineral acid, the free electron pair being shielded by a proton in the corresponding pyridinium (XLI) and piperidinium (XLIII) ions. Kinetic studies by Devereux, Payne and Peeling[264] have shown that "unshielded" nitrogen atoms in association with an aromatic ring as in pyridine (XL) and NN-dimethylaniline (XLIV), do not exert toxic effects and that these are only exhibited by saturated systems as in piperidine (XLII) and NN-dimethylcyclohexylamine (XLV). Thus catalyst poisoning observed with



(XLIV)

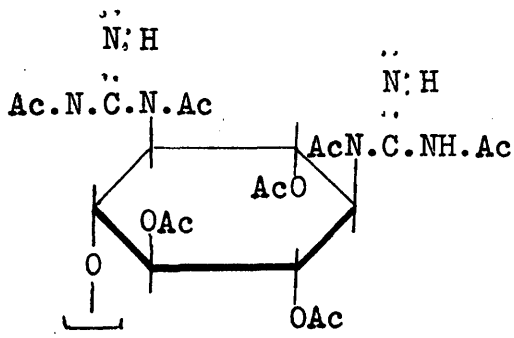


(XLV)

pyridine and other aromatic bases results from the formation

of saturated bases in the course of hydrogenation.

Undeca-acetylstreptomycin, like octa-acetylstreptidine (XXXIXb) which is capable of forming a dihydrobromide[262], acts as a base having "unshielded" nitrogen atoms in the guanidine groups adjacent to a saturated system (XXXVIIa).



(XXXVIIa)

Consequently the availability of nitrogen lone electron pairs in undeca-acetylstreptomycin for co-ordinate-bond formation with a platinum catalyst, could account for its failure to hydrogenate in methanol or ethanol and would explain the successful use of glacial acetic acid.

The resultant undeca-acetyldihydrostreptomycin was characterised by conversion to the known dodeca-acetyldihydrostreptomycin.

Tritylation of Undeca-acetyldihydrostreptomycin.

Although triphenylmethyl ethers may be synthesised either (a) from tritanol by reaction with an alcohol in the

presence of an acid catalyst[265] or (b) from triphenylmethyl chloride by reaction with an alkoxide[261] or (c) from triphenylmethyl chloride and an alcohol[266], the tritylation of carbohydrates is commonly carried out by the last method.

(a) $(C_6H_5)_3C.OH + ROH \rightleftharpoons (C_6H_5)_3C.OR + H_2O$

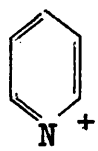
(b) $(C_6H_5)_3C.Cl + ROK \longrightarrow (C_6H_5)_3COR + KCl$

(c) $(C_6H_5)_3C.Cl + ROH \rightleftharpoons (C_6H_5)_3C.OR + HCl$

where R is an alkyl or aryl radical.

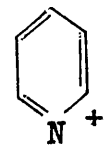
Many carbohydrates and in particular their acetylated derivatives are soluble in pyridine. Dry pyridine was, therefore, a most suitable solvent for the reaction[266] which is, consequently, applicable to acid sensitive substances such as glycosides. Moreover, pyridine helps in the removal of hydrogen chloride liberated in the reaction by formation of pyridine hydrochloride. The velocity of tritylation reaction is influenced by the hydroxyl containing component[267] and the temperature which may vary in the case of primary hydroxyl groups from several days at room temperature to a few hours at a higher temperature.[268] Hockett, Fletcher and Ames[269] have investigated the velocity of tritylation quantitatively. With four moles of triphenylmethyl chloride at room temperature, half tritylation was achieved after fifty eight hours and with eight moles of triphenylmethyl chloride, slightly better results were obtained.

Undeca-acetyldihydrostreptomycin was therefore, tritylated in pyridine at room temperature for eight days using eight moles of triphenylmethyl chloride. In preliminary experiments much lower yields were obtained when tritylation was carried out with four moles of triphenylmethyl chloride or for shorter times. The residue remaining after removal of pyridine in vacuo and washing with ether, was dissolved in chloroform. After keeping in the refrigerator for two hours, crystals of pyridinium triphenylmethyl chloride[270] (XLVI) (m.p. 172-173°) were separated.



$(C_6H_5)_3C.Cl^-$

(XLVI)



$H.Cl^-$

(XLVII)

At this stage pyridine hydrochloride (XLVII), which is sufficiently acidic to de-tritylate the product in polar solvents,[271] could not be washed off either with water or cold solution of sodium bicarbonate because the solubility of the trityl ether of undeca-acetyldihydrostreptomycin (XXXVIId) was found to be greater in water than in chloroform. The crude amorphous product was, therefore, obtained by precipitation from chloroform-ether and dried in vacuo.

The amorphous product was deacetylated[262,272] by dissolving in dry methanol previously boiled and cooled to remove dissolved carbon dioxide and immediately saturating the solution at 0° with ammonia. This method avoided the possibility of de-tritylation, since alcoholysis of trityl ether occurs very rapidly in polar solvent under acidic conditions[271]. After twenty four hours at room temperature, the solution was evaporated in vacuo and the residue was washed with ether to remove the decomposition products of pyridine hydrochloride and pyridinium triphenylmethyl chloride, such as pyridine, triphenyl carbinol and triphenylmethyl amine[270]. The residue was, then thoroughly triturated and washed with chloroform to remove acetamide[262]. The separation of ammonium chloride formed from pyridine hydrochloride during deacetylation, was affected by dissolving the chloroform-washed residue in NN-dimethyl formamide and filtering off the insoluble salt. The crude deacetylated product was recovered from NN-dimethyl formamide by precipitation in ether.

At this stage N-acetyltrityldihydrostreptomycin (XXXVIIIa) was likely to be contaminated with N-acetyldihydrostreptomycin (XXXVIIIb) and purification was achieved by chromatography in ethanol on a column of neutral alumina previously washed with methanol and ethanol. Elution with

ethanol, with ethanol-methanol and finally with methanol, was very slow and was followed spectrophotometrically by the observation of optical density of the eluates at 260 mμ. Elution with methanol-ethanol gave a series of fractions which showed varying optical density at 260 mμ. The eluates collected with 20 per cent methanol in ethanol gave a residue showing the most intense maximum at 259 mμ, and although this product could not be crystallised, it was found to be analytically pure. Ascending paper chromatography[273] using 2.5 per cent p-toluene-sulphonic acid in butanol saturated with water gave Rf values of 0.34 for N-acetyldihydrostreptomycin and 0.16 for its trityl ether. The ultraviolet absorption maximum of N-acetyltrityldihydrostreptomycin at 259 mμ had a molecular extinction coefficient of 974.7. This is in good agreement with the observed value 1010.3 obtained by summation of the maximum at 260 mμ of triphenylmethyl chloride ($\epsilon_{max.}$ = 770) and absorption at 259 mμ of N-acetyldihydrostreptomycin (ϵ = 240.3). Further confirmation of the product as N-acetyltrityldihydrostreptomycin was obtained from analysis of its picrate.

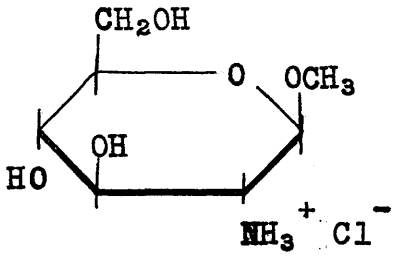
Deacetylation of Dodeca-acetyldihydrostreptomycin and Undeca-acetylstreptomycin.

A strict comparative study of the biological activity of N-acetyltrityldihydrostreptomycin necessitated the preparation of N-acetyldihydrostreptomycin (XXXVIIIb) and N-acetylstreptomycin (XXXVIIIc). The deacetylation of dodeca-acetyldihydrostreptomycin (XXXVIIc) and undeca-acetylstreptomycin (XXXVIIa) was accomplished in dry methanol at 0° saturated with dry ammonia[262,272]. After keeping at room temperature from 18 to 24 hours, the solution in both cases was evaporated to dryness in vacuo. The residue was washed with chloroform to remove acetamide yielding an amorphous product contaminated with a small amount of the carbonate[262]. The insoluble carbonate was filtered off from the methanol solution which afforded amorphous N-acetyldihydrostreptomycin as well as N-acetylstreptomycin on pouring into dry ether.

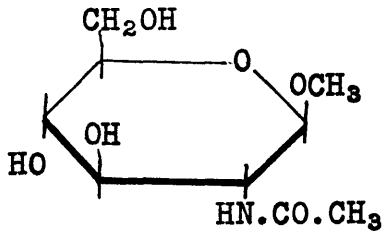
The N-acetyl derivatives are analogous to N-hydroxymethylstreptomycin[237] (XXXII), which has a biological activity comparable to streptomycin. Since the neurotoxicity was found to be significantly decreased in N-hydroxymethylstreptomycin, it would also be interesting to assess the toxic manifestations of N-acetyl derivatives.

The high resistance of the N-acetyl group to hydrolysis by methanolic ammonia is of interest. Other deacetylating agents such as methoxides of sodium[274], potassium and barium[275], and even stronger alkalis, such as, barium hydroxide and sodium hydroxide[276] have also been demonstrated to produce N-acetylglucosaminides from their fully acetylated derivatives. But recently Baker, Schaub and Kissman[277] have achieved N-deacetylation by refluxing N-acetyl nucleosides with 0.5 N barium hydroxide at 100°. Under these drastic alkaline conditions, N-acetylstreptomycin is, undoubtedly, degraded to yield streptamine and maltol[260].

The relative stability of the N-acetyl group to alkaline hydrolysis compared with the O-acetyl group, has also been observed with acidic reagents[278] when more vigorous conditions are necessary for N-deacetylation than O-deacetylation. Acidic hydrolysis of other O-alkylglycosidic derivatives of 2-acetamido-2-deoxy-D-glucose has also been investigated. Thus Moggridge and Neuberger[279] demonstrated that methyl β-D-glucosaminide (XLVIII) is hydrolysed much more slowly by acid than N-acetyl methyl β-D-glucosaminide (XLIX), in which the nitrogen atom carried no charge. Recently Foster, Horton and Stacey[280] have investigated the

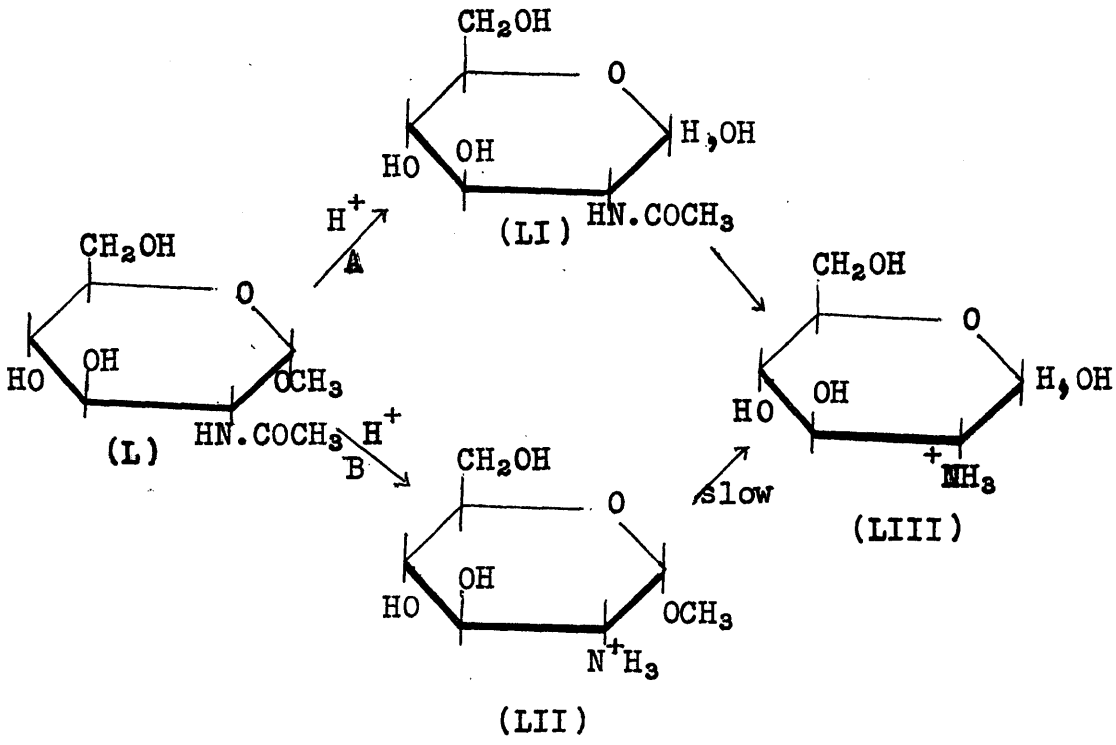


(XLVIII)



(XLIX)

acid hydrolysis of glycosidic derivatives of 2-amino-2--deoxy-glucose. They have demonstrated that incomplete glycosidic hydrolysis was due to the existence of two reaction pathways which are influenced by the glycosidic and the N-substituents. However, the acid hydrolysis of methyl 2-acetamido-2-deoxy-α-D-glucopyranoside (L) was found to occur by both pathways, A and B as the final hydrolysate, on chromatography, showed the absence of the initial glycoside (L) and the presence of components corresponding to 2-amino-2-deoxy-D-glucose (LIII) and methyl 2-amino-2-deoxy-α-D-glucopyranoside (LII) respectively. The slow hydrolysis of compound (LII) to compound (LIII) is influenced by the positive charge on the basic group and conforms to the general rule that α-glycopyranosides are more resistant to acid hydrolysis than their β-anomers.[286]



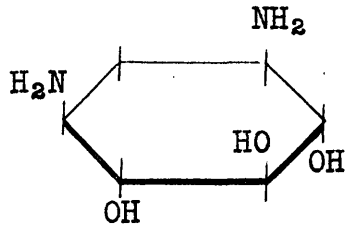
It is evident, therefore, that on these grounds alone, any attempt to remove the N-acetyl group in N-acetyl dihydrostreptomycin or N-acetylstreptomycin under either alkaline or acidic conditions, would in all probability result in cleavage of the α-L-glucosaminide linkage. Even greater instability of the β-L-strepto-biosaminide linkage under acid conditions and the instability of streptose and also the streptidine-guanidino groups to alkali have already been discussed.

Synthesis of Streptidine Glycosides.

Whilst the aldehyde group of streptose is, undoubtedly, important for biological activity, the probable role of the strongly basic guanidino groups has also been subjected to investigation. Abraham and Duthie[206] examined the influence of pH of the test media upon antibacterial activity of streptomycin and observed that the biological activity of streptomycin decreases with increase in the acidity of the medium. From this they concluded that the antibiotic in its cationic form, possibly, competes with hydrogen ions for the same sites on the bacterial cells. On the other hand, basic degradation products of streptomycin such as streptidine and streptobiosamine are reported to be biologically inactive either individually[282] or in admixture[283]. Streptobiosamine which comprises the basic N-methyl-L-glucosamine linked glycosidically to streptose and thus incorporates the important aldehyde group, is inactive and this may be attributed to the lack of the strongly basic streptidine units.

Stenlake[284] has suggested that the streptobiosamine fragment of streptomycin is merely responsible for concentrating and possibly orientating streptidine at the required site of action. This hypothesis is supported by the evidence of aminohexoses, possibly, D-glucosamine as constituent unit of

both the lipoid-bond and somatic polysaccharides of Mycobacteria tuberculosis[32,42]. This, too, is apparently underlined by the structures of the antituberculous antibiotics such as neomycin[242] and kanamycin[259] (XXXVI) which are derivatives of deoxystreptamine (LIV).



(LIV)

Wolfrom and Polglase[233] degraded dihydrostreptomycin trihydrochloride with barium hydroxide solution to streptamine dihydrostreptobiosaminide (XXVIII) which in its crude form contaminated with barium chloride, was said to be inactive biologically. Since the commencement of this work, a further communication on the same subject by Bodanszky[234] has become available[285] in which both strepturea dihydrostreptobiosaminide (XXVII) and streptamine dihydrostreptobiosaminide (XXVIII) are shown to be inactive. The same compounds have been prepared during the present work independently of Bodanszky by the controlled hydrolysis[224,286] of dihydrostreptomycin with barium hydroxide in a series of

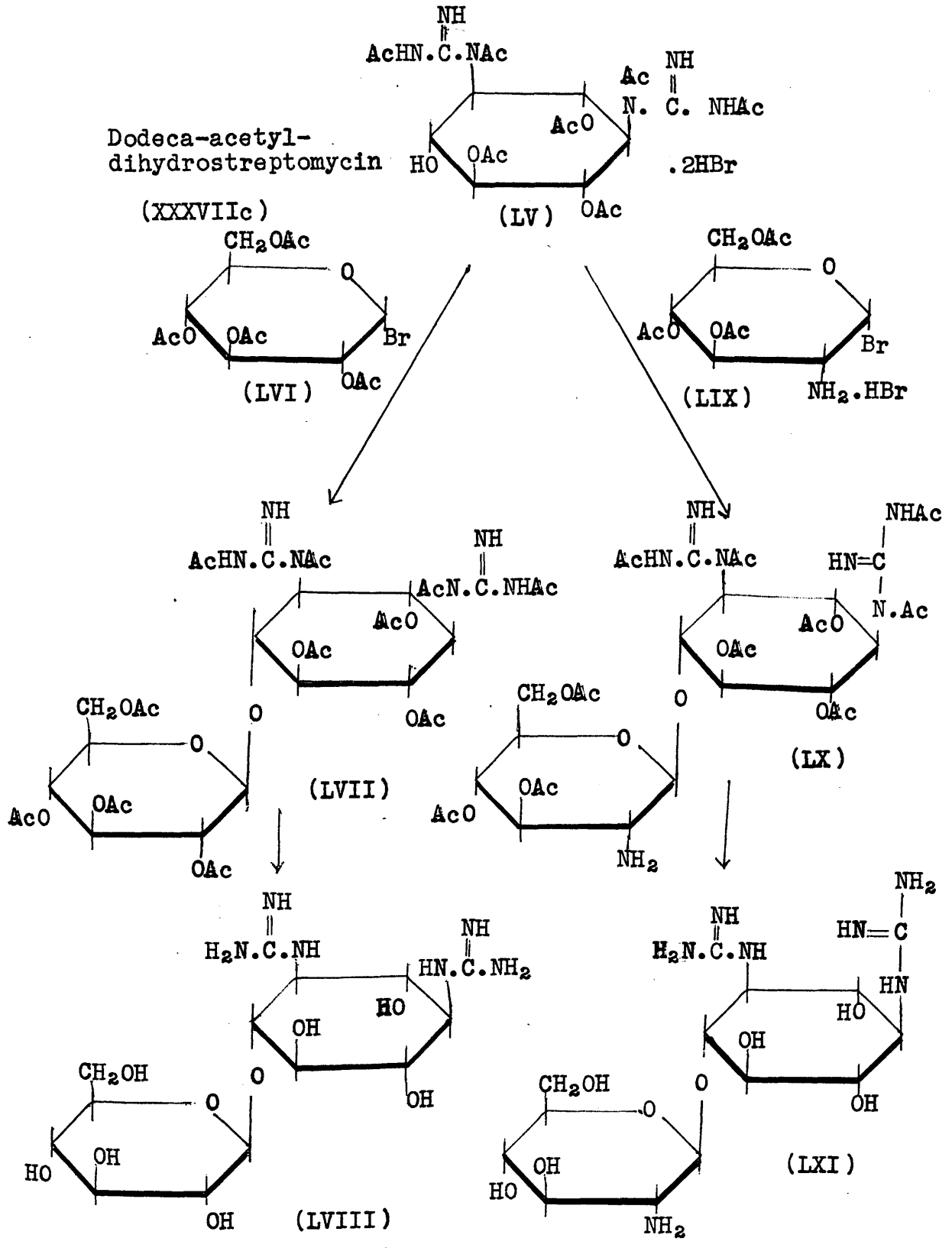
experiments to establish conditions for the degradation of streptidine glucosaminide (LXI).

The diamino- and diguanidino- phenyl glucosides (XXIX) and corresponding glucosaminides (XXX) have been synthesised[235] and found to be ineffective *in vitro* against *Myco. tuberculosis* but the respective streptidine and streptamine glycosides have not been examined.

During the present investigation, streptidine β-D--glucoside (LVIII) and streptidine β-D-glucosaminide (LXI) have been synthesised from hepta-acetylstreptidine dihydrobromide (LV) and O-acylglycopyranosyl bromides as outlined in the scheme on page 76.

Hepta-acetylstreptidine Dihydrobromide

Dodeca-acetyldihydrostreptomycin (XXXVIIc) was hydrolysed at about 0° with 31 per cent solution of hydrobromic acid in glacial acetic acid[287a]. Hepta-acetylstreptidine dihydrobromide (LV) was deposited slowly as a sticky residue. It was observed that if the solution was kept at room temperature, a very slight residue was deposited which did not increase even upon cooling. Attempts to isolate the hydrolysis products by chromatography with various solvents using charcoal, celite and mixtures of these, were without success.

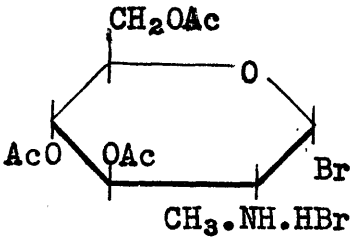

Dodeca-acetyl-
dihydrostreptomycin
(XXXVIIc)
(LV)
.2HBr
(LVI)
(LIX)
(LVII)
(LX)
(LVIII)
(LXI)

After decanting the chloroform solution, the residue was washed with absolute chloroform to remove unhydrolysed dodeca-acetyldihydrostreptomycin and then with ether to further effect removal of excess of acidic reagents. The residue so obtained was still found to be contaminated with the reactants. Consequently, the excess of acids were removed by repeated addition and evaporation of chloroform, and, finally by drying the residue *in vacuo* over potassium hydroxide. The decolourised methanolic solution of the dried residue afforded a white hygroscopic micro-crystalline precipitate when slowly stirred into dry ether. The precipitate was washed with dry ether and then with absolute chloroform to remove any trace of dodeca-acetyldihydrostreptomycin and finally with dry ether. The dried sample, m.p. 180-183° (decomp.) was found to be optically active and in this respect resembled heptabenzoyl-streptidine[262]. Hepta-acetylstreptidine dihydrobromide was confirmed by conversion to hepta-acetylstreptidine with silver oxide and also by acetylation in pyridine with acetic anhydride, to octa-acetylstreptidine[288] (XXXIXb). Hepta-acetylstreptidine dihydrobromide was also obtained from undeca-acetylstreptomycin (XXXVIIa) by the same method.

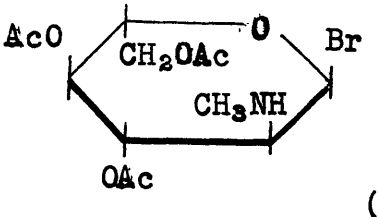
O-Acetylglycopyranosyl Bromides.

O-acetylglycosyl halides have been extensively employed in the synthesis of glycosides in preference to O-benzoylglycosyl halides because of the ease with which they can be prepared and deacetylated. The stability of O-acetylglycosyl halides decreases in the order, fluorides, chlorides, bromides and iodides. The fluorides can be stored for long periods without decomposition whereas iodides decompose even at 0°. The reactivity of O-acetylglycosyl halides, on the other hand, is in the reverse order to that of their stability. Since iodides decompose too easily and fluorides react with great difficulty, bromides and chlorides are most commonly employed.

Consequently tetra-O-acetyl-α-D-glucopyranosyl bromide[289] (LVI) (acetobromoglucose), tri-O-acetyl-2-amino-2-deoxy-α-D-glucopyranosyl bromide hydrobromide[290,291] (LIX) (acetobromoglucosamine hydrobromide), tri-O-acetyl-2-methylamino-2-deoxy--α-D-glucopyranosyl bromide hydrobromide (LXII) and tri-O--acetyl-2-methylamino-2-deoxy-α-L-glucopyranosyl bromide hydrobromide (LXIII) have been prepared as intermediates in the synthesis of streptidine glucosides.



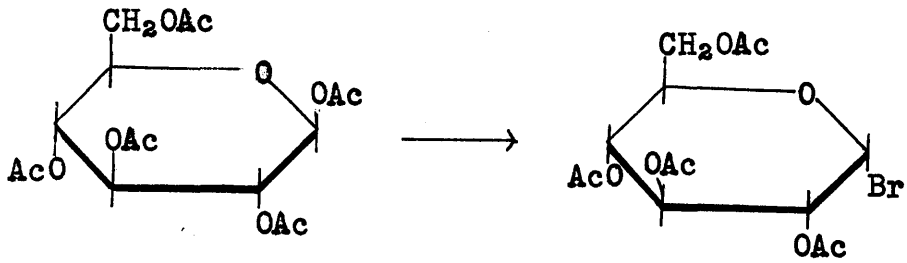
(LXII)



(LXIII)

In the cyclic form of aldose sugars, the hemiacetal hydroxy group on the anomeric carbon atom has a greatly enhanced reactivity over that exhibited by the primary and secondary alcoholic groups. This reactivity is attributed to spatial asymmetry and by the nature and relative positions of the groups on the neighbouring carbon atoms. By the proper choice of reagents, such groups as hydroxy, halogen, acyloxy, alkoxy, nitrate, phosphate and sulphate may be substituted for each other on the reducing carbon of acetylated sugars. In the preparation of aceto-bromoglucose[289], therefore, the acetoxy group on the reducing carbon of penta-O-acetyl-β-D-glucopyranose[287b] (LXIV) was replaced by bromine using hydrobromic acid in glacial acetic acid[287a], the substitution being accompanied by a Walden inversion.

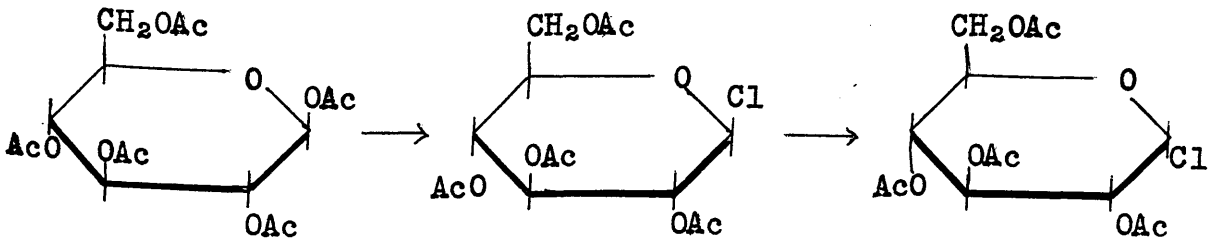
The prolonged action of hydrogen bromide on penta-O-acetyl β-D-glucopyranose has been reported to produce dibromide by replacement of acetoxy groups at $C_{(1)}$ and $C_{(6)}$,



(LXIV) (LVI)

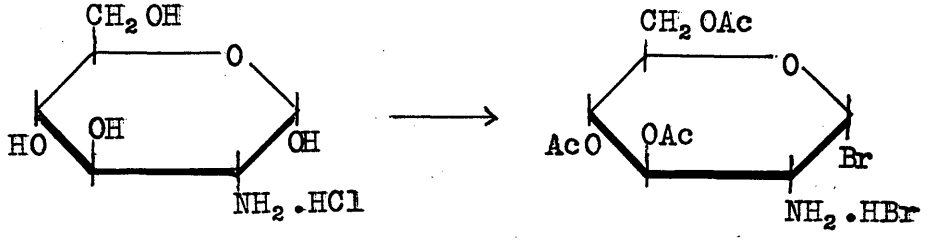
but this can be largely avoided by careful control of the reaction time[292].

Other reagents have also been employed for the preparation of β-D-acetylglycosyl halides. The reaction of liquid hydrogen fluoride with acetylated sugars yields O-acetyl-glycosyl fluorides[293], and phosphorus pentachloride and aluminium chloride[294] in chloroform are suitable for preparation of the corresponding chlorides. Lemieux and Brice[295] have shown that penta-O-acetyl-β-D-glucopyranose reacts rapidly with titanium tetrachloride to form tetra-O-acetyl-glucopyranosyl chloride (LXV) which rearranges to its α-anomer (LXVI).



(LXIV) (LXV) (LXVI)

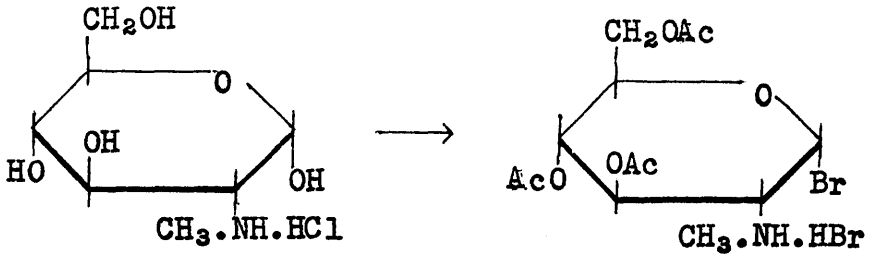
The use of acetyl chloride[296] for the preparation of O-acylglycosyl halide has not gained importance because of the difficulty in controlling the reaction. However, acetyl bromide has been employed successfully for the preparation of tri-O-acetyl-2-amino-2-deoxy-α-D-glucopyranosyl bromide hydrobromide (LIX) since it permits acetylation without N-acetylation. Irvine, McNicoll and Hynd[290] treated D-glucosamine hydrochloride (LXVII) with acetyl bromide at 60° for thirty minutes whereas Fodor and Otvos[291] conducted the same reaction at 25-30° for three days, obtaining thereby analytically pure acetobromoglucosamine hydrobromide (LIX).



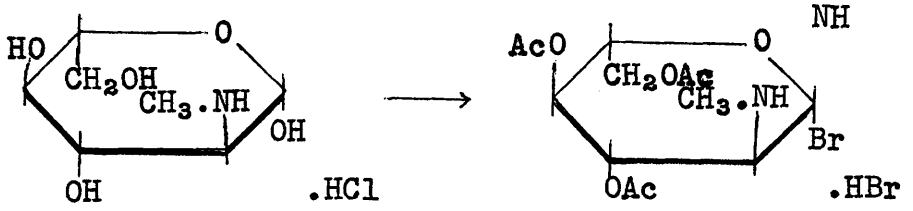
(LXVII) (LIX)

It has been observed in the present work that much less decomposition of the product occurs by following the procedure of Fodor and Otvos[291] and that old samples of acetyl bromide reacted vigorously initially but ultimately caused extensive decomposition of the product. This is, possibly, due to the presence of traces of water in the acetyl bromide owing to its hygroscopic nature.

The method of Fodor and Otvos was adopted for the preparation of tri-O-acetyl-2-amino-2-deoxy-α-D-glucopyranosyl bromide hydrobromide (LIX), tri-O-acetyl-2-methylamino-2--deoxy-α-D-glucopyranosyl bromide hydrobromide (LXII) and tri-O-acetyl-2-methylamino-2-deoxy-α-L-glucopyranosyl bromide hydrobromide (LXIII) from D-glucosamine hydrochloride (LXVII), α-D-N-methylglucosamine hydrochloride (LXVIII) and α-L-N-methylglucosamine hydrochloride (LXIX) respectively.



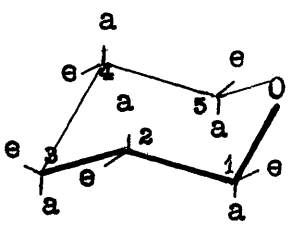
(LXVIII) (LXII)



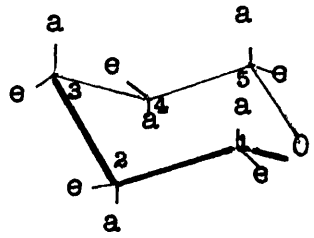
(LXIX) (LXIII)

In the formation of O-acetylglycosyl halides, two anomers are possible. Most of the stable O-acetylglycosyl halides in the aldohexoses have the α-D- configuration while with many aldopentoses, the stable forms have the β-D- configuration. Based on conformational studies, Hassel and Ottar[297] have advanced a theory to explain the greater

stability of one of the two anomeric forms of O-acetyl-D--glucopyranosyl halides. Theoretically, the pyranose ring is capable of eight strainless ring conformations, six boat and two staggered or chair forms. On the premise that the boat form of cyclohexane is considerably less stable than the chair form, the boat forms can be neglected and one of the two chair forms (LXX, LXXI) will be favoured.



(LXX)



(LXXI)

In the O-acetylglycopyranosyl halides, the anomers in which the relative position of the halogen (X) on $C_{(1)}$ and the larger group ($-CH_2OH$) on $C_{(5)}$ can be represented in the two chair forms by

$$-X_{\underline{a}},\ -CH_2OAc_{\underline{e}} \rightleftharpoons -X_{\underline{e}},\ -CH_2OAc_{\underline{a}}$$

is more stable than the anomer which can be represented by

$$-X_{\underline{e}},\ -CH_2OAc_{\underline{e}} \rightleftharpoons -X_{\underline{a}},\ -CH_2OAc_{\underline{a}}$$

where e and a refer to the equatorial or axial positions of the substituents.

Thus O-acetylglycosyl halides of the hexoses show a strong preference for the α-1, 5-trans configuration. This

behaviour which governs the stability of the anomeric halides is known as Hassel-Ottar effect[298]. The stability is, thus, correlated with the conformations[299] of α-D- and α-L-O-acetylglucopyranosyl bromides.

As pointed out earlier (page 78), O-acetylglucopyranosyl bromides are less stable than the corresponding chlorides and fluorides. Likewise, acetobromoglucosamine hydrobromide has been reported to decompose slowly at room temperature[235]. Consequently all the O-acetylglucopyranosyl bromides prepared during the present work were used in the synthesis of glycosides as soon as possible after their preparation.

Condensation of Hepta-acetylstreptidine Dihydrobromide and O-acetylglycopyranosyl Bromides.

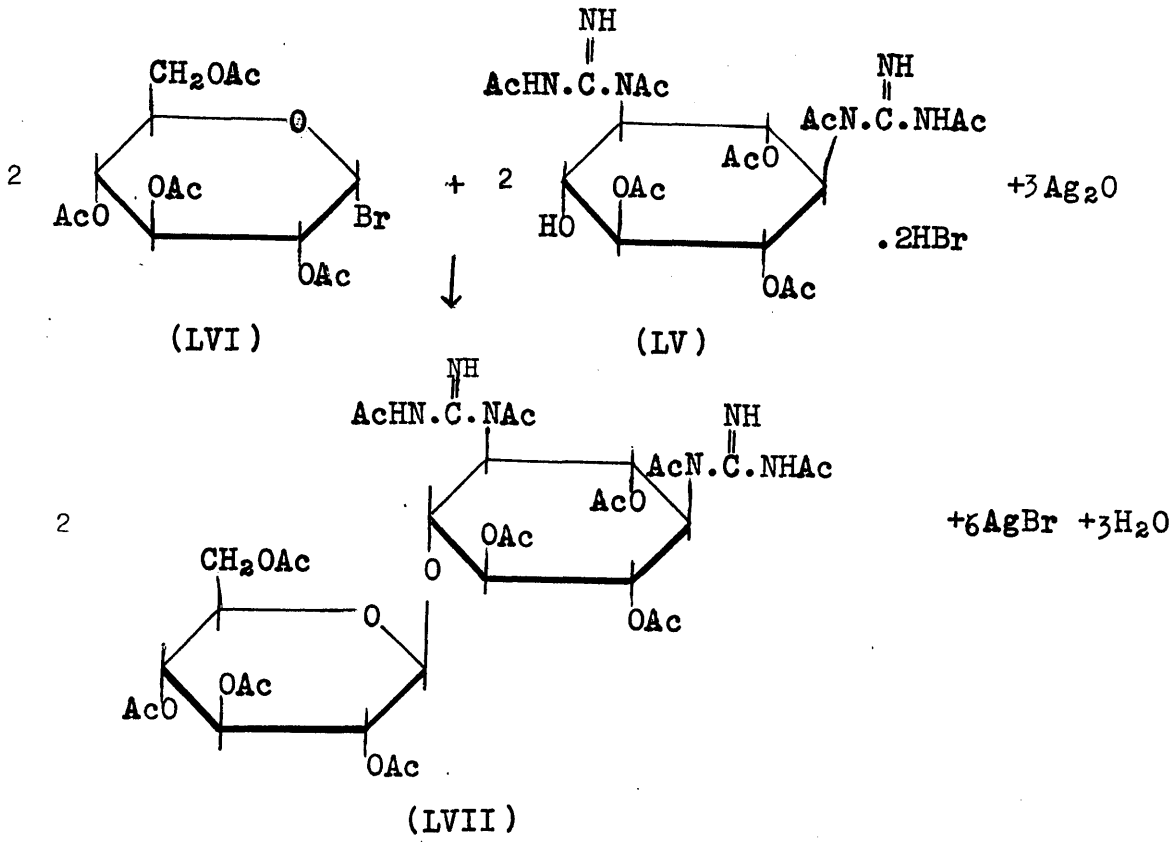
The Koenigs-Knorr reaction[300] which is widely used for the synthesis of glycosides, consists of the interaction of a substance containing a free hydroxyl group with an acetylglycosyl halide in the presence of an "acid acceptor" to speed up the reaction and prevent the deacetylation of the product.

Normally under the conditions of the Koenigs-Knorr reaction when an O-acetylglycosyl halide reacts with a free hydroxyl group, Walden inversion occurs. Since the

O-acetylglycopyranosyl halides used during the present work belonged to the alpha-series, the linkages formed in the glycosides prepared by the Koenigs-Knorr reaction using silver salts were, therefore considered to be of the beta-configuration.

Hepta-acetylstreptidine dihydrobromide and the respective O-acetylglycopyranosyl bromide in equimolecular proportions were dissolved in NN-dimethyl-formamide. After the addition of each substance to the solvent, the flask was evacuated to ensure the removal of traces of moisture from the reaction mixture. Silver oxide and quinoline were then added simultaneously and the water liberated during the reaction was removed under continuous high vacuum along with NN-dimethylformamide. When necessary, more NN-dimethyl formamide was added to keep the reactants in solution during the reaction. The general reaction may be represented as on page 86.

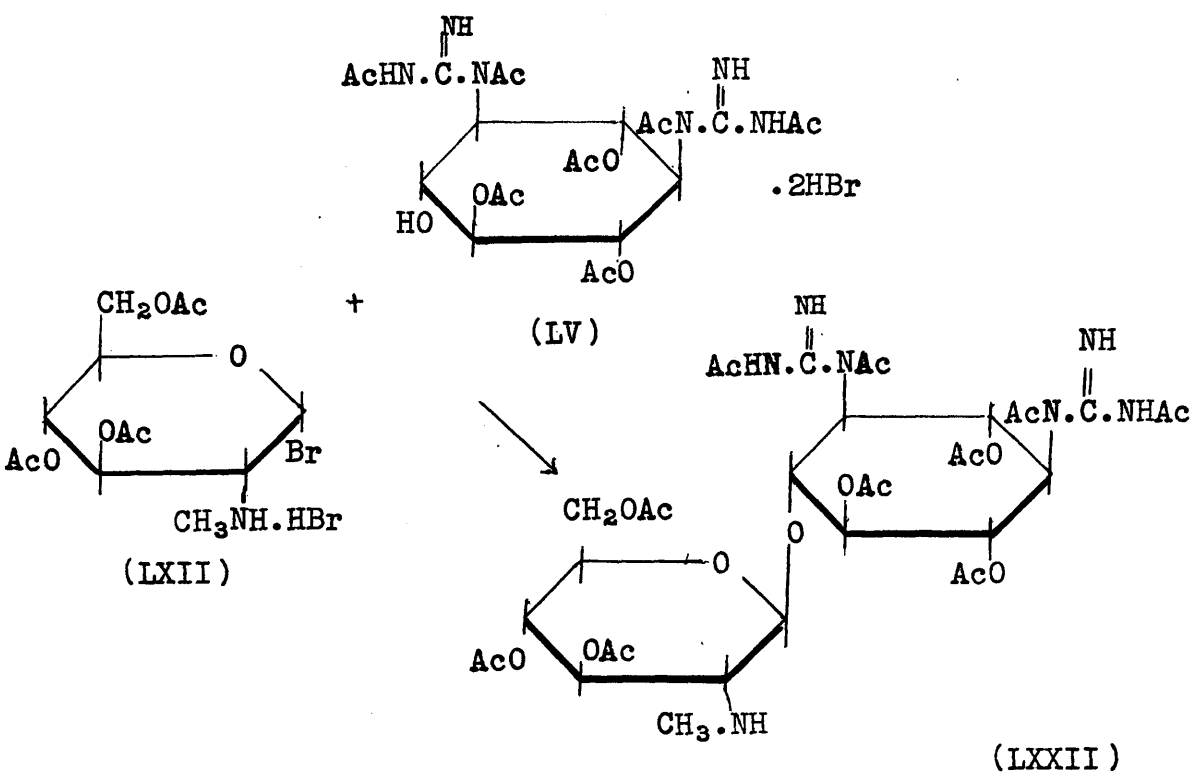
After the reaction was completed, the excess of NN-dimethylformamide was distilled off in high vacuum. The semi-solid mass was triturated and washed with dry ether to remove unreacted O-acetylglycopyranosyl bromide and quinoline, the latter having been added to act as a catalyst during the course of the reaction. The residue was, then, extracted

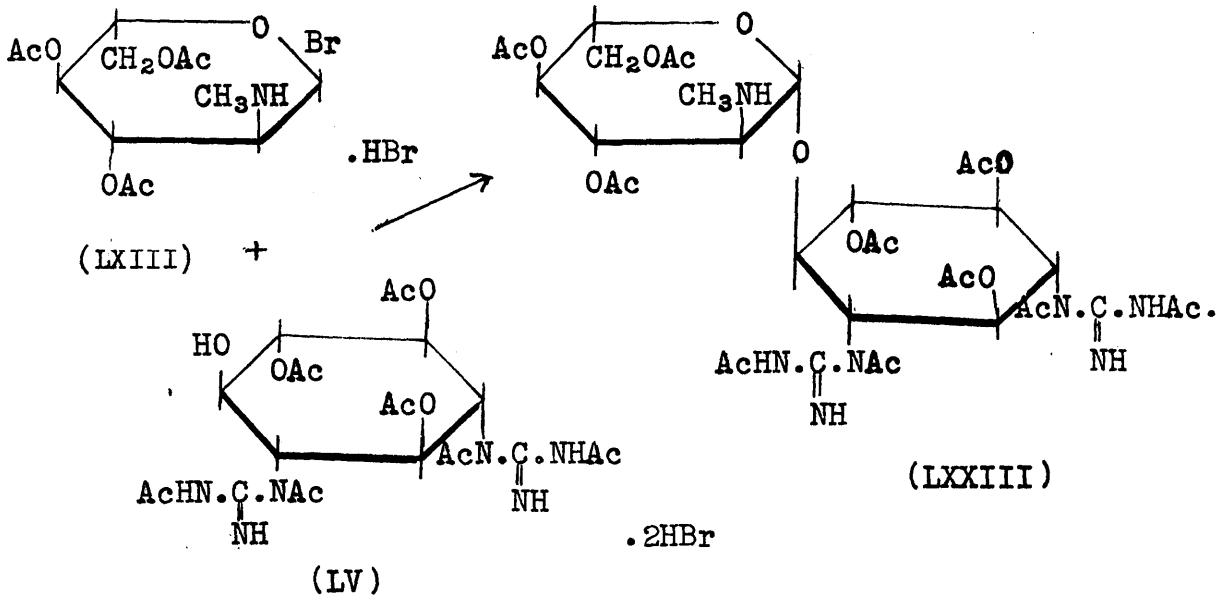


with absolute chloroform which dissolved the β-glycoside only since hepta-acetylstreptidine was found to be insoluble in absolute chloroform. The chloroform extract in each case was dark red in colour and seemed to contain some colloidal silver. The silver salts were removed as silver sulphide by passing hydrogen sulphide into a cold chloroform solution of the glycoside. Further purification by chromatographic adsorption was attempted but without success. The chloroform extract was redissolved in acetone and decolourised with charcoal. The acetone

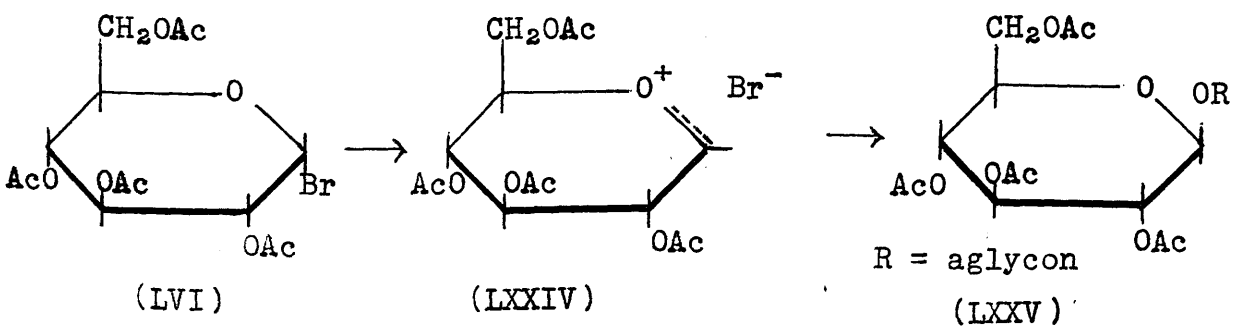
solution on pouring into ether or petroleum ether (b.p. 40-60°) afforded either an amorphous or in some cases microcrystalline precipitate.

By following the general series of reactions four glycosides were prepared from hepta-acetylstreptidine dihydrobromide (LV), namely undeca-acetylstreptidine β-D--glucopyranoside (LVII), deca-acetylstreptidine 2-amino-2--deoxy-β-D-glucopyranoside (LX), deca-acetylstreptidine 2-methylamino-2-deoxy-β-D-glucopyranoside (LXXII) and deca-acetylstreptidine 2-methylamino-2-deoxy-β-L-glucopyranoside (LXXIII).



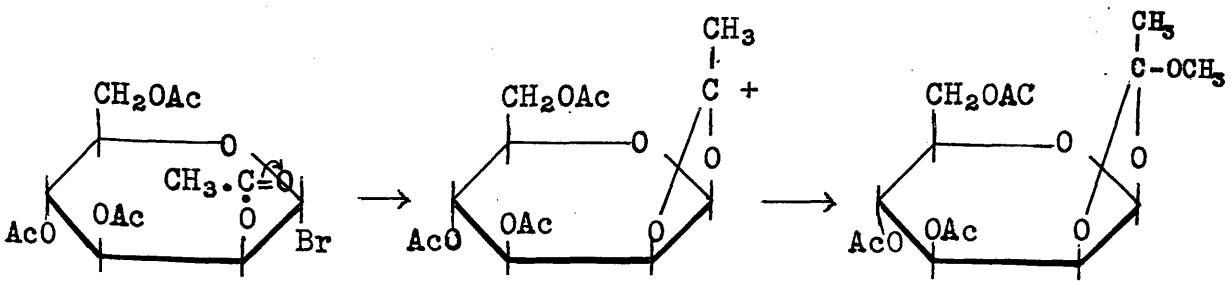


The mechanism of formation of glycosides by the Koenigs-Knorr reaction is based on the general theory of displacement reactions as suggested by Frush and Isbell[301]. According to this mechanism, the ion or molecule replacing the halogen must approach the carbon atom from the opposite side to that occupied by the halogen in the presence of excess of silver carbonate or silver oxide.



This substitution follows first order kinetics accompanied by a Walden inversion yielding the acetylglycoside (LXXV) of configuration opposite to that of the parent halide. The ionization of halide to form a cation (LXXIV) is considered to be the rate controlling step[302,303]. The cation in turn combines with the hydroxyl group of the aglycon as rapidly as it is formed.

The substitution of halogen at $C_{(1)}$ of the O-acetylglycosyl halide is influenced by neighbouring groups i.e. at $C_{(2)}$. According to Pacsu[304], and Frush and Isbell[301], in O-acetylglycosyl halides having the halogen at $C_{(1)}$ *trans* to the acetoxy group at $C_{(2)}$ (tetra-O-acetyl-α-D-manno-pyranosyl bromide (LXXVI)), the O-acetyl group may attack the back face of $C_{(1)}$ as the halogen recedes, thus forming an orthoester carbonium ion (LXXVII). Under slightly basic conditions, the alkoxy radical then attacks this carbonium ion to form the stable orthoester (LXXVIII).



(LXXVI) (LXXVII) (LXXVIII)

Besides the alkyl orthoacetate (LXXVIII), a small amount of alkyl β-D-glucopyranoside may be formed from the *trans*-isomer without participation of the 2-*O*-acetyl group.

In an *O*-acetylglycosyl halide having the halogen *cis* to the neighbouring acetyl group, for instance, acetobromoglucose (LVI), steric conditions make reaction between $C_{(1)}$ and the neighbouring acetyl group impossible and no orthoester is formed. The halogen is, therefore, replaced readily by nucleophilic group from the environment accompanied by Walden inversion.

The Koenigs-Knorr reaction is normally carried out in a solvent which will dissolve the acetylglycosyl halide and the substance carrying the free hydroxyl group. The solvents most commonly used are chloroform, benzene, carbon tetrachloride, ether, dioxane and xylene. During the present work, difficulty was experienced in the choice of the proper solvent. None of the above-mentioned solvents gave any glycoside and the starting material was recovered in each case. This may be attributed to the insolubility of hepta-acetylstreptidine in these solvents. Consequently, NN-dimethyl-formamide was selected and found to be a good solvent for the reactants as well as for the final product and also inert under the experimental conditions.

The yield of glycoside is affected not only by the nature of the solvent but also by the type of condensing agent, its amount and the time of contact. Helferich and Wedemeyer[305] prepared a number of glycosides by using the more readily available catalysts or condensing agents such as zinc oxide, cadmium oxide, mercuric oxide, zinc acetate, mercuric cyanide and mercuric bromide under various conditions. They suggested that the most effective catalyst was mercuric cyanide which yielded β-glycoside, though certain other mercuric salts such as mercuric acetate have been reported to give α-glycosides[306] also. During the present work, however, attempts to prepare the glucoside using mercuric cyanide were unsuccessful.

Although Koenigs and Knorr[300] and Ness, Fletcher and Hudson[307] have carried out the reaction for the preparation of simple glycosides without the use of condensing agent, these cases are exceptions to the general rule. Normally, a catalyst or condensing agent is required to act as an acid acceptor and accordingly silver carbonate and silver nitrate were first used by Koenigs and Knorr[300]. Silver carbonate and silver oxide are still extensively used in the synthesis of β-glycosides. Pyridine[308] alone is unsuitable since it reacts with acetobromoglucose yielding tetra-O-acetyl-glucopyranosyl pyridinium bromide. Using

quinoline as the condensing agent, however, Helferich and Bredereck[309] isolated a small yield of α-glycoside. Likewise, it was observed that when hepta-acetylstreptidine dihydrobromide was treated with acetobromoglucose in the presence of quinoline alone, a partially deacetylated product, tetra-O-acetylstreptidine α-D-glucoside was obtained, the α-configuration being given on the basis of optical rotation. Since the yield was very small, this procedure was not employed for the preparation of other glycosides.

Later on, using silver oxide and quinoline together as condensing agents[310], streptidine β-glucoside was prepared in very small yield. This low yield could be attributed to the presence of the byproduct, water, itself a hydroxylic compound, in the reaction mixture.[311] This may, then, react preferentially with the O-acetylglycosyl halide to give a third hydroxy compound (tetra-O-acetyl-D-glucopyranose) which will then react with more of the halide.

The amount of the product lost in these side reactions can be cut down by decreasing the active water content of the reaction to a minimum with various internal desiccants such as anhydrous sodium sulphate[312], calcium chloride,[313] anhydrous magnesium perchlorate,[314] and finely divided calcium sulphate hemihydrate (Drierite)[315]. The properties of drierite such as inertness to nearly all materials except water and

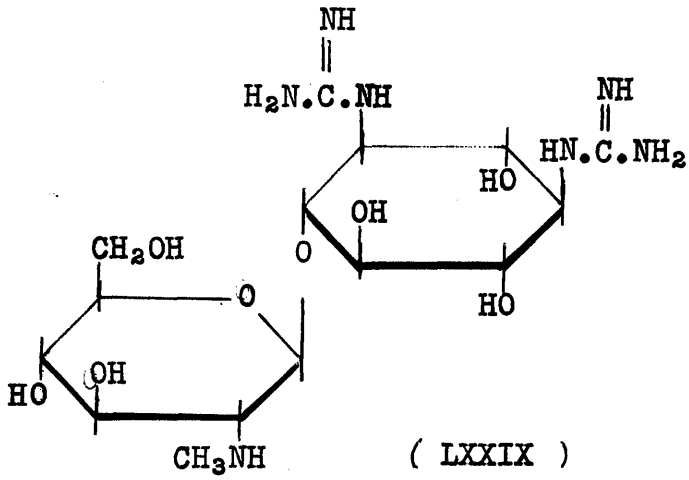
insolubility in all the common solvents, make it an ideal internal desiccant for use at normal or moderately elevated temperatures. Though the yields of glycosides were improved in many instances[315], it did not give encouraging results in the present study since the reaction with water could not be entirely eliminated[311]; secondly, the velocity of condensation was possibily diminished.[316]

The use of an entraining agent has, also, been suggested for the removal of water from the reaction mixture[317], the reaction being conducted at elevated temperature or under vacuum. After a series of experiments, it was found that the water formed during the reaction could best be removed by carrying out the reaction under high vacuum. This method has, also, proved useful in the preparation of the remaining glycosides.

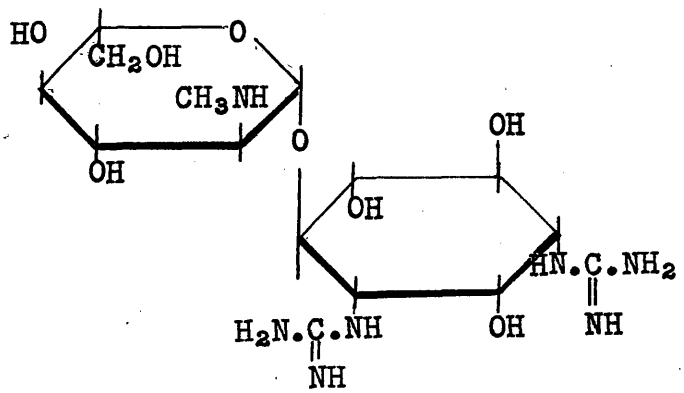
Deacetylation of Acetylstreptidine β-Glycosides.

Deacetylation of acetylstreptidine β-glycosides was carried out in dry methanol at 0° saturated with dry ammonia[262,272] as explained earlier (page 69) for dodeca-acetyldihydrostreptomycin and undeca-acetylstreptomycin. Undeca-acetylstreptidine β-D-glucopyranoside (LVII), deca-acetylstreptidine 2-amino-2-deoxy-β-D-glucopyranoside (LX), deca-acetylstreptidine 2-methylamino-2-deoxy-β-D-glucopyranoside (LXXII) and deca-acetylstreptidine 2-methylamino-2-deoxy-β-L-glucopyranoside (LXXIII) were deacetylated in this way to streptidine β-D-

-glucopyranoside (LVIII), streptidine 2-amino-2-deoxy-β-D--glucopyranoside (LXI), streptidine 2-methylamino-2-deoxy-β--D-glucopyranoside (LXXIX) and streptidine 2-methylamino-2--deoxy-β-L-glucopyranoside (LXXX). These bases were purified by conversion to the hydrochloride or sulphate and further characterised by preparing the respective picrate or helianthate.



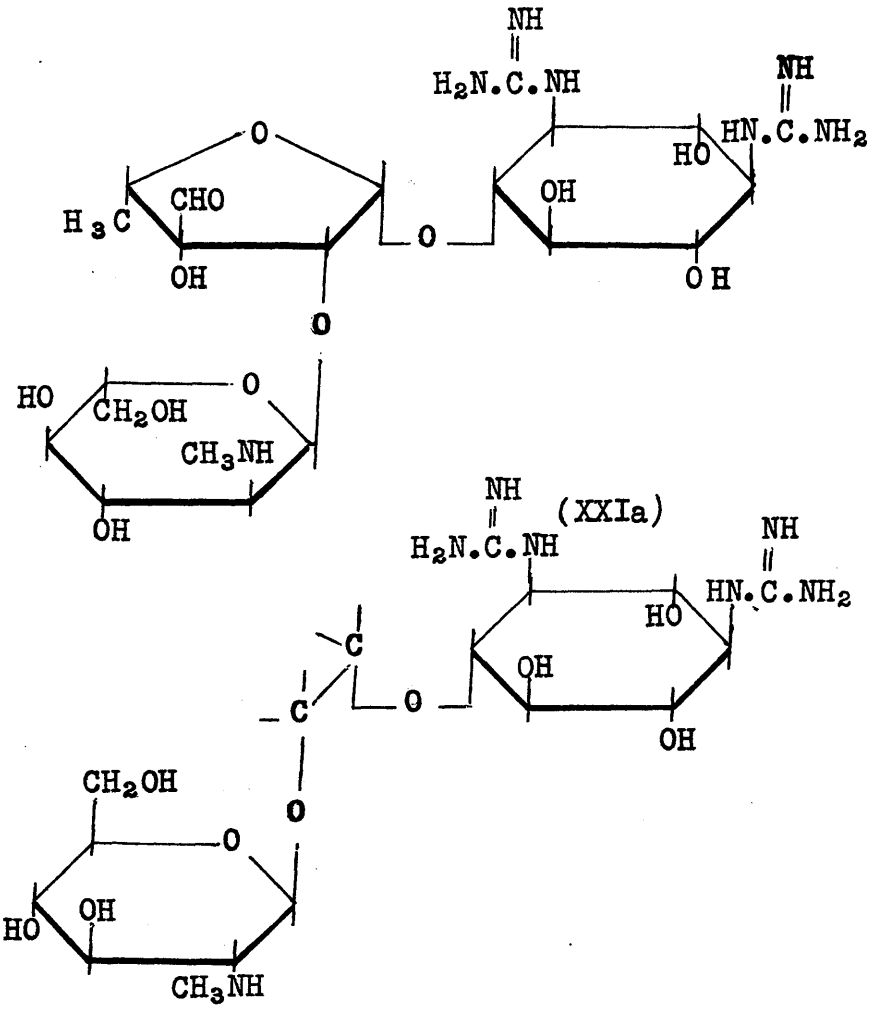
(LXXIX)



(LXXX)

Synthesis of Streptidine-oxyethyl β-D-Glucopyranoside.

The preparation of streptidine oxyethyl β-D-glucopyranoside was undertaken as a model compound for the preparation of streptidine-oxyethyl 2-N-methylamino-2-deoxy-β-D-glucopyranoside (LXXXI) which bears a structural analogy to the streptomycin molecule (XXIa) as shown below. Two basic units of streptomycin



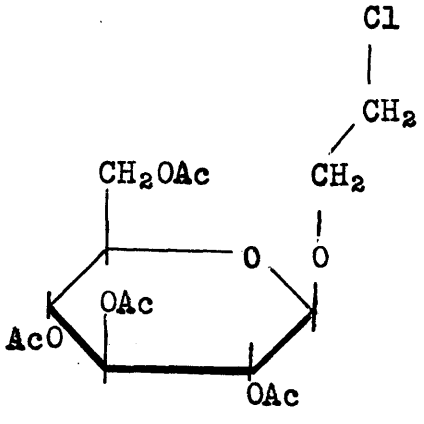
(LXXXI)

molecule i.e. streptidine and N-methyl-glucosamine fragments could, therefore, be separated by an ethoxy group which apart from the extra-cyclic carbonyl group essentially represents the streptose fragment as far as the linkages of the two basic fragments are concerned. Thus streptidine oxyethyl 2-N-methylamino-2-deoxy-β-D-glucopyranoside may possess the characteristic shape of the streptomycin molecule essential for the biological activity of the antibiotic.

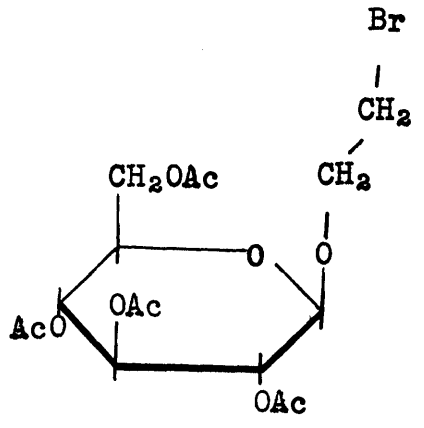
For the preparation of streptidine-oxyethyl β-D-glucopyranoside, streptidine and glucose fragments of streptidine β-D-glucoside (LVIII) were required to be separated by an ethoxy group ($-O-CH_2-CH_2-$). This could be achieved either by linking the β-hydroxy-ethyl group ($HO-CH_2-CH_2-$) to the free hydroxyl group of hepta-acetylstreptidine dihydrobromide (LV) or by replacing the halogen of acetobromoglucose at $C_{(1)}$ with β-hydroxy-ethoxy group ($HO-CH_2-CH_2-O-$) and thus condensing the two units. One approach to the former route might lie through the reaction of ethylene oxide with hepta-acetylstreptidine under acidic conditions[318], but this would, undoubtedly, lead to the deacetylation of the latter. The alternative route which seemed more feasible was, therefore, followed.

In a preliminary investigation, ethylene chlorhydrin and ethylene bromhydrin were condensed with acetobromoglucose to form tetra-O-acetyl-(2-chloroethyl) β-D-glucopyranoside [319]

(LXXXII) and tetra-O-acetyl-(2-bromoethyl) β-D-glucopyranoside[319] (LXXXIII) respectively. Neither of these substances gave



(LXXXII)



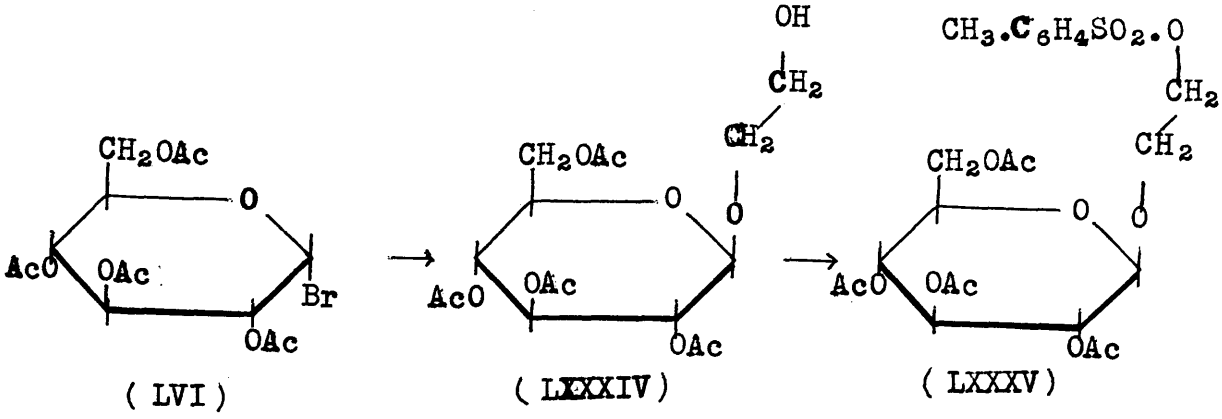
(LXXXIII)

any condensed product in reaction with a model compound, cyclohexanol. This failure is attributed to the lower reactivity of the alkyl halide halogen compared with the halogen at the reducing carbon of O-acetylglycosyl halides.

A search of the literature at this juncture revealed that toxyloxy group could be replaced by methoxy[320] and benzylphenoxy[321] groups using sodium hydride and other condensing agents. The reactivity of tosyl esters has been studies in detail by Tipson[322] and found to be in the order: ethanol> β-ethoxyethanol> cyclohexanol. This is further supported by the observation of Wiggins and Wood[323] that in general a tosyl group attached to a primary hydroxyl group

reacts more readily than when attached to a secondary hydroxyl group. It is evident, therefore, that the tosyl ester of tetra-O-acetyl-(2-hydroxyethyl) β-D-glucopyranoside (LXXXIV) will be more reactive than the corresponding ester of hepta-acetylstreptidine.

For the preparation of the tosyl ester, acetobromoglucose (LVI) was condensed with ethylene glycol by the procedure of Fischer and Fischer[324] to yield tetra-O-acetyl-(2-hydroxyethyl) β-D-glucopyranoside (LXXXIV). This was further treated with dry p-toluenesulphonyl chloride in dry pyridine, the latter serving both as solvent and also to neutralize the hydrochloric acid formed. After reaction at 0° for two hours, the excess of p-toluenesulphonyl chloride was hydrolysed slowly with cold water and subsequently removed along with other by-products by adding more cold water. The product, tetra-O-acetyl-(2-tosyloxyethyl) β-D--glucopyranoside (LXXXV), after purification was crystallised from ether as colourless needles, m.p. 109-110°, $[\alpha]_D^{18}$ - 13.1°.



(LVI) (LXXXIV) (LXXXV)

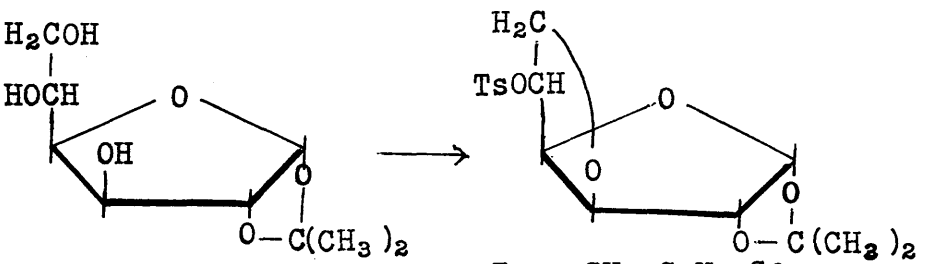
The reaction of the tosyl ester (LXXXV) with hepta-acetylstreptidine dihydrobromide in the presence of sodium hydride[321] seemed to be unworkable since alkaline conditions would in all probability deacetylate the reactants, whilst the reaction conditions used by Heyl, Herr and Centolella[320] were found to be too mild to start the reaction.

However, it has been observed that during the preparation of sulphonic esters in the presence of pyridine, a number of side reactions occur including ether formation[326] (LXXXVI). Such reactions are favoured only under rather drastic or prolonged conditions. Likewise, intramolecular

$$CH_3.C_6H_4.SO_2.OR + R'OH \longrightarrow ROR' + CH_3.C_6H_4SO_3H$$

(LXXXVI)

formation of a cyclic ether ("dehydration") has also been shown to occur when 1,2-O-isopropylidene-D-glucofuranose (LXXXVII) was treated with excess of p-toluenesulphonyl chloride in boiling pyridine-chloroform for eight hours. The product isolated was 3,6-anhydro-1,2-O-isopropylidene-5--O-tosyl-D-glucose (LXXXVIII) instead of 3,5,6-tri-O-tosyl derivative which was obtained at 40°[327, 328].

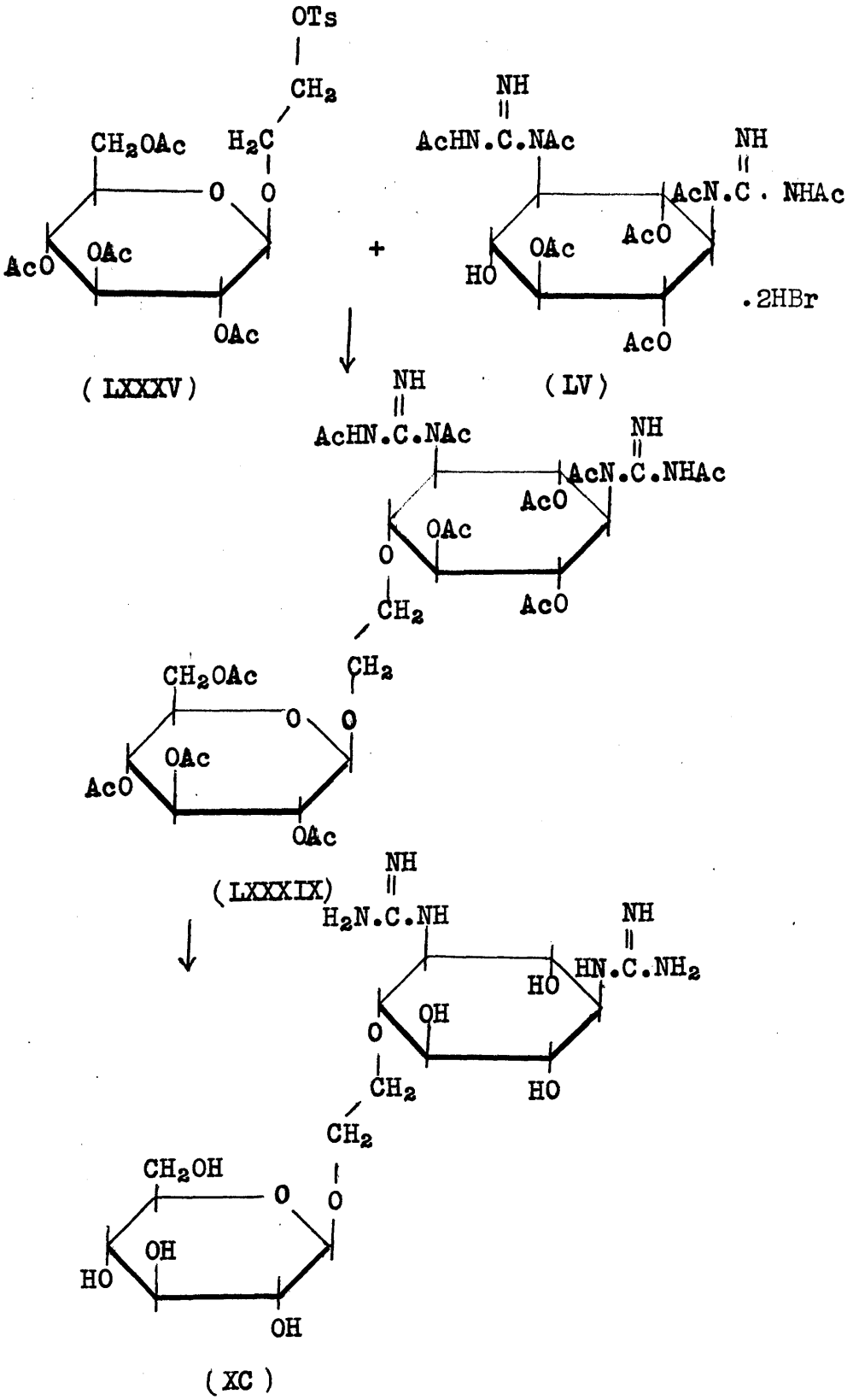


(LXXXVII) (LXXXVIII)

Ts = $CH_3.C_6H_4.SO_2$-

It seemed to be possible, therefore, that ether formation may be accomplished from the tosyl ester under such drastic and prolonged conditions. Consequently hepta--acetylstreptidine dihydrobromide (LV) and tetra-O-acetyl--2-(tosyloxyethyl) β-D-glucopyranoside (LXXXV) were allowed to react in NN-dimethylformamide in the presence of Drierite to ensure freedom from moisture, and silver oxide to remove the hydrogen bromide and to neutralize the liberated p-toluenesulphonic acid which is sufficiently acidic to deacetylate[278] the reaction products. After a series of experiments, the reaction was conducted at 95° for eight hours with continuous stirring. The product undeca-acetyl-streptidine-oxyethyl β-D-glucopyranoside (LXXXIX) was purified and deacetylated as for streptidine β-D-glucopyranoside (LVIII). The deacetylated product, streptidine-oxyethyl β-D--glucoside (XC) was confirmed by conversion to its crystalline dihelianthate.

The low yields of the intermediates in the preparation of streptidine-oxyethyl β-D-glucopyranoside necessitated the repetition of the experiment many times to collect convenient quantities of the intermediates. Consequently, the preparation of streptidine-oxyethyl 2-methylamino-2-deoxy-β-D-glucopyranoside was abandoned.


OTs
CH2
CH2OAc
H2C
O
O
AcO
OAc
OAc
(LXXXV)
+
NH
AcHN.C.NAc
NH
AcN.C.NHAc
AcO
OAc
HO
AcO
.2HBr
(LV)
NH
AcHN.C.NAc
NH
AcN.C.NHAc
AcO
OAc
O
CH2
AcO
CH2OAc
CH2
O
O
OAc
AcO
OAc
(LXXXIX)
NH
H2N.C.NH
NH
HN.C.NH2
HO
OH
O
CH2
HO
CH2
CH2OH
O
O
OH
HO
OH
(XC)

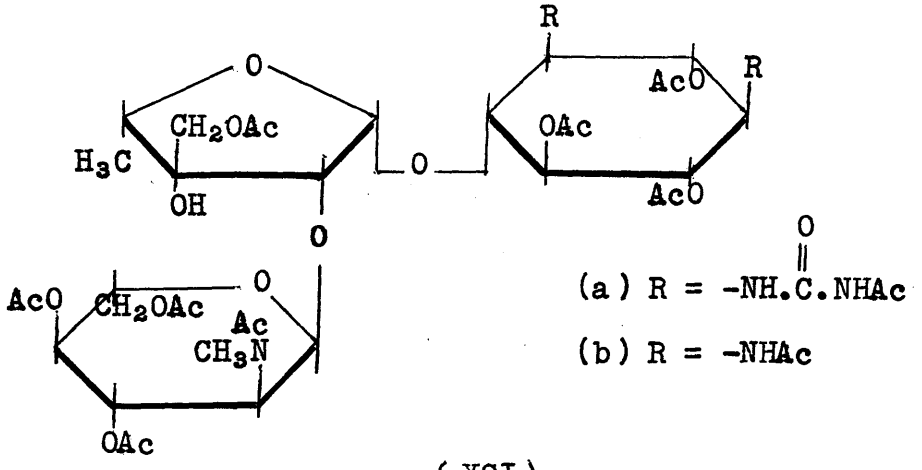
Degradation of Dihydrostreptomycin Sulphate and Streptidine 2-amino-2-deoxy-β-D-glucopyranoside Sulphate.

As stated earlier (page 74) dihydrostreptomycin sulphate was converted into strepturea and streptamine derivatives by controlled hydrolysis with barium hydroxide solution in order to ascertain the optimum conditions for the degradation of streptidine 2-amino-2-deoxy-β-D-glucopyranoside sulphate to its corresponding strepturea and streptamine derivatives.

Like alkaline hydrolysis of streptidine[224,286], dihydrostreptomycin sulphate was degraded with 0.35N barium hydroxide under reflux for different periods. After one hour refluxing, two moles of ammonia per mole of dihydrostreptomycin were liberated and the excess of barium hydroxide was precipitated as barium carbonate with solid carbon dioxide. The neutral pale yellow filtrate after decolourisation with charcoal, afforded a micro-crystalline strepturea β-L-dihydrostreptobiosaminide (XXVII), $[\alpha]_D^{19}$ - 90°(in water). Treatment with charcoal also facilitated the removal of traces of undecomposed dihydrostreptomycin. The strepturea derivative was characterised by acetylation using the two stage procedure of Wolfrom and his coworkers[222], crystalline deca-acetylstrepturea β-L-dihydrostreptobiosaminide (XCIa) being obtained.

Degradation of dihydrostreptomycin sulphate with 0.35N

barium hydroxide under reflux for twenty four hours in the same way afforded streptamine β-L-dihydrostreptobiosaminide (XXVIII). The latter showed the presence of primary amino groups with ninhydrin solution[329] and the absence of guanidine or urea groups was demonstrated with alkaline solution of potassium ferricyanide and sodium nitroprusside[330]. The crude product was purified by adsorption on charcoal and subsequent elution with methanol acidified with hydrochloric acid, yielding the hydrochloride, m.p. 194-196° and $[\alpha]_D^{17}$ - 88.6° (in water). Its identity was confirmed by acetylation following a procedure similar to that used for deca-acetyl-strepturea β-L-dihydrostreptobiosaminide. The crystalline product, deca-acetylstreptamine β-L-dihydrostreptobiosaminide (XCIb) showed m.p. and optical rotation comparable to a similar product cited by Wolfrom and Polglase[233].

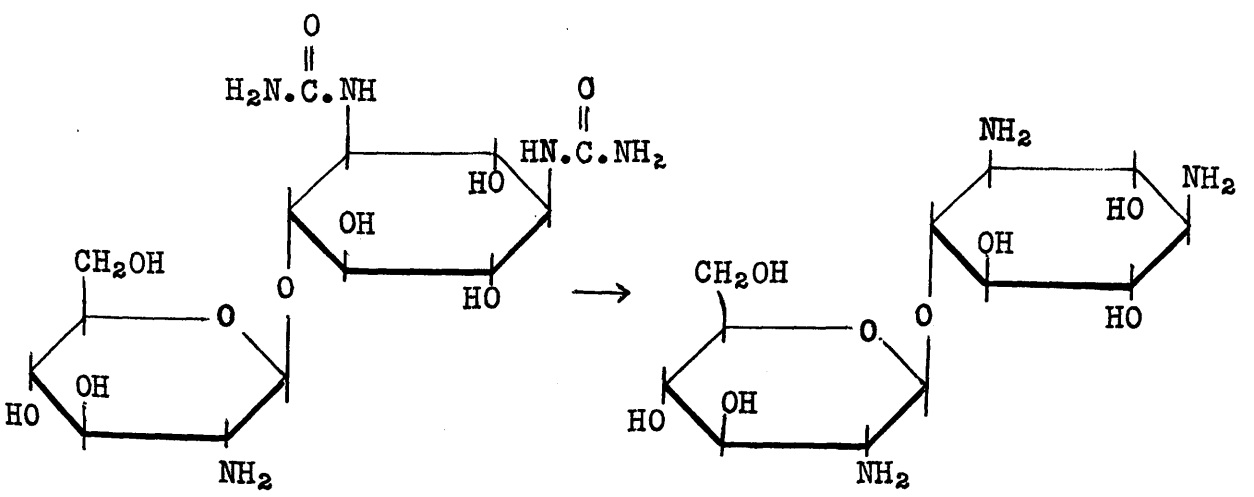


(XCI)

Streptidine 2-amino-2-deoxy-β-D-glucopyranoside sulphate was degraded similarly by stepwise hydrolysis with 0.35N barium hydroxide to strepturea 2-amino-2-deoxy-β-D-glucopyranoside (XCII) and streptamine 2-amino-2-deoxy-β-D-glucopyranoside (XCIII).

(Streptidine 2-amino-2-deoxy-β-D-glucopyranoside.)

(LXI)



(XCII) (XCIII)

Attempted Anomerization of Dodeca-acetyl-dihydrostreptomycin.

The relationship of biological activity and stereoisomerism of some antibiotics has been studied in the past. The stereoisomer of penicillin[331] synthesised from L-penicillamine was found to be devoid of antibacterial activity. Of the four

possible stereoisomers of chloramphenicol[332], only the natural antibiotic, the D(-) threo-isomer inhibits bacterial growth and protein synthesis while its L(+)erythro-stereoisomer is only a very weak growth inhibitor[333]. Similarly in the case of cycloserine, the natural D-isomer[255] is more active than the synthetic L-isomer[334]. Thus the change in configuration affords a means of studying the importance of molecular shape of the antibiotic. The anomerization of glycosidic linkages in dihydrostreptomycin would alter the whole shape of the molecule without effecting the nature of the constituent units, thus, producing new isomers.

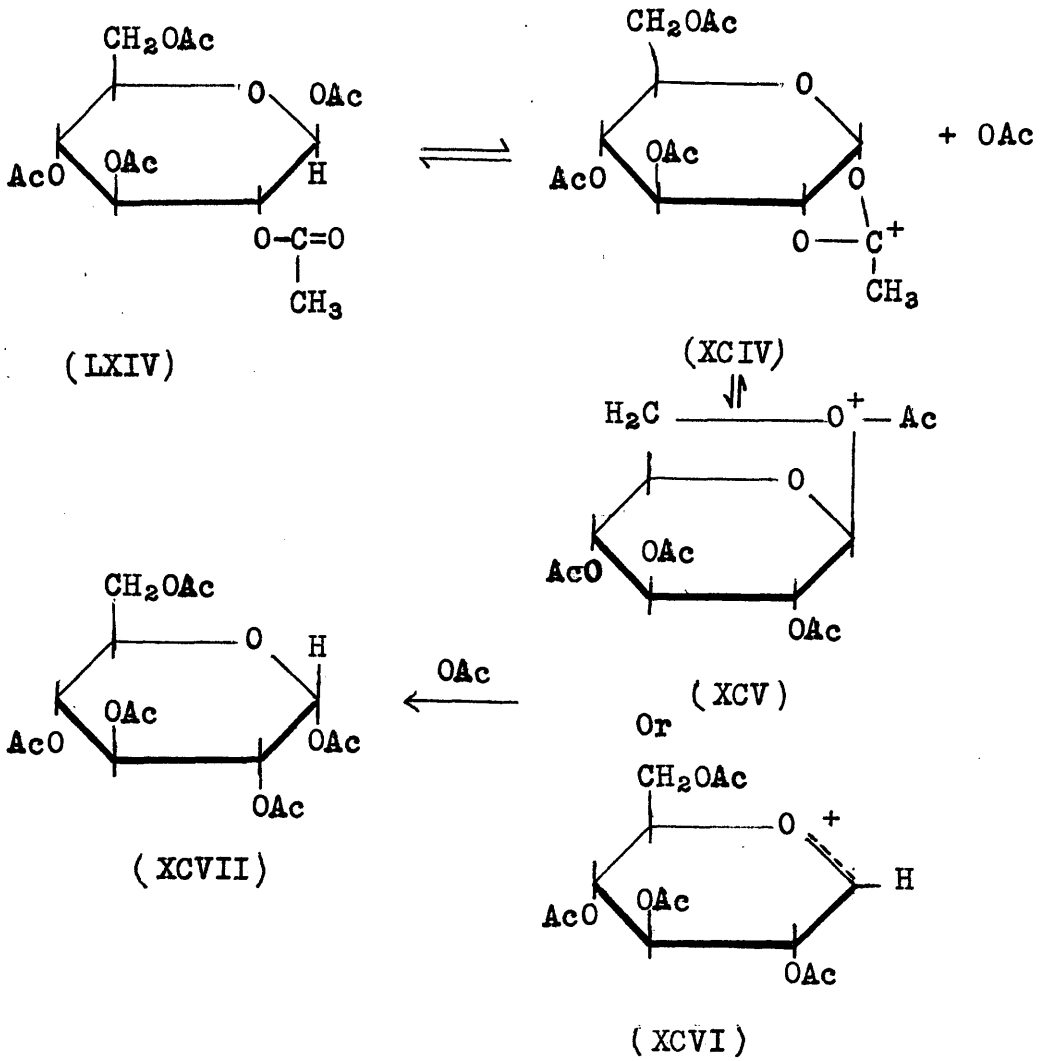
Dihydrostreptomycin has been shown to possess an α-linkage between N-methyl-glucosamine and dihydrostreptose, and a β-linkage between dihydrostreptose and streptidine units[222]. The former linkage is comparatively much more stable in acidic solutions than the latter, drastic acidic conditions being necessary to cleave the α-linkage[226]. In order to accomplish the conversion of the β-linkage in dihydrostreptomycin to a more stable α-linkage, anomerization of dodeca-acetyl-dihydrostreptomycin was attempted in non-aqueous media with acidic catalysts such as boron trifluoride[335], stannic chloride[336] titanium tetrachloride[336] and a mixture of sulphuric acid and acetic anhydride[337] by the usual procedures. In each case the final product was found to be either the starting material,

the hydrolysed product or an unidentified decomposed substance. Anomerization with sodium hydroxide[338], an alkaline catalyst in a mixture of dry dioxane and dry ether resulted in partial deacetylation of dodeca-acetyldihydrostreptomycin to a product which on re-acetylation with acetic anhydride in pyridine afforded the starting material.

The inability to anomerize the β-linkage in dodeca--acetyldihydrostreptomycin to the α-form seems to conform with the mechanism of anomerization which is thought to involve participation of the neighbouring acetoxy group, since this is absent in the dihydrostreptose moiety. This mechanism has been investigated by Lemieux and his coworkers.

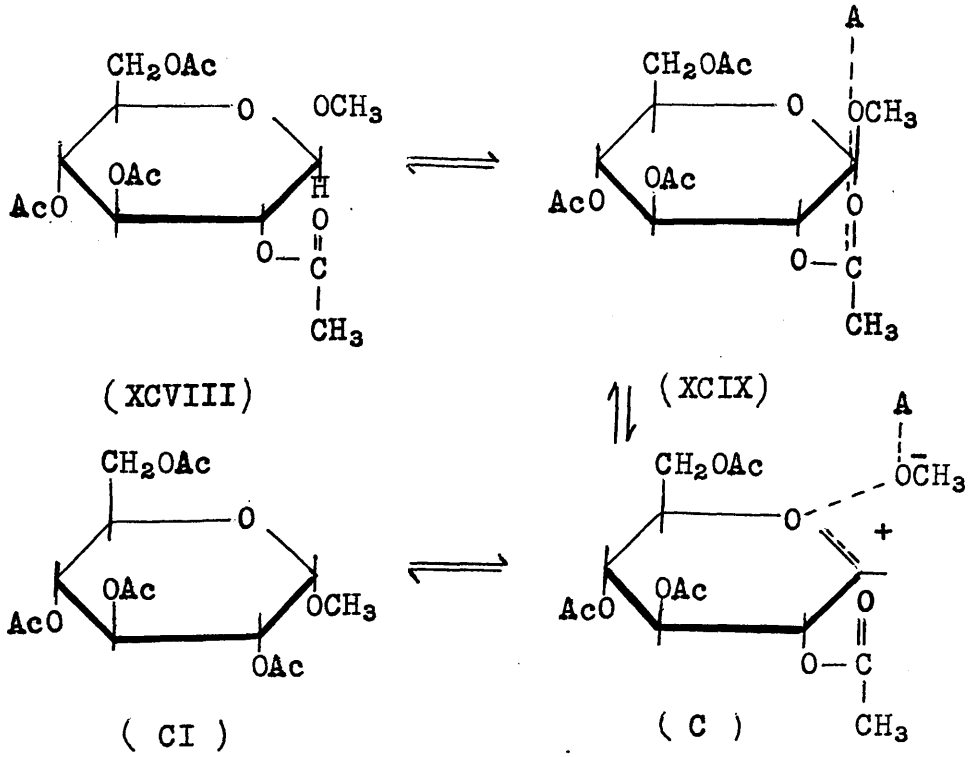
Lemieux and Brice[295,339] have anomerized penta-O-acetyl β-D-glucopyranose (LXIV) in chloroform solution by introducing ^{14}C-labelled acetate ion into the reaction mixture. Since the $C_{(1)}$-to-acetoxy group bond of β-D-glucopyranose penta acetate is highly labile[295], the most favourable route for exchange of acetate ion is by dissociation of the $C_{(1)}$-to-acetoxy group bond. The starting material was, therefore, found to equiliberate very rapidly with the labelled acetate ions (XCIV) formed by the participitation of $C_{(2)}$-acetoxy group. The formation of this 1,2-α-cyclic ion (XCIV) is followed by a slower reaction to form a second intermediate such as 1,6-orthoester ion (XCV) or a carbonium ion (XCVI) which was assumed to be the rate-controlling stage for the

β→α conversion. This new intermediate particularly (XCV) combines with the labelled acetate ion to form labelled penta-O--acetyl α-D-glucopyranose (XCVII). During these transformation which are reversible reactions, the formation of highly complex and labile substrate molecules, inevitably leads to a number of side reactions and it is difficult to obtain a true equilibrium.



Lindberg[340], on the other hand, anomerized a mixture of tetra-O-acetyl-isopropyl β-D-glucopyranoside and hepta-O-acetyl-ethyl β-cellobioside using titanium tetrachloride as the catalyst and obtained α-anomers in 66 and 75 per cent yield. On the basis of this study, he deduced that the glycosidic linkage is not completely broken down.

Lemieux and his collaborators[341,342], however, have recently postulated that tetra-O-acetyl-methyl β-D-glucopyranoside (XCVIII) is transformed to an ortho-ester (XCIX) under the influence of the acid catalyst, A. The participation of $C_{(2)}$-acetoxy group weakens the $C_{(1)}$-to-methoxyl group bond and the transition state (XCIX) readily leads to the ion-pair



intermediate ($\overline{C}$). The collapse of the ion-pair then results in the formation of tetra-O-acetyl-methyl α-D-gluco-pyranoside (CI).

EXPERIMENTAL

The author wishes to thank Dr. A. C. Syme, Mr. W. McCorkindale, Miss P. Adams and Miss M. Buchanan of the College and Drs. Weiler and Strauss, Oxford for carrying out the micro-analyses.

Melting points are uncorrected. Ultraviolet absorption spectra were determined on a Hilger Uvispek photoelectric spectrophotometer (Model H.700.303) fitted with a fused silica prism.

Materials

Dihydrostreptomycin sulphate and streptomycin sulphate used in the present investigation was the medicinal grade supplied by Glaxo Laboratories Ltd.

Reagents

Silver oxide was freshly prepared from silver nitrate and potassium hydroxide, washed thoroughly with water and acetone, and dried over phosphorus pentoxide.

"Drierite", [315] finely divided calcium sulphate hemihydrate was dried by heating for two hours at 230-250° before use.

NN-dimethylformamide was kept over "Drierite" for twenty four hours and distilled before use. Pyridine and quinoline were refluxed over potassium hydroxide and then distilled. Chloroform was washed repeatedly with water, dried over calcium chloride and distilled before use.

Dihydrostreptomycin

Dihydrostreptomycin sulphate (5.94 g.) was dissolved in water (60 ml.) and the solution passed through a column (25 x 1.8 cm.) of anion exchange resin (De-Acidite F.F.). The column was washed with water (ca. 50 ml.). The combined eluates were freeze-dried giving the sulphate-free white amorphous base (4.05 g., 85%), m.p. 193-195° (decomp., micro block), $[\alpha]_D^{20}$ - 94° (c 1.06 in water).

Dodeca-acetyldihydrostreptomycin

(a) (Based on the method of Wolfrom, Cron, De Walt and Husband[222]).

Dihydrostreptomycin (9.41 g.) was stirred in dry methanol (250 ml.) and pyridine (60 ml.). Acetic anhydride (100 ml.) was added in 5 ml. portions with vigorous stirring over a period of 45 minutes. The solution was maintained for one hour at room temperature and then concentrated below 50° in vacuo. The viscous residue (ca. 10 ml.) was treated with dry ether to give a white precipitate which was washed twice with dry ether. The hygroscopic precipitate was stirred with pyridine (100 ml.) and acetic anhydride (100 ml.), the latter being added in small amounts. The resultant solution

was kept at room temperature for forty four hours, and then heated at 50° for five hours. After cooling, the solution was poured onto crushed ice (1500 g.) with stirring. The aqueous solution was extracted with chloroform (5 x 80 ml.) and the chloroform extract evaporated to a small volume (ca. 15 ml.) in vacuo below 50°. The syrupy mass on trituration with crushed ice (150 g.) yielded a cream coloured solid (ca. 15 g.). Extraction of the aqueous solution with chloroform afforded some more solid (ca. 3 g.). The combined product was dissolved in chloroform (100 ml.), the solution dried ($CaCl_2$) and evaporated to a semi-solid residue which was redissolved in benzene (100 ml.). The benzene solution was filtered and added slowly to light petroleum (b.p. 80-100°) (1500 ml.). The product reprecipitated from benzene and light petroleum (b.p. 80-100°) and dried in a vacuum desiccator gave dodeca--acetyldihydrostreptomycin (14.1 g., 80.2%), m.p. 152-155° (decomp.) sintered at 130°, $[\alpha]_D^{20}$ - 67.6° (c 1.086 in chloroform). Wolfrom et al[222] give m.p. 153-155°, $[\alpha]_D^{20}$ - 67°.

Found: C, 48.0; H, 5.9; N, 8.5,

Calculated for $C_{45}H_{65}O_{24}N_7.2H_2O$, C, 48.1; H, 6.2; N, 8.7%

(b) Dihydrostreptomycin sulphate (10.25 g.) was stirred in dry methanol (200 ml.) and pyridine (60 ml.). Acetic anhydride (100 ml.) was added in 5 ml. amounts with constant stirring over a period of fifty minutes. Fused sodium acetate (10.47 g.) was incorporated and stirring continued for a further twenty four hours. Sodium sulphate was removed and the filtrate concentrated in vacuo below 50° to a semi-solid mass which was triturated and washed with ether. The solid mass after drying was further treated with acetic anhydride and pyridine and worked up as in method (a) (yield 11.4 g., 75%), m.p. 152-155° (decomp.) sintered at 130°.

Undeca-acetylstreptomycin

Streptomycin sulphate (5.1 g.) was acetylated as in method (b) for dihydrostreptomycin sulphate yielding undeca-acetylstreptomycin (5.3 g., 71%), m.p. 168-170° (decomp.) with sintering at 145°, $[\alpha]_D^{21}$ - 64.5° (c 1.1 in chloroform).

Found:	C, 47.9; H, 5.6; N, 9.0,
$C_{43}H_{61}O_{23}N_7 . 2H_2O$ requires	C, 47.8; H, 6.1; N, 9.1%.

Undeca-acetyldihydrostreptomycin

A solution of undeca-acetylstreptomycin (1.05 g.) in glacial acetic acid was shaken in an atmosphere of

hydrogen with platinum oxide[228] (0.157 g.) at room temperature and atmospheric pressure. The platinum oxide was hydrogenated prior to the addition of undeca-acetylstreptomycin. After eighteen hours, 20 ml. of hydrogen had been taken up (1 mole). The decanted solution was filtered through a sintered-glass funnel and the acetic acid removed in vacuo below 50° leaving a viscous residue which on trituration with dry ether afforded a white solid (0.95 g.). This was washed thoroughly with dry ether and dried in a vacuum desiccator over potassium hydroxide, to yield undeca--acetyldihydrostreptomycin, m.p. 156-158° (decomp.) with sintering at 130-135°, $[\alpha]_D^{21}$ - 58.4° (c 1.1 in chloroform).

Found:	C, 47.1;	H, 6.7;	N, 9.5,
$C_{43}H_{63}O_{23}N_7 \cdot 2H_2O$ requires	C, 47.7;	H, 6.2;	N, 9.05%

Undeca-acetyldihydrostreptomycin did not reduce Fehling's solution on boiling.

Acetylation of Undeca-acetyldihydrostreptomycin to Dodeca-acetyldihydrostreptomycin

Undeca-acetyldihydrostreptomycin (0.25 g.) was acetylated in pyridine (10 ml.) with acetic anhydride (10 ml.) at room temperature for forty eight hours,

followed by heating at 50° for four hours with stirring. The resultant solution was concentrated *in vacuo* below 50° to a viscous residue which on trituration with dry ether afforded a white amorphous substance. After thorough washing with dry ether and drying in a vacuum desiccator, it was dissolved in chloroform (5 ml.) and added slowly to petroleum ether (b.p. 60-80°) (50 ml.) with stirring. The white amorphous precipitate was dried in a vacuum desiccator to give dodeca-acetyldihydrostreptomycin m.p. 152-155° (decomp.), $[\alpha]_D$ - 66.9° (c 0.88 in chloroform). A sample dried at 100° for three hours *in vacuo* was analysed.

Found: N, 9.2,

Calculated for $C_{45}H_{65}O_{24}N_7$, N, 9.0%

O-α-2-N-acetyl-N-methylamino-2-deoxy-L-glucopyranosyl--(1→2)-O-β-3-C-trityloxymethyl-5-deoxy-L-lyxofuranosido--(1→4)-1,3-diguanidino-2,4,5,6-tetrahydroxycyclohexane. (N-Acetyltrityldihydrostreptomycin).

Undeca-acetyldihydrostreptomycin (3.684 g., 0.0034 mole) dried over phosphorus pentoxide was dissolved in dry pyridine (30 ml.) and trityl chloride (7.876 g, 0.028 mole) added. The resultant solution was kept in

a dark place for eight days when the solution attained reddish brown colour. The pyridine was removed *in vacuo* at room temperature and the residue was extracted with ether. The ether-insoluble buff-coloured residue was dissolved in chloroform (50 ml.) and the solution was concentrated to *ca.* 20 ml. After keeping in the refrigerator for two hours, crystals of pyridinium trityl chloride[270] (2.33 g., m.p. 172–173°) were collected. The chloroform solution was added slowly to ten times its volume of dry ether and the precipitate washed with ether and dried *in vacuo* (yield 4.6 g.).

This amorphous product was dissolved in dry methanol (20 ml.) previously boiled and cooled. The solution was saturated with ammonia at 0° and was kept at room temperature for twenty four hours. The solvent was removed *in vacuo* at room temperature, and the residue washed with ether and chloroform. The chloroform-insoluble residue (3.71 g.) was dissolved in NN-dimethylformamide (15 ml.). [The insoluble residue (0.24 g.) was identified to be ammonium chloride by qualitative tests]. The NN-dimethylformamide solution was stirred into dry ether (150 ml.) and the precipitate thus obtained fractionated by chromatography, a slurry of the above

crude product in ethanol (25 ml.) being poured on to the top of a column (30 x 1.9 cm.) of neutral ethanol--washed alumina (75 g.). The column was eluted as recorded in table 1, by using mixtures of ethanol and methanol in various proportions. The eluates were collected in a continuous fraction collector in 50 ml. quantities. The optical density of each 50 ml. fraction was measured at 259 mμ. and the next solvent was used when the last fraction of the previous solvent showed zero optical density. The combined solutions in each case were concentrated in vacuo below 45°.

Table 1

Fraction	Solvent	Volume collected in ml.	Wt. of product in g.	$E^{1cm.}_{1\%}$ at 259mμ in methanol
1	Ethanol	1000	-	-
2	Ethanol–methanol 4:1	2100	0.376	10.8
3	Ethanol–methanol 1:1	1250	0.35	6.62
4	Ethanol–methanol 1:4	1150	0.55	4.38
5	Methanol	800	0.15	3.38

Eluates obtained with a mixture of ethanol and methanol (4:1) showed the greatest optical density at 259 mμ. and gave N-acetyltrityldihydrostreptomycin, m.p. 179–181° (decomp.), $[\alpha]_D^{21}$ - 69.3° (c 1.5 in water).

Found: N, 11.25,

$C_{42}H_{57}O_{13}N_7$ requires N, 11.3%

(Consistent analysis could not be obtained for C and H).

Paper chromatography using toluene-p-sulphonic acid (2.5 per cent) in n-butanol saturated with water showed a single spot (R_f value 0.16) as compared to R_f value 0.34 for N-acetyldihydrostreptomycin when sprayed with potassium ferricyanide-sodium nitroprusside solution.[330]

N-acetyltrityldihydrostreptomycin Dipicrate

N-acetyltrityldihydrostreptomycin (73 mg.) was dissolved in water (1 ml.) and a saturated solution of picric acid (7 ml.) representing 67 mg. of picric acid was added slowly. The solution rapidly became turbid and was kept in the refrigerator for two hours, yielding N-acetyltrityldihydrostreptomycin dipicrate (from aqueous methanol), m.p. 178–180° (decomp.).

Found: N, 13.3,

$C_{54}H_{63}O_{27}N_{13}\cdot 2H_2O$ requires N, 13.3%

O-α-2-N-acetyl-N-methylamino-2-deoxy-L-glucopyranosyl-
-(1→2)-O-β-3-C-hydroxymethyl-5-deoxy-L-lyxofuranosido-
-(1→4)-1,3-diguanidino-2,4,5,6-tetrahydroxycyclohexane.
(N-acetyldihydrostreptomycin).

Dodeca-acetyldihydrostreptomycin (1.01 g.) was dissolved in dry methanol (10 ml.) previously boiled and cooled. The solution was saturated with dry ammonia at 0° and was kept at room temperature for twenty four hours. Methanol and ammonia were evaporated off *in vacuo* at room temperature, and the residue washed with chloroform. Addition of the chloroform-insoluble residue in dry methanol (5 ml.) to dry ether (50 ml.) gave N-acetyldihydrostreptomycin as a white precipitate (0.466 g., 82%), m.p. 190-192° (decomp., micro-block), $[\alpha]_D^{20}$ - 91.6° (c 1.01 in water).

Found: C, 42.2; H, 7.1; N, 15.1,

$C_{23}H_{43}O_{13}N_7.1\frac{1}{2}H_2O$ requires C, 42.4; H, 7.1; N, 15.02%

N-Acetyldihydrostreptomycin Dipicrate

N-acetyldihydrostreptomycin (37 mg.) was dissolved in water (0.5 ml.). A solution of picric acid (4 ml.) representing 40 mg. of picric acid was added slowly. After two hours at 0° N-acetyldihydrostreptomycin dipicrate was obtained as yellow prisms (from aqueous methanol), yield 25 mg., m.p. 168-170° (decomp.).

Found: C, 36.2; H, 4.2; N, 16.7,

$C_{35}H_{49}O_{27}N_{13}.2H_2O$ requires C, 37.5; H, 4.8; N, 16.3%

O-α-2-N-acetyl-N-methylamino-2-deoxy-L-glucopyranosyl-(1 → 2)-O-β-3-C-formyl-5-deoxy-L-lyxofuranosido-(1→ 4)-1,3-diguanidino-2,4,5,6-tetrahydroxycyclohexane. (N-Acetylstreptomycin).

Undeca-acetylstreptomycin (0.82 g.) was deacetylated by the procedure used for N-acetyldihydrostreptomycin. N-acetylstreptomycin was obtained as white amorphous precipitate (0.40 g., 81.2%), m.p. 180-182° (decomp., micro-block), $[\alpha]_D^{20}$ - 87.1° (c 1.67 in water).

Found: C, 42.3; H, 7.1; N, 15.6,

$C_{23}H_{41}O_{13}N_7.1\frac{1}{2}H_2O$ requires C, 42.5; H, 6.8; N, 15.1%

Hydrolysis of Dodeca-acetyldihydrostreptomycin to 1,3-Bis (diacetylguanidino)-4-hydroxy-2,5,6-triacetoxycyclohexane Dihydrobromide. (Hepta-acetylstreptidine Dihydrobromide).

Dodeca-acetyldihydrostreptomycin (2.053 g., 0.0019 mole) in absolute chloroform (30 ml.), was cooled in ice and a 31 per cent solution of hydrogen bromide[287(a)] in glacial acetic acid (1.3 ml., 0.0058 mole) was added with stirring. The solution on keeping for twenty four hours in the refrigerator deposited a sticky residue. The chloroform was decanted off and the residue washed with absolute chloroform (2 x 10 ml.). The decanted chloroform solution with more

31 per cent solution of hydrogen bromide in glacial acetic acid (0.4 ml., 0.0019 mole), yielded an additional amount of similar residue during the next twenty four hours.

The residue in each case was washed with dry ether (15 ml.). The traces of acid were removed by adding chloroform (10 ml.) and evaporating it *in vacuo*. This process was repeated a second time. The dry yellowish residues (1.068 g.) were combined, dissolved in methanol (10 ml.), treated with charcoal (0.2 g.) and the colourless solution added with stirring to dry ether (100 ml.). The white micro-crystalline precipitate was collected on a sintered-glass funnel, and washed with dry ether, absolute chloroform (2 x 10 ml.) and finally with dry ether to yield *hepta-acetylstreptidine dihydrobromide* (0.728 g., 54 per cent) which did not crystallise from common organic solvents but had m.p. 180-183° (decomp.), $[\alpha]_D^{19}$ - 5.4° (c 0.918 in methanol).

Found: C, 35.6; H, 4.7; N, 11.4;
Br, 22.0; acetyl, ^{278}41,

$C_{22}H_{32}O_{11}N_6.2HBr.H_2O$ requires C, 35.9; H, 4.9; N, 11.4;
Br, 22.2; acetyl, 40.9%

The product was insoluble in absolute chloroform, ether and petroleum ether but soluble in ethanol, methanol and NN-dimethylformamide.

The dihydrobromide (m.p. 180-183°) (decomp.) was also obtained from undeca-acetylstreptomycin by the same method.

Found: N, 11.25,

$C_{22}H_{32}O_{11}N_6.2HBr.H_2O$ requires N, 11.4%

1,3-Bis(diacetylguanidino)-4-hydroxy-2,5,6-triacetoxy-cyclohexane. (Hepta-acetylstreptidine).

Hepta-acetylstreptidine dihydrobromide (0.2 g.) in methanol (3 ml.) was treated with silver oxide (0.19 g.) and the filtrate added to dry ether (50 ml.). The white precipitate, thus, obtained was dried in vacuo to yield hepta-acetylstreptidine m.p. 195-197° (decomp., micro-block).

Found: N, 14.9,

$C_{22}H_{32}O_{11}N_6$ requires N, 15.1%

The product was soluble in methanol, ethanol and NN-dimethylformamide but insolbule in absolute chloroform.

Octa-acetylstreptidine.

Hepta-acetylstreptidine dihydrobromide (0.41 g.) was acetylated in pyridine (5 ml.) with acetic anhydride at room temperature for twenty four hours, followed by heating at 50° for one hour. The solution was evaporated in vacuo to a viscous residue and the product triturated and washed with dry ether to yield octa-acetylstreptidine (from chloroform-ether) (0.232 g.), m.p. 260-262° (micro-block). Peck et al[224]

give m.p. 260-262° (micro-block).

Found: N, 13.8,

Calculated for $C_{24}H_{34}O_{12}N_6$ N, 14.04%

Penta-O-acetyl-β-D-glucopyranose was prepared from D-glucose by the method given by Vogel[287(b)], m.p. 130-132°, $[\alpha]_D^{18}$ + 3.6 (c 1.1 in chloroform).

2,3,4,6-Tetra-O-acetyl-α-D-glucopyranosyl Bromide, (acetobromoglucose) was prepared by the procedure of Jeremias, Lucas and Mackenzie[289], yield 85%, m.p. 87-88°, $[\alpha]_D^{19}$ + 198° (c 1.03 in chloroform). Jeremias et al[289] give m.p. 88-89°.

The product underwent decomposition on storage at room temperature. It was, therefore, kept under petroleum ether (b.p. 40-60°) and was recrystallised from carbon tetrachloride-petroleum ether (b.p. 40-60°) before use.

Tetra-acetyl-[4-O-α-(1,3-diguanidino-2,5,6-trihydroxycyclohexyl) D-glucopyranoside], (Tetra-acetylstreptidine α-D-glucopyranoside)

Hepta-acetylstreptidine dihydrobromide (0.453 g., 0.00063 mole) and acetobromoglucose (0.264 g., 0.00064 mole) were dissolved in dry quinoline (1.841 g.). After keeping at 5° for eighteen hours, the excess of quinoline was washed off with dry ether by trituration. The ether-insoluble residue was shaken with silver oxide in chloroform (10 ml.) and

filtered. The filtrate on evaporation gave a residue which was washed with ether and extracted with absolute chloroform (5 x 10 ml.). The chloroform extract on evaporation *in vacuo* gave an amorphous *product* (0.056 g.) which was purified by precipitation from chloroform-ether and then from acetone-petroleum ether (b.p. 40-60°), (yield, 0.037 g., 10%). It did not crystallise from common organic solvents. It was soluble in chloroform, ethanol, methanol and NN-dimethylformamide but insoluble in ether and petroleum ether, m.p. 140-142° (decomp., micro-block), $[\alpha]_D^{19}$ + 15.6° (c 0.74 in chloroform).

Found: C, 41.9; H, 5.55; N, 13.1,

$C_{22}H_{36}O_{13}N_6.2H_2O$ requires C, 42.0; H, 6.4; N, 13.4%

α-Configuration for this product was assigned on the basis of optical rotation.

4-O-β-[1,3-bis(diacetylguanidino)-2,5,6-triacetoxycyclohexyl] tetra-O-acetyl-D-glucopyranoside. (Undeca-acetylstreptidine β-D-glucopyranoside).

Hepta-acetylstreptidine dihydrobromide (0.67 g., 0.00093 mole) was dissolved in NN-dimethylformamide (10 ml.) in a flask wrapped in a black paper. The solution was kept *in vacuo* (0.2 mm.) for fifteen minutes with constant stirring. Acetobromoglucose (0.378 g. 0.00092 mole) was added. The solution was again maintained *in vacuo* for ten minutes.

Silver oxide (0.604 g.) and quinoline (4 drops) were added simultaneously. The water liberated during the reaction was removed under vacuum (0.2 mm.). NN-dimethylformamide was added when necessary during the reaction period (8 hours) to maintain the volume.

After the reaction the solvent was distilled off in vacuo at room temperature. The viscous residue was triturated and washed with dry ether (5 x 10 ml.) and extracted with absolute chloroform (10 x 10 ml.). The chloroform solution was kept in the refrigerator overnight and hydrogen sulphide passed into the cold solution. The filtrate was evaporated and the tan-coloured residue precipitated from chloroform-ether. The precipitate was redissolved in acetone and the solution decolourised with charcoal (0.150 g.). The filtrate when poured slowly into petroleum ether (40-60°) afforded undeca-acetylstreptidine β-D-glucopyranoside as a micro-crystalline precipitate which was dried in vacuo (yield 0.184 g., 22%), m.p. 164-167° (decomp.) $[\alpha]_D^{18}$ - 25.5° (c 2.56 in chloroform).

Found: C, 46.2; H, 6.3; N, 9.5,

$C_{36}H_{50}O_{20}N_6.2H_2O$ requires C, 46.9; H, 5.9; N, 9.1%

Attempted Condensation of Hepta-acetylstreptidine Dihydrobromide and acetobromoglucose.

(a) (Using quinoline as a condensing agent in the presence of Drierite[315]).

Hepta-acetylstreptidine dihydrobromide (0.457 g., 0.00063 mole) was dissolved in NN-dimethylformamide (5 ml.) in a flask protected from light. The solution was stirred with Drierite (1.08 g.) for thirty minutes. Silver oxide (0.516 g.) and quinoline (2 drops) were added simultaneously and stirring continued for ten minutes. Acetobromoglucose (0.284 g., 0.00069 mole) was added and the mixture was stirred for eight hours at room temperature. After the reaction, most of NN-dimethylformamide was distilled off in vacuo (0.2 mm.) at room temperature. The residue was washed with ether (5 x 10 ml.) and extracted with chloroform (5 x 10 ml.). The chloroform extract on evaporation in vacuo gave 0.01 g. of tan-coloured residue (2% yield) (possibly an impure product). The methanol extract of the chloroform residue afforded 0.251 g. of hepta-acetylstreptidine m.p. 193-196°.

(b) (Using mercuric cyanide[305] as condensing agent).

Hepta-acetylstreptidine dihydrobromide (0.14 g., 0.00019 mole) and acetobromoglucose (0.082 g., 0.0002 mole) were dissolved in NN-dimethylformamide (10 ml.). Mercuric cyanide (0.104 g.) was added. Immediately the optical rotation was observed and it bacame constant (+1.64° → + 0.252°) after forty two hours. The reaction mixture was added to dry ether (30 ml.) and kept in the refrigerator. A sticky residue was

deposited which became amorphous on trituration with chloroform. It gave tests for Hg^{++}, CN^{-} and Br^{-}. On purification from methanol-ether it afforded a residue (0.126 g.) still contaminated with traces of impurities. Further purification with hydrogen sulphide yielded an amorphous substance which did not melt below 300°. Further purification was without any success.

4-O-β-(1,3-diguanidino-2,5,6-trihydroxycyclohexyl) D-glucopyranoside. (Streptidine β-D-glucopyranoside).

Undeca-acetylstreptidine β-D-glucopyranoside (0.528 g.) was dissolved in dry methanol (5 ml.). The solution was saturated with ammonia at 0° and kept for 15 hours at room temperature. The solvent was removed *in vacuo* at room temperature and the residue washed with chloroform (3 x 20 ml.). The chloroform-insoluble residue was redissolved in methanol (5 ml.), filtered and the filtrate added slowly to dry ether (50 ml.). The amorphous base could not be crystallised from common organic solvents and was dried in a vacuum desiccator to yield streptidine β-D-glucopyranoside (0.178 g., 70%), m.p. 170-172° (decomp., micro-block) $[\alpha]_D^{20}$ - 11.4° (c 0.615 in methanol).

Found: C, 37.8; H, 7.0; N, 18.6,

$C_{14}H_{28}O_9N_6.H_2O$ requires C, 38.0; H, 6.8; N, 19.0%

Streptidine β-D-glucopyranoside Dipicrate

Streptidine β-D-glucopyranoside (64 mg.) was dissolved in water (2 ml.). A cold saturated solution of picric acid (6 ml. containing 70 mg. of picric acid) was added slowly and the turbid solution kept in the refrigerator for a few hours. Crystals were collected and washed with cold water (1 ml.). Recrystallisation from aqueous methanol yielded streptidine β-D-glucopyranoside dipicrate, m.p. 260-262° (decomp., micro-block).

Found: C, 33.4; H, 4.3; N, 18.4,

$C_{26}H_{34}O_{23}N_{12}.2H_2O$ requires C, 34.0; H, 4.2; N, 18.3%

3,4,6-Tri-O-acetyl-2-amino-2-deoxy-α-D-glucopyranosyl Bromide Hydrobromide, (Acetobromoglucosamine Hydrobromide), was prepared essentially by the methods of Irvine, McNicoll and Hynd[290], and Fodor and Ötvös[291].

Acetyl bromide (15.4 g., 0.127 mole) was stirred with D-glucosamine hydrochloride (5.2 g., 0.024 mole) for three days at room temperature. Excess of the reagent was removed in vacuo. The dry residue was washed with ether (25 ml.) and then dissolved in warm chloroform (50 ml.). The filtered chloroform solution was concentrated to ca. 35 ml., diluted with dry ether to turbidity and kept in the refrigerator. The crystalline product thus obtained was recrystallised from chloroform-ether (white needles) (yield 6.01 g., 55 per cent),

m.p. 151-153° (decomp.) $[\alpha]_D^{22}$+148° (c 1.05 in acetone) Irvine, McNicoll and Hynd[290] give m.p. 149-150°, $[\alpha]_D^{22}$ + 148.4 (c 1.05 in acetone).

4-O-β-[1,3-bis(diacetylguanidino)-2,5,6-tri-acetoxycyclohexyl] tri-O-acetyl-2-amino-2-deoxy-D-glucopyranoside. (Deca-acetylstreptidine 2-amino-2-deoxy-β-D--glucopyranoside).

Hepta-acetylstreptidine dihydrobromide (1.652 g., 0.0023 mole) were dissolved in NN-dimethylformamide (15 ml.) in a flask protected from light. The solution was kept *in vacuo* (0.2 mm.) for fifteen minutes with constant stirring. Tri-O-acetyl-2-amino-2-deoxy-α-D-glucopyranosyl bromide hydrobromide (1.06 g., 0.00236 mole) was added and the solution was again evacuated as before. Silver oxide (3.018 g.) and dry quinoline (0.2 ml.) added simultaneously. The reaction was conducted as for the preparation of undeca-acetylstreptidine β-D-glucoside using proportionately larger quantities of solvents. The product (0.4 g., 20.6%) was crystallised from methanol-ether; m.p. 132-134° (decomp.), $[\alpha]_D^{19}$ - 12° (c 0.88 in chloroform).

Found: C, 46.3; H, 6.4; N, 11.3,

$C_{34}H_{49}O_{18}N_7.2H_2O$ requires C, 46.4; H, 6.1; N, 11.1%

4-O-β-(1,3-diguanidino-2,5,6-trihydroxycyclohexyl) 2-amino-2-deoxy-D-glucopyranoside Trihydrochloride. (Streptidine 2-amino-2-deoxy-β-D-glucopyranoside Trihydrochloride).

Deca-acetylstreptidine 2-amino-2-deoxy-β-D--glucopyranoside (1.145 g.) was dissolved in dry methanol (10 ml.). The solution was saturated at 0° with dry ammonia. After keeping at room temperature for eighteen hours, the solvent was evaporated off *in vacuo*. The residue was extracted with chloroform (3 x 20 ml.) and the chloroform-insoluble residue was taken up in dry methanol (5 ml.). The filtered solution was slowly poured into dry ether (50 ml.) yielding an amorphous white precipitate (0.50 g., 87.3%).

A part of this precipitate (120 mg.) was dissolved in water (5 ml.) and was treated with charcoal (1 g.) and centrifuged to separate the aqueous solution. The charcoal was washed twice with water and then with dry methanol. It was suspended in dry methanol (5 ml.) and acidified with N methanolic hydrochloric acid. After shaking for a few minutes, the methanolic solution was separated by centrifugation. The charcoal was washed twice with methanol (10 ml.) and the combined

methanolic solutions were filtered and concentrated *in vacuo* to *ca.* 5 ml. The hygroscopic product (80 mg.) was obtained by precipitation in dry ether (75 ml.), m.p. 160–162° (decomp.), $[\alpha]_D^{19}$ – 17.6° (c 1.02 in water).

A sample for analysis was dried at 80° for three hours *in vacuo*.

Found: C, 32.0; H, 5.8; N, 17.8,

$C_{14}H_{29}O_8N_7.3HCl$ requires C, 31.6; H, 6.05; N, 18.4%

Streptidine 2-amino-2-deoxy-β-D-glucopyranoside sulphate.

The crude base (80 mg.) obtained in the previous experiment was dissolved in dry methanol (5 ml.) and the solution cooled and acidified with N sulphuric acid to pH 3. After fifteen minutes the suspension was centrifuged and washed with dry methanol and dry ether, and the product dried in a vacuum desiccator to yield streptidine β-D-glucosaminide sulphate (70 mg.), $[\alpha]_D^{20}$ – 17.1° (c 1.07 in water). For analysis the sample was dried at 80° for three hours.

Found: N, 17.2,

$C_{14}H_{29}O_8N_7.1\frac{1}{2}H_2SO_4$ requires N, 17.2%

Streptidine 2-amino-2-deoxy-β-D-glucopyranoside Trihelianthate.

The crude base (120 mg., 0.00028 mole) obtained in

the preparation of streptidine 2-amino-2-deoxy-β-D--glucopyranoside trihydrochloride was dissolved in water (2 ml.), the solution acidified with N/10 hydrochloric acid and methyl orange (301 mg., 0.0003 mole) dissolved in 26 ml. of 50 per cent aqueous methanol at 60°, added slowly. The flocculent precipitate was redissolved at 50° with the addition of 10 ml. of 50 per cent aqueous methanol. After setting aside at room temperature overnight needle-like crystals of streptidine β-D-glucosaminide trihelianthate were deposited. These were separated and washed with 50 per cent aqueous methanol (2 ml.) and dried (yield 96 mg.), m.p. 236-239° (decomp.).

Found: C, 48.0; H, 5.9; N, 16.0; S, 6.7,

$C_{56}H_{77}O_{17}N_{16}S_3 \cdot 2H_2O$ requires C, 48.75; H, 5.6; N, 16.3; S, 7.0%

2-N-Methylamino-2-deoxy-α-D-glucopyranose Hydrochloride

was prepared by the method of Kuehl, Flynn, Holly, Mozingo and Folkers.[226]

3,4,6-Tri-O-acetyl-2-N-methylamino-2-deoxy-α-D--glucopyranosyl Bromide Hydrobromide.

Acetyl bromide (6.15 g., 0.05 mole) was stirred with 2-N-methylamino-2-deoxy-α-D-glucopyranose hydrochloride (3.1 g., 0.00135 mole) for two days at room

temperature. Excess of the reagent was removed *in vacuo* and the residue was washed with dry ether (25 ml.). The residue was, then, dissolved in chloroform (50 ml.) and filtered. The filtrate was concentrated to about 10 ml. and poured into dry ether with stirring. The precipitate after washing with dry ether, was dried *in vacuo* over phosphorus pentoxide. Attempts to crystallise this hygroscopic product from non-alcoholic solvents were without success, (yield 2.1 g., 33.6%), m.p. 120-122° (decomp.), $[\alpha]_D^{23}$ + 149.5° (c 1.25 in acetone).

Found: C, 32.7; H, 4.7; N, 3.1; Br, 32.9,

$C_{13}H_{20}O_7NBr.HBr$ requires C, 32.4; H, 4.8; N, 2.9; Br, 33.2%

4-O-β-[1,3-bis(diacetylguanidino)-2,5,6-tri-acetoxycyclohexyl] tri-O-acetyl-2-N-methylamino-2-deoxy-D-glucopyranoside. (Deca-acetylstreptidine 2-N-methylamino-2-deoxy-β-D--glucopyranoside).

Hepta-acetylstreptidine dihydrobromide (1.35 g., 0.0018 mole) was dissolved in freshly distilled NN-dimethylformamide (10 ml.) in a flask protected from light. After maintaining the solution under high vacuum for fifteen minutes, tri-O-acetyl-2-N-methylamino--2-deoxy-α-D-glucopyranosyl bromide hydrobromide

(0.8 g., 0.00175 mole) was added and the solution was again kept under vacuum for ten minutes. Silver oxide (1.57 g.) and dry quinoline (0.15 ml.) were added simultaneously. The reaction was conducted as for undeca-acetylstreptidine β-D-glucopyranoside using proportionately larger quantities of solvents.
The product (0.3 g., 18.5%) could not be crystallised, m.p. 168-171° (decomp.), $[\alpha]_D^{20}$ - 11° (c 0.9 in chloroform).
Found: C, 46.1; H, 5.4; N, 10.6,
$C_{35}H_{51}O_{18}N_7 . 2H_2O$ requires C, 47.0; H, 6.2; N, 11.0%

4-O-β-(1,3-diguanidino-2,5,6-trihydroxycyclohexyl) 2-N-methylamino-2-deoxy-D-glucopyranoside carbonate. (Streptidine 2-N-methylamino-2-deoxy-β-D-glucopyranoside Carbonate).

The deca-acetate obtained in the previous experiment was dissolved in dry methanol (10 ml.) and deacetylated as described for streptidine β-D-glucopyranoside.
The chloroform-insoluble amorphous product (0.240 g., 77%) gave $[\alpha]_D^{22}$ - 16° (c 0.95 in water), and decomposed slowly above 180°.
Found: C, 36.15; H, 6.3; N, 18.2,
$C_{15}H_{31}O_8N_7 . 1\frac{1}{2} H_2CO_3 . H_2O$ requires C, 36.1 ; H, 6.6; N, 17.9%

Streptidine 2-N-methylamino-2-deoxy-β-D-glucopyranoside Sulphate.

The carbonate (125 mg.) obtained in the previous

experiment was dissolved in water (2 ml.) and the solution acidified to pH 4.8 with N/10 sulphuric acid. The filtered solution was freeze-dried and further drying was affected over phosphorus pentoxide (yield 100 mg.), $[\alpha]_D^{20}$ - 16.5° (c 0.8 in water).

Found: N, 16.4,

$C_{15}H_{31}O_8N_7 \cdot 1\frac{1}{2} H_2SO_4$ requires N, 16.8%

Streptidine 2-N-methylamino-2-deoxy-β-D-glucopyranoside Tripicrate.

The carbonate (30 mg.) was dissolved in water (0.5 ml.) and to it was added slowly picric acid (39 mg.) in water (3.6 ml.). After several hours in the refrigerator, the crystalline tripicrate (20 mg.) was collected, m.p. 269-271° (decomp., micro-block).

Found: C, 33.4; H, 4.2; N, 18.8,

$C_{33}H_{40}O_{29}N_{16} \cdot 2H_2O$ requires C, 34.2; H, 3.8; N, 19.3%

2-N-Methylamino-2-deoxy-α-L-glucopyranose Hydrochloride, was prepared by the procedure of Kuehl, Flynn, Holly, Mozingo and Folkers.[226]

3,4,6-Tri-O-acetyl-2-N-methylamino-2-deoxy-α-L-glucopyranosyl Bromide Hydrobromide.

Acetyl bromide (3.3 g., 0.027 mole) was stirred with

2-N-methylamino-2-deoxy-α-L-glucopyranose hydrochloride (1.165 g., 0.0051 mole) at room temperature for twenty four hours. It was purified by the procedure used for 3,4,6-tri-O-acetyl-2-N-methylamino-2-deoxy-D-glucopyranosyl bromide hydrobromide using proportionate quantities of the solvents. The amorphous hygroscopic powder (1.1 g., 47%) gave m.p. 114-116° (decomp.), $[\alpha]_D^{22}$ - 148° (c 0.9 in acetone).

Found: N, 3.0; Br, 32.7,

$C_{13}H_{20}O_7NBr.HBr.H_2O$ requires N, 2.9; Br, 33.2%

4-O-β-[1,3-bis(diacetylguanidino)-2,5,6-triacetoxycyclohexyl] tri-O-acetyl-2-N-methylamino-2-deoxy-L-glucopyranoside. (Deca-acetylstreptidine 2-N-methylamino-2-deoxy-β-L--glucopyranoside).

Hepta-acetylstreptidine (1.40 g., 0.0019 mole) was dissolved in NN-dimethylformamide (10 ml.) in a flask protected from light. After keeping the solution under high vacuum for fifteen minutes with continuous stirring, 3,4,6-tri-O-acetyl-2-N-methylamino-2-deoxy-α--L-glucopyranosyl bromide hydrobromide (0.9 g., 0.0019 mole) was added and the solution again maintained under high vacuum for ten minutes. Silver oxide (1.25 g.) and dry quinoline (0.12 ml.) were added simultaneously. The reaction was conducted as for undeca-acetylstreptidine

β-D-glucopyranoside using proportionately larger quantities of solvents. The product (0.51 g., 30%) could only be obtained in an amorphous condition and had m.p. 171-173° (decomp.), $[\alpha]_D^{21}$ - 20° (c 8.4 in chloroform).

Found: C, 46.5; H, 5.5; N, 11.1,

$C_{35}H_{51}O_{18}N_7.2H_2O$ requires C, 47.0; H, 6.2; N, 11.0%

4-O-β-(1,3-diguanidino-2,5,6-trihydroxycyclohexyl) 2-N-methylamino-2-deoxy-L-glucopyranoside Trihydrochloride. (Streptidine 2-N-methylamino-2-deoxy-β-L-glucopyranoside Trihydrochloride).

The deca-acetate (0.41 g.) obtained in the previous experiment was dissolved in dry methanol (10 ml.) and deacetylated by the procedure used for streptidine β-D-glucopyranoside yielding 0.175 g. of the amorphous base.

The amorphous precipitate was redissolved in water 3 ml. and the solution acidified with N/10 hydrochloric acid to pH 4.8. After decolourisation (charcoal), the solution was freeze-dried giving the white hygroscopic trihydrochloride, m.p. 178-180° (decomp.), $[\alpha]_D^{22}$ - 29.5° (c 0.8 in water).

Found: C, 31.3; H, 6.2; N, 17.5,

$C_{15}H_{31}O_8N_7.3HCl.H_2O$ requires C, 31.9; H, 6.4; N, 17.4%

Streptidine 2-N-methylamino-2-deoxy-β-L-glucopyranoside Tripicrate.

The amorphous base (25 mg.) obtained in the previous experiment was dissolved in water (0.5 ml.) and to it was added cold saturated solution of picric acid (3 ml.) at 45°. The mixture when cooled in the refrigerator yielded the crystalline tripicrate (aqueous methanol), m.p. 268-270° (decomp., micro-block).

Found: C, 33.6; H, 3.5; N, 18.9,

$C_{33}H_{40}O_{29}N_{16}.2H_2O$ requires C, 34.2; H, 3.8; N, 19.3%

2-Hydroxyethyl tetra-O-acetyl-β-D-glucopyranoside was prepared from acetobromoglucose by the method of Fischer and Fischer[324]; yield, 46%, m.p. 100-102°, $[\alpha]_D^{19}$ - 26° (c 0.88 in chloroform). Fischer and Fischer give m.p. 101-103°, $[\alpha]_D^{16}$ - 26°.

2-Toluene-p-sulphonyloxyethyl tetra-O-acetyl-β-D--glucopyranoside. (2-Tosyloxyethyl tetra-O-acetyl β-D-glucopyranoside).

2-Hydroxyethyl tetra-O-acetyl-β-D-glucopyranoside (3.03 g., 0.0078 mole) was dissolved in dry pyridine (3 ml.) and cooled in ice. A solution of dry toluene-p--sulphonyl chloride[325] (1.631 g., 0.0085 mole) in dry pyridine (3 ml.) was added slowly. The solution was kept at 0° for two and a half hours. After the reaction

ice-cooled water (60 drops) was stirred in over a period of fifteen minutes, followed by a further addition of cold water (50 ml.). The white precipitate, after trituration, was collected and washed with cold water (2 x 20 ml.). The precipitate, dried in a vacuum desiccator, was dissolved in chloroform (25 ml.), the solution was dried ($CaCl_2$) and evaporated. The viscous residue was dissolved in warm dry ether (200 ml.) and cooled slowly to 0° to yield white needles of 2-toluene-p-sulphonyloxyethyl tetra-O--acetyl-β-D-glucopyranoside (2.298 g., 54.5%), m.p. 109-110°, $[\alpha]_D^{18}$ - 13.1° (c 2.0 in chloroform) $\lambda_{max.}$ 225 (ϵ 5947), 262 (ϵ 297.5) in ethanol.

Found: C, 50.4; H, 5.6; S, 5.4,

$C_{23}H_{30}O_{13}S$ requires C, 50.5; H, 5.5; S, 5.9%

4-[1,3-bis(diacetylguanidino)-2,5,6-tri-acetoxycyclohexyl]--oxyethyl tetra-O-acetyl-β-D-glucopyranoside. (Undeca-acetylstreptidine-oxyethyl-β-D-glucopyranoside).

Hepta-acetylstreptidine dihydrobromide (2.185 g., 0.003 mole) was dissolved in NN-dimethylformamide (10 ml.) in a flask wrapped in a black paper. The solution was kept in vacuo (0.2 mm.) for fifteen minutes. Drierite (3.01 g.) and silver oxide (2.5 g.) were added and the mixture was stirred for thirty minutes. 2-Toluene-p-

sulphonyloxyethyl tetra-O-acetyl-β-D-glucopyranoside (1.515 g., 0.0029 mole) in two portions was added after thirty minutes interval. The mixture was stirred at 95° for eight hours. After the reaction, the solvent was evaporated off leaving a viscous residue which was triturated and extracted with dry ether (5 x 20 ml.). The ether extract afforded 0.25 g. of unreacted crystalline 2-tosyloxyethyl tetra-O-acetyl β-D-glucopyranoside. The ether-insoluble residue was extracted with absolute chloroform (5 x 10 ml.). The reddish brown chloroform extract was kept in the refrigerator overnight and hydrogen sulphide then passed into the cold solution. The filtrate was concentrated *in vacuo* to about 10 ml. and poured slowly into dry ether (100 ml.). The tan-coloured precipitate was dissolved in acetone (100 ml.) and the solution filtered, decolourised with charcoal (0.3 g.) and poured slowly into dry ether (100 ml.) yielding 1.38 g. of amorphous product, m.p. 168-171° (decomp., micro-block).

Found: N, 9.5,

$C_{38}H_{54}O_{21}N_6$ requires N, 9.0%

Further purification by chromatography on Silene EF and Celite[222] (5:1 by weight) in various solvents did not improve the above constants, neither could crystallisation be achieved. This product was, consequently used in the

next experiment.

4-[1,3-diguanidino-2,5,6-trihydroxycyclohexyl)-oxyethyl β-D-glucopyranoside. (Streptidine-oxyethyl β-D--glucopyranoside).

Undeca-acetylstreptidine-oxyethyl β-D-glucopyranoside (1.05 g.) was dissolved in dry methanol (10 ml.) and deacetylated as described for streptidine β-D-glucopyranoside to yield streptidine-oxyethyl β-D-glucopyranoside (0.41 g., 73%), m.p. 160-162° (decomp.), $[\alpha]_D^{22}$ - 18° (c 2.7 in water).

Found: C, 39.8; H, 6.2; N, 16.4,

$C_{16}H_{32}O_{10}N_6 \cdot H_2O$ requires C, 39.5; H, 7.0; N, 17.3%

The attempts to crystallise the product were without success.

Streptidine-oxyethyl β-D-glucopyranoside Dihydrochloride.

The glucoside (0.15 g.) obtained in the previous experiment was dissolved in dry methanol (3 ml.) and the solution cooled in ice mixture. It was acidified with 2N hydrochloric acid (1.6 ml.) and slowly added to dry ether (50 ml.). The white precipitate was washed with dry ether and dried in a vacuum desiccator yielding the white hygroscopic dihydrochloride, m.p. 190-192° (decomp.). A sample for analysis was dried at 80° in vacuo for three hours.

Found: N, 15.6,

$C_{16}H_{32}O_{10}N_6 \cdot 2HCl$ requires N, 15.5%

Streptidine-oxyethyl β-D-glucopyranoside Dihelianthate.

A solution of methyl orange (0.15 g.) in water (8 ml.) at 70° was added to the solution of the base (0.12 g.) in 80 per cent aqueous methanol (2 ml.) at 40°. The solution was allowed to cool at room temperature when crystallisation commenced and then kept in the refrigerator overnight. The crystals were filtered off and washed with a few ml. of cold water. It was recrystallised from hot 33 per cent aqueous methanol yielding 0.15 g. of the crystalline product, m.p. 240-243° (decomp., micro-block).

Found: C, 46.6; H, 5.9; N, 15.5; S, 6.1,

$C_{44}H_{64}O_{16}N_{12}S_2 \cdot 2H_2O$ requires C, 47.3; H, 6.1; N, 15.1; S, 5.7%

Attempted Condensation of 2-Bromoethyl tetra-O-acetyl--β-D-glucopyranoside and 2-Chloroethyl tetra-O-acetyl--β-D-glucopyranoside with Cyclohexanol.

2-Bromoethyl tetra-O-acetyl-β-D-glucopyranoside[319] (0.477 g.) was added to cyclohexanol (2 ml.) (dried over Na_2SO_4 and distilled). Addition of absolute chloroform afforded a clear solution. Quinoline (4 drops) and silver oxide (0.55 g.) were added simultaneously and the

mixture was stirred for eighteen hours. The silver salts were removed by filtration and washed with absolute chloroform (25 ml.). The chloroform solution on evaporation yielded a clear liquid. The addition of light petroleum (50 ml.) (b.p. 40-60°) to this liquid afforded a white precipitate (0.4 g.) m.p. 115-116° (from ethanol) of unreacted glucoside. The filtrate after precipitation of glucoside was evaporated in vacuo. The viscous liquid gave $\eta_D^{22.5} = 1.469$ (Heilbron[343] gives $\eta_D^{22} = 1.465$ for cyclohexanol)

Similarly starting materials were obtained by using 2-chloroethyl tetra-O-acetyl-β-D-glucopyranoside[319] and cyclohexanol.

Strepturea β-L-Dihydrostreptobiosaminide.

Dihydrostreptomycin sulphate (0.756 g.) in water (5 ml.), was refluxed for one hour[224,286] with 0.35N barium hydroxide solution (25 ml.). The ammonia was distilled off in a stream of nitrogen into a flask containing 20 ml. of N/10 hydrochloric acid and found to be equivalent to two moles. Excess barium hydroxide was precipitated by the addition of solid carbon dioxide and the solution centrifuged, filtered and decolourised (charcoal). The resulting solution was freeze-dried giving a white solid (0.51 g., 84.3%). Micro-crystalline product was obtained from aqueous methanol and ethanol

and dried *in vacuo* at 60° for three hours, decomposed above 280° and had $[\alpha]_D^{19}$ - 90.1° (c 1.32 in water).

Found: C, 40.8; H, 7.5; N, 11.6,

$C_{21}H_{39}O_{14}N_5 \cdot 2H_2O$ requires C, 40.6; H, 7.0; N, 11.3%

Deca-acetylstrepturea β-L-Dihydrostreptobiosaminide

Stretpurea β-L-dihydrostreptobiosaminide (0.26 g.) was suspended in dry methanol (10 ml.) and pyridine (5 ml.). Acetic anhydride (5 ml.) was stirred in slowly. The substance dissolved in about half an hour and the solvent removed *in vacuo* below 50°. The residue was thoroughly washed with dry ether. The white hygroscopic solid was again acetylated in pyridine (5 ml.) with acetic anhydride (5 ml.), the latter being stirred in slowly. The substance dissolved in about 1.5 hour and the solution was kept at room temperature for 40 hours with occasional shaking. The reaction was completed by heating the solution at 45° for 4 hours with stirring. The solvents were removed *in vacuo* below 45° and the residue triturated and washed with petroleum ether (b.p. 40–60°), gave a white solid which was soluble in methanol and partially soluble in chloroform, ethanol and benzene. The product crystallised from methanol–ethanol gave deca-acetylstrepturea β-L-dihydrostreptobiosaminide (0.097 g.), m.p. 256–258°, $[\alpha]_D^{19}$ - 73.3°

(c 0.96 in methanol).

Found: C, 47.4; H, 6.0; N, 7.3,

$C_{41}H_{59}O_{24}N_5 \cdot 2H_2O$ requires C, 47.3; H, 6.1; N, 6.7%

Streptamine β-L-dihydrostreptobiosaminide Trihydrochloride

Dihydrostreptomycin sulphate (5.125 g.) was heated under reflux, over a water-bath with 0.35N barium hydroxide (350 ml.) for twenty four hours.[224,286] The cooled solution was neutralized with solid carbon dioxide, filtered and evaporated *in vacuo* below 50° to yield a pale yellow solid (2.85 g.) which gave a positive test for primary amino group with ninhydrin solution[329] and negative tests for guanidino and urea groups with sodium nitroprusside, potassium ferricyanide solution[330]. The base was purified by adsorption on charcoal (2 g.), impurities being removed by washing with water (3 x 10 ml.) and methanol (3 x 10 ml.). The charcoal was suspended in methanol (15 ml.) at about 10° and treated with N methanolic hydrochloric acid (3 ml.), stirred vigorously for ten minutes, filtered and the charcoal washed with methanol (2 x 10 ml.). The combined filtrates were concentrated *in vacuo* to 10 ml. and added to dry ether. The white precipitate (0.25 g.) was washed twice with dry ether and dried in a desiccator

to yield the trihydrochloride, m.p. 194–196° (decomp.), $[\alpha]_D^{17}$ – 88.6° (c 0.94 in water). A sample for analysis was dried at 80° for three hours.

Found: C, 36.3; H, 6.7; N, 7.2,

$C_{19}H_{37}O_{12}N_3.3HCl.H_2O$ requires C, 36.4; H, 6.75; N, 6.7%

Deca-acetylstreptamine β-L-dihydrostreptobiosaminide

The base (0.3 g.) obtained in the previous experiment was acetylated as for deca-acetylstrepturea β-L-dihydrostreptobiosaminide. The product crystallised from methanol-ethanol gave deca-acetylstreptamine β-L--dihydrostreptobiosaminide (0.192 g.), m.p. 260–262°, $[\alpha]_D^{20}$ – 83° (c 1.0 in methanol). Wolfrom and Polglase[233] give m.p. 261.5–262.5° and $[\alpha]_D^{23}$ – 84°.

4-O-(1,3-diureido-2,5,6-trihydroxycyclohexyl) 2-amino-2--deoxy-β-D-glucopyranoside sulphate. (Strepturea 2-amino-2-deoxy-β-D-glucopyranoside sulphate).

A solution of streptidine 2-amino-2-deoxy-β-D--glucopyranoside sulphate (0.42 g.) in water (3 ml.) and 0.35 N barium hydroxide (15 ml.) were refluxed over a water-bath for one hour. Excess barium hydroxide was precipitated by the addition of solid carbon dioxide. The solution was centrifuged, filtered and decolourised (charcoal). The resulting solution was freeze-dried

giving a white powder which showed the presence of barium salts, though in very small amounts. The substance was, therefore, redissolved in water (2 ml.), cooled and acidified with N sulphuric acid to pH 4.9. The precipitate of barium sulphate was removed by centrifugation. The filtered solution was diluted with methanol (25 ml.) and precipitation of the sulphate was facilitated by the addition of dry ether (100 ml.) and by keeping in the refrigerator overnight. The ether solution was decanted and the residue was washed with dry ether and dried *in vacuo* over phosphorus pentoxide to yield the product (0.25 g., 71%), m.p. decomposed slowly above 220°.

Found: C, 32.6; H, 5.9; N, 13.8,

$C_{14}H_{27}O_{10}N_5 \cdot \frac{1}{2} H_2SO_4 \cdot 2H_2O$ requires C, 33.0; H, 6.3; N, 13.7%

4-O-(1,3-diamino-2,5,6-trihydroxycyclohexyl) 2-amino-2-deoxy-β-D-glucopyranoside. (Streptamine 2-amino-2-deoxy-β-D-glucopyranoside).

Streptamine 2-amino-2-deoxy-β-D-glucopyranoside (0.452 g.) was heated under reflux over a water bath with 0.35N barium hydroxide (50 ml.) for twenty four hours. Excess of barium hydroxide was precipitated with solid carbon dioxide. The precipitate was centrifuged and the solution filtered. The filtrate was

freeze-dried. The amorphous substance showed the presence of primary amino groups with ninhydrin solution[329] but gave negative tests for guanidine and urea groups with sodium nitroprusside, potassium ferricyanide solution.[330] The freeze-dried substance was redissolved in water (5 ml.) and the solution cooled. Traces of barium salts were removed by acidification with N/10 sulphuric acid to pH 4. Barium sulphate was centrifuged and the solution was decolourised (charcoal). The colourless solution was passed through a small column of De-Acidite FF (5 g.) and the column was washed with water (10 ml.). The combined eluates were freeze-dried giving a white amorphous base (0.181 g., 67%), m.p. 160-162° (decomp.). A sample for analysis was dried at 80° in vacuo for three hours

Found: C, 42.2; H, 7.1; N, 12.5,

$C_{12}H_{25}O_8N_3$ requires C, 42.5; H, 7.4; N, 12.4%

Attempted Anomerization of Dodeca-acetyldihydrostreptomycin

(a) A freshly distilled solution of boron trifluoride-etherate[335] complex in absolute chloroform (10 ml.) was added to a solution of dodeca-acetyldihydrostreptomycin (0.102 g.) in absolute chloroform (10 ml.). There was only a slight decrease in optical rotation over a period

of eighteen hours. The chloroform solution was washed with cold water, cold saturated solution of sodium bicarbonate and dried (Na_2SO_4). Removal of the solvent and purification of the residue gave only starting material m.p. 150-153° (decomp.), $[\alpha]_D^{20}$ - 60° (in chloroform).

(b) A solution of stannic chloride[336] (0.0279 g.) in absolute chloroform (10 ml.) was added to dodeca-acetyldihydrostreptomycin, (0.2 g.). The precipitate which separated after two days was removed by centrifuging and the chloroform washed with ice-cold water and dried (Na_2SO_4). Removal of the solvent and precipitation of the residue in acetone with petroleum ether (b.p. 80-100°) gave a product which did not melt below 300° and was not, therefore, investigated further.

(c) A 4 per cent solution of titanium tetrachloride[336] in chloroform (2 ml.) was added to dodeca-acetyldihydro-streptomycin (0.201 g.) in absolute chloroform (2 ml.). The mixture was refluxed for six hours, cooled, poured on to ice and extracted with chloroform. Removal of the solvent gave only impure starting material.

(d) Dodeca-acetyldihydrostreptomycin (0.1 g.) in acetic anhydride (2 ml.) was treated with a mixture of

concentrated sulphuric acid (0.25 ml.) in acetic anhydride[337] (*ca.* 2.3 ml.) and the volume adjusted to 5 ml. with acetic anhydride. The optical rotation reached a constant value after an hour and a half. The reaction mixture was poured on to the crushed ice (150 g.) containing sodium acetate (2.5 g.) and extracted with chloroform as before. Removal of the solvent and extraction of the residue with dry ether gave tetra-acetylbisdeoxystreptobiosaminide[344] (24 mg.) m.p. 159-161°, $[\alpha]_D^{20}$ - 85.8° (c 0.8 in chloroform) and a very small ether-insoluble portion which was probably hepta-acetylstreptidine, $[\alpha]_D^{20}$ - 3° (methanol).

(e) Drierite (1.01 g.) and sodium hydroxide[338] (0.111 g.) was shaken in a mixture of dry dioxane (5 ml.) and dry ether (5 ml.). Dodeca-acetyldihydrostreptomycin (0.1 g.) was added and the whole shaken for a further period of eight hours. The dioxane-ether insoluble portion was taken up in dry methanol (10 ml.) containing acetic anhydride (2 ml.) and filtered. Evaporation of the filtrate to dryness gave a crude residue containing sodium acetate and some partially deacetylated material from which 60 mg. of starting material m.p. 148-150°, $[\alpha]_D^{20}$ - 62° (in chloroform) was recovered on reacetylation with acetic anhydride in pyridine.

B A C T E R I O L O G I C A L

R E S U L T S

Tuberculostatic Activity of Streptomycin Derivatives.

The *in vitro* evaluation of the antitubercular activity of some of the compounds synthesised in this work was carried out by Glaxo Laboratories Ltd. to whom the author wishes to express his thanks.

Each compound was dissolved in water (1000μg. ml.), the solution diluted in Dubos' liquid medium and the dilution inoculated with *Mycobacterium tuberculosis* (human strain 666). The results were recorded after fourteen days incubation at 37° and again after twenty eight days and are shown in tables 2 and 3. For the purpose of comparison, the test was also carried out on streptomycin.

Table 2

Inhibition of Mycobacterium tuberculosis in

Compound
Streptomycin (XXIa)
N-acetylstreptomycin(XXXVIIIc)
N-acetyldihydrostreptomycin (XXXVIIIb)
N-acetyltrityldihydrostreptomycin (XXXVIIIa)
Undeca-acetylstreptomycin (XXXVIIa)
Dodeca-acetyldihydrostreptomycin (XXXVIIc)
Streptidine β-D-glucopyranoside (LVIII)
Streptidine 2-amino-2-deoxy-β-D-glucopyranoside sulphate (LXI)
Streptidine 2-N-methylamino-2-deoxy-β-D--glucopyranoside sulphate (LXXIX)
Streptidine 2-N-methylamino-2-deoxy-β-L--glucopyranoside trihydrochloride (LXXX)
Streptidine-oxyethyl β-D-glucopyranoside (XC)
Strepturea dihydrostreptobiosaminide (XXVII)

Dubos' Liquid Medium after incubation at 37° for 14 days.

Concentration μg./ml.											
100	50	25	12.5	6.25	3.12	1.56	0.78	.4	.2	.1	.05
-	-	-	-	-	-	-	-	-	-	++	++
-	-	-	-	-	-	-	+	+	++	++	++
-	-	-	-	-	-	-	+	++	++	++	++
-	-	+	+	++	++	++	++	++	++	++	++
++	++	++	++	++	++	++	++	++	++	++	++
++	++	++	++	++	++	++	++	++	++	++	++
-	-	+	++	++	++	++	++	++	++	++	++
++	++	++	++	++	++	++	++	++	++	++	++
++	++	++	++	++	++	++	++	++	++	++	++
++	++	++	++	++	++	++	++	++	++	++	++
++	++	++	++	++	++	++	++	++	++	++	++
-	-	-	±	+	++	++	++	++	++	++	++

Table 2 (contd.)

Compound
Streptamine dihydrostreptobiosaminide-trihydrochloride (XXVIII)
Strepturea 2-amino-2-deoxy β-D-glucopyranoside sulphate (XCII)
Streptamine 2-amino-2-deoxy β-D-glucopyranoside (XCIII)

Table 3

Inhibition of Mycobacterium tuberculosis in

Compound
Streptomycin (XXIa)
N-acetylstreptomycin (XXXVIIIc)
N-acetyldihydrostreptomycin (XXXVIIIb)
N-acetyltrityldihydrostreptomycin (XXXVIIIa)

Concentration μg./ml.											
100	50	25	12.5	6.25	3.12	1.56	0.78	.4	.2	.1	.05
++	++	++	++	++	++	++	++	++	++	++	++
++	++	++	++	++	++	++	++	++	++	++	++
++	++	++	++	++	++	++	++	++	++	++	++

Table 3

Dubos' Liquid Medium/after incubation at 37° for 28 days.

Concentration μg./ml.											
100	50	25	12.5	6.25	3.12	1.56	0.78	.4	.2	.1	.05
-	-	-	-	-	-	-	-	±	+	++	++
-	-	-	-	-	-	+	++	++	++	++	++
-	-	-	-	±	+	+	++	++	++	++	++
-	+	++	++	++	++	++	++	++	++	++	++

Conclusion

The results recorded in table 2 and table 3 show that whereas N-acetylstreptomycin and N-acetyldihydrostreptomycin are somewhat less active than streptomycin itself, activity is significantly less in N-acetyltrityldihydrostreptomycin. This, therefore, lends further support for the view that microbiological oxidation of dihydrostreptomycin to streptomycin is a pre-requisite for the activity of the former and the streptose-aldehyde group is essential for the activity of the latter.

Substitution of the streptobiosamine fragment in the streptomycin molecule by such carbohydrates as D-glucose, D-glucosamine, N-methyl-α-D-glucosamine, N-methyl-α-L-glucosamine and 2-hydroxyethyl β-D-glucoside, leads to the abolition of tuberculostatic activity. This further stresses the importance of the streptose unit for the activity of the antibiotic. Further, the conversion of guanidino groups to either ureido or amino groups abolishes the activity of dihydrostreptomycin. As was anticipated, none of the acetylated intermediates showed significant activity.

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