Summary of Thesis presented in fulfilment of the requirements for the Degree of Doctor of Philosophy of Glasgow University.

# "THE CHEMISTRY OF SOME STREPTOMYCIN DERIVATIVES"

by

IAIN J. McGILVERAY

"The Chemistry of some Streptomycin Derivatives"

The history of the chemotherapy of tuberculosis is reviewed, with emphasis on the newer experimental drugs such as ethambutol, kanamycin and 4,4'-di-isoamyl oxythiocarbanilide. The recognised regimens in tuberculo-therapy are discussed briefly in order that the current status of streptomycin may be appreciated.

The chemistry of the aminoglycoside antibiotics is reviewed in detail, evidence of the recently assigned structures of the neomycin-paromomycin group and the kanamycins being cited as well as the more classical degradation and structural determination of streptomycin. Suggested biogenetic pathways and recent mode of action studies are noted, the introduction being completed by an inventory of synthetic derivatives of this group of antibiotics.

The remaining points of the chemistry of dihydrostreptomycin to be elucidated are discussed. This includes confirmation of the configurati of the glycosidic linkages, and direct proof of the nature and ring form of dihydrostreptose, the central moiety of dihydrostreptomycin.

Early workers degraded the trisaccharide dihydrostreptomycin with methanolic hydrogen chloride into the diguanidinoinositol, streptidine, and the methyl glycoside of the glucosaminide, dihydrostreptobiosaminide, the latter is composed of the methyl lyxofuranoside, dihydrostreptose, linked glycosidically to the hexosamine, N-methyl-L-glucosamine. Furthe acid degradation, however, led to destruction of the dihydrostreptose and this was not isolated.

D-glucosaminides are known to be very stable to acid hydrolytic conditions, a stability attributed to the positive charge on the amino nitrogen group causing repulsion of the proton and suppressing formation of the intermediate carbonium ion. N-Substitution of the methyl D-glucosaminides with an electron withdrawing group has been shown to facilitate glycosidic cleavage; the formation of an electronically neutral substituted amino group, such as the acetamido group, apparently allowing protonation of the glycosidic bond. Hydrolysis of the N-substituted glucosaminides, however, is accompanied by removal of the substituting group, and the rate at which this occurs at the expense of glycosidic hydrolysis is apparently dependent on the electrophilic properties of the substituting group.

In this present work dihydrostreptose was obtained from the L-glucosaminide methyl dihydrostreptobiosaminide by application of the above theories. The N-(2,4-dinitrophenyl)-(DNP), N-acetyl- and, N-tosyl- derivatives of methyl dihydrostreptobiosaminide have been prepared, the N-acetyl derivative has been obtained by a variety of routes. Samples of N-methyl-D-and L-glucosamine and some novel N-acyl derivatives have also been prepared for comparison by paper chromatography with various hydrolysis products.

The acid hydrolysis of methyl N-(2,4-dinitrophenyl)-dihydrostreptobiosaminide has been investigated in detail; paper chromatographic result showed that hydrolysis had occurred with 2N hydrochloric acid. The hydrolysis of methyl N-acetyldihydrostreptobiosaminide yielded a small sample of dihydrostreptose, which was characterised as the free sugar and

the monoacetate. Methyl N-acetylstreptobiosaminide dimethylacetal the analogous derivative of streptomycin under the same acid conditions gave only N-methyl-L-glucosamine, the streptose moiety being degraded.

Deamination of methyl dihydrostreptobiosaminide with 1,2,3-indane trione hydrate was attempted unsuccessfully, as was cation-exchange hydrolysis of this compound.

Enzymatic cleavage of methyl N-acetyldihydrostreptobiosaminide was also unsuccessful under the conditions tried.

The second section of the thesis is devoted to glycosides of dihydrostreptobiosaminide. Methods of glycosidation, the Koenigs-Knorr, the Helferich and the Fischer syntheses are reviewed. Transglycosidation the acid-catalysed replacement of the methyl by another aglycone was attempted with methyl dihydrostreptobiosaminide and yielded the benzyl, phenyl, β-bromoethyl, cyclohexyl, m-cresyl and anisyl glycosides. No mechanism has heretofore been proposed for this substitution. An examination of its relation to the Fischer glycoside synthesis suggests that the furanose ring of dihydrostreptose would facilitate the reaction and that the ease of glycosidation provides indirect evidence of the nature of this ring in dihydrostreptobiosaminide.

The phenyl and β-bromoethyl dihydrostreptobiosaminides have been tested in vitro against Mycobacterium tuberculosis.

Nuclear Magnetic Resonance studies of various dihydrostreptomycin derivatives are also interpreted.

# THE CHEMISTRY OF SOME STREPTOMYCIN DERIVATIVES

T H E S I S

submitted to

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IAIN J. McGILVERAY

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C O N T E N T S.

APPENDIX II

## INTRODUCTION

Tuberculosis has been a major scourge of mankind throughout recorded history but a dramatic decline in mortality from this disease has been evident in the Western World over the past two decades. The death rate per 100,000 from all forms of tuberculosis fell in Scotland from 64 in 1941 to 37 in 1951 and 8.5 in 1961[1]. In England and Wales over the same period tuberculosis deaths regressed from over 28,000 to 13,800 and 3,300. There were 45,000 notified cases in 1951 falling to 22,000 in 1962[2].

This decline represents a joint triumph for chemotherapy and public health. It can be attributed both to the introduction of the major antituberculous drugs and to the extensive mass radiography campaigns contributing to early diagnosis of pulmonary tuberculosis. No finer tribute may be paid to the success of this work than the closure of many tuberculosis sanitoria throughout the Western World.

On the distaff side however there are the great human problems of the East where living conditions with malnutrition aid the dissemination of this wasting disease. Diagnosis and treatment are often too late and generally too expensive. Statistics for 1960 give the respective death rates per 100,000 caused by tuberculosis as 35 in Japan 64 in Hong Kong and for comparison 8.9 in[3] England and Wales.

Again there is the problem of resistance where the causative organism Mycobacterium tuberculosis develops strains intractable to all three major drugs. Newer drugs such as cycloserine are more toxic, a serious factor in the long term administration necessary to combat the disease, and the present mortality rate still leaves much to be desired.

It is evident that the chemotherapy of tuberculosis has not been as successful as with most other infections. The need for an agent which would be effective on short term therapy and lead to eradication of the disease is long felt. The examination of the chemistry of antitubercular antibiotics in complete stereochemical detail has hitherto been mainly of academic interest. According to Waksman[4] eventually a firmer link must be forged between structure and mode of action, before chemotherapy can become a completely rational discipline. The work of this thesis it is hoped will be a contribution towards this end.

# TUBERCULOSIS

## The Causative Organism

Mycobacterium tuberculosis the causative organism of tuberculosis, first described and isolated by Koch[5] in 1882 in his classical treatise, belongs together with the leprosy bacillus Myco. leprae to the family Mycobacteriaceae. The organisms are Gram-positive, essentially pathogenic, non-motile, aerobic, non-spore forming rods; they are readily distinguished on staining by their unique acid-fastness, the tuberculosis bacillus being the most strongly acid fast of the genus[6].

There are several kinds of tubercle bacilli varying according to the animal infected, the more important being var. hominus and var. bovis both of which are infective to man. In 1937 a third mammalian type murine bacillus was isolated[7], which has been utilised for vaccines (Vole vaccine)[8,9].

The property of acid fastness reflects fundamental underlying chemical properties of the organism which have been studied more extensively perhaps, than for any other micro-organism. It is attributed to a lipopolysaccharide composed of a mycolic acid (q.v.) and a complex carbohydrate[10a]. The characteristic is most pronounced in the lipoids of the cytoplasmic membrane and in the internal granules of the fixed cell[11]. Some younger cells lack this property which is

most pronounced in mature forms[12].

The unclassified Mycobacteria are a source of increasing concern. Since they interfere with diagnosis and may even cause similar lesions to Myco. tuberculosis but be resistant to chemotherapy[13,14,15].

The Disease

The host tissues which usually become infected with Myco. tuberculosis via the respiratory tract[16] normally react against the organism in characteristic fashion. The bacilli are first ingested by a normal monocyte, which alters to form an epitheloid cell. Several of these form a cluster within 3 to 4 days representing the beginnings of a tubercle, the typical lesion of the disease. If the hosts resistance allows, this cluster grows progressively causing pressure to be exerted on surrounding host cells, which thereby die of nutritional deficiencies. This has been studied in the rabbit ear chamber[17]. Numbers of adjacent tubercles coalesce, and the enclosed host tissue dies and becomes necrotic. This tissue which would be expected to undergo liquifaction by means of phagocytic enzymes only undergoes partial autolysis to a cheese like mass caseation. The mechanism of caseation however is not fully understood. Sabin[18] and her colleagues showed that injection of certain lipid fractions from Myco. tuberculosis caused a similar formation of caseous tissue to occur, though as Rich[19] remarks, the amounts of lipid

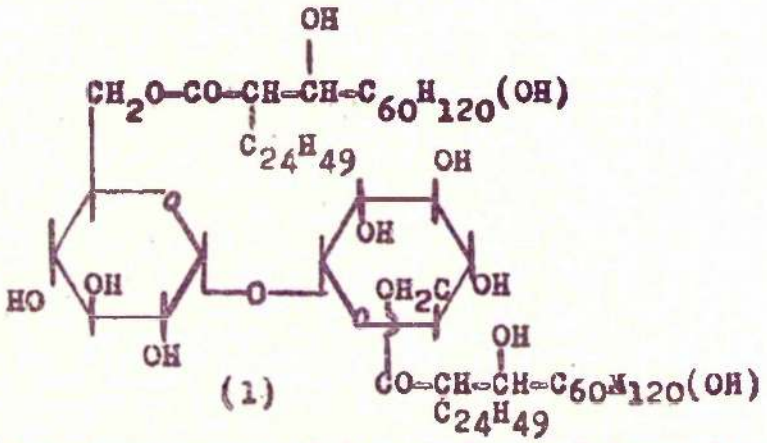
employed in these experiments are much greater than those present in the tubercle. Caseation may rapidly involve the lung and other infected organs, and if the tubercle is in the lung the necrosis may extend until it invades and breaks through a wall or bronchus. Caseous material containing millions of living bacilli is then discharged with the sputum by coughing, and a large cavity is left behind. The disease is then said to have reached the cavitation stage. The cavity generally tends to heal leaving a scar, which becomes fibrous (fibrosis), the main constituent being collagen. Later the cavities may calcify to leave life long evidence of the occurrence of disease - the calcification stage, which often signifies approach towards arrest[10b].

The nature and formation of the tuberculous lesions has been described and reviewed elsewhere[10c,19b,20].

Brownlee has reviewed the biochemical reactions concerned in the exceedingly complex host-parasite relation[20]. Four major factors are involved.

Cord factor: Tubercle bacilli contain a lipid substance which enables them to reproduce and establish tubercles in the host. This substance named "cord factor" by Bloch[21] has been the subject of extensive chemical studies, mainly by Lederer and his associates and was reviewed in 1961[22]. The product isolated from the wax fractions of the bacillus, (Strains $H_{37}R_v$ and B.C.G.) is purified by repeated

chromatography on a silica column yielding an almost colourless wax, melting at 40°C $[\alpha]_D^{25}$ +30[23,24]. Dogradation studies showed the presence of two molecules of a high molecular weight, $\beta$ -hydroxy acid with a long $\alpha$ side chain a "mycolic acid" characteristic of Mycobacteria[25] together with one molecule of $\alpha$-trehalose a non-reducing glycoside. Further painstaking infrared and chromatographic studies revealed the structure of "cord factor" as 6,6'-di-O-mycoloyl-$\alpha$,$\alpha$-trehalose (1)[26].




(1)

Various syntheses of "cord factor" have been achieved which give biological activity indistinguishable from the natural product[27,28].

Chemotactic Substances: Tubercule bacilli contain substances which resist degradation by the body's ordinary defence mechanism. Instead of being ingested by polymorphs and carried into the lymph nodes for digestion, they are absorbed in situ by monocytes which may be turned, along with neighbouring monocytes into a tubercle. The specific substances involving the chemotaxis of monocytes has not been pinpointed. Work by Sabin[18] using various lipid fractions of Mycobacteria observed alteration of monocytes. It is now known that

the branch chain fatty acids present in the lipopolysaccharide eg. mycolic acid are active in this way. Delauney and his co-workers demonstrated that the peptide-glycolipid Wax D produces similar cellular modifications[29]. According to Brownlee this may be a non specific effect of the fatty acids[20].

Hypersensitivity Response: The complex delayed hypersensitivity response typified by the Tuberculin Reaction.

Multiplication of the bacillus releases a product of metabolism which induces hypersensitivity of adjacent cells with the result that an otherwise innocuous product becomes a poison responsible for the death of cells. This remarkable host-parasite relation is responsible for most of the clinical manifestations of the disease.

Choucron[30] and Raffel[31] established that a mixture of purified chloroform soluble wax together with protein from the bacillus induced on injection to guinea pigs, the tuberculin type of sensitivity to the protein. The protein from which the wax was removed failed in the induction of the response[32]. From further studies by Raffel in association with Lederer[33] into the nature of the adjuvant wax it was concluded that Wax D lipopolysaccharide composed of mycolic acids esterified with polysaccharide were most active. Raffel has reviewed hypersensitivity response in detail (1953)[34].

The toxicity of "cord factor" also plays an important role in lowering host resistance. A single small injection of "cord factor", which by itself has no deleterious effect, causes tuberculous infections to progress more rapidly than in controls, mice being the test animals[35]. A large injection (5 - 10 µg) of "cord factor" or a series of smaller ones kills the adult mice within 5 to 8 days, pulmonary damage being the most conspicuous symptom.

Acquired Resistance: This may develop in the host to modify the course of the disease. The labile antigen which reduces this resistance is not as yet unidentified.

## THE CHEMOTHERAPY OF TUBERCULOSIS.

The organism Mycobacterium tuberculosis is well known for its slow rate of division[36] and also for its ability to enter a resting phase of metabolism[19c]. As the antituberculous drugs are all active on growing organisms, being bacteriostatic rather than bacteriocidal, this constitutes a major problem in chemotherapy of the disease and is responsible for relapses. The early concept of a continous protective lipoid capsule to explain the ability of the organism to survive in adverse conditions e.g. acidic and alkaline has been rejected, as many water-soluble, non-lipoid-soluble molecules are ingested for metabolism[20] and all effective tuberculosats known are associated with water-solubility rather than lipoid-solubility[37].

The lesions of tuberculosis where the bacilli are fairly central require that an effective drug has sufficient diffusability to penetrate to this site of action. Many studies have been devoted to this requirement[10d]. In general, adequate blood supply is a most important factor. Recent tubercles are more susceptible as they have greater blood circulation and therefore higher drug concentration. In caseous tissue there is no blood supply and penetration is mainly dependent on passive diffusion.

Having achieved an effective concentration in tissue the agent has then to enter the bacterial cells within the phagocytes in inhibitory concentrations. Mackaness[38] using tissue culture techniques

showed that much higher concentrations of drug are necessary to inhibit the growth of intracellular bacilli than those in tissue fluids, though some drugs possibly due to diffusion characteristics, are more effective than others in this respect.

The criteria for the ideal antituberculous drug may be summarised as follows.

1. It should be toxic for the tubercle bacillus, non-toxic for the host.
2. It should be readily administered preferably orally.
3. It should enter the tubercle and attain the site of action in tubercle bacilli avoiding complications of the disease.
4. It should assist the patient to achieve the non infective state and prevent relapse.
5. The agent should accomplish these ends as rapidly as possible.

The drugs at present available for the treatment of tuberculosis depart from these criteria in at least three ways; they are suppressive rather than eradicative making long term treatment necessary (over two years after the disappearance of active tuberculosis); resistant strains of the tubercle bacillus emerge making the value of subsequent treatment doubtful; they are frequently ineffective against chromogenic strains a variety showing up more often. Fitzpatrick[39] has been searching for drugs for short term therapy in experimental tuberculosis, a fresh approach which has not yet had marked success.

## History of the Chemotherapy of Tuberculosis

Table 1.

| | | |
|---|---|---|
| 1882 | Causative organism discovered | Koch |
| 1938 | Sulphonamides tested in experimental tuberculosis | Rich |
| 1939 | Dapsone ameliorative in experimental tuberculosis | Rist, Buttle. |
| 1942 | Promin (Sulphone) found effective in guinea pig tuberculosis. | Feldman |
| 1944 | Streptomycin very effective in human tuberculosis | Wakeman |
| 1946 | P.A.S. effective against human tuberculosis | Lehmann |
| | Amithiozone (Tibione) active against human tuberculosis | Various Workers |
| | | Domagk |
| 1951 | Isoniazid highly effective in human tuberculosis | Various Workers |
| | Viomycin found to be active in human tuberculosis | Finlay |
| 1952 | Pyrazinamide effective against human tuberculosis | Kushner |
| 1955 | Cycloserine proved active against human tuberculosis | Harned, Epstein. |
| 1958 | Kanamycin active against tuberculosis | Umezawa |
| 1959 | Ethionamide shown active against human tuberculosis | Brouet |
| 1960 | 4,4-isoAmylthiocarbanilide active against human tuberculosis | Buu-Hoi |
| 1961-2 | Ethambutol in experimental murine tuberculosis | Wilkinson et al. |

## HISTORICAL.

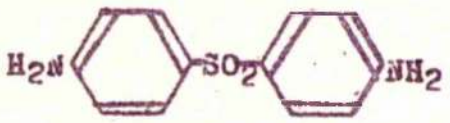
The search for an agent capable of inhibiting the organism or arresting the disease without undue toxicity to the host began at the time of Koch's[5] discovery of *Mycobacterium tuberculosis* as the causative organism. Ehrlichs pioneer contribution to antimicrobial chemotherapy with the antispirochaete arsenical Salvarsan intensified this search, he himself experimenting with many dyestuffs, but to no avail.

The time-lapse before the advent of the sulphonamides, *via* dyestuffs twenty five years later[40] masks the extensive detailed fundamental studies of the organism and the disease as well as the despairing efforts to find curative properties in anything with demonstrated activity against any disease.

Even into the 1930's such medieval forms of treatment as mud baths[41], raw spleen[42], intravenous charcoal[43], were being quoted as alleviating the disease. Gold and other heavy metals salts[44] vitamins, calcium salts and quinine were among the more conventional forms of therapy[45] but none of the host of agents tried was shown to be, unequivocally antituberculous.

With the introduction of the sulphonamides, many bacterial diseases came under effective control for the first time. The sulphonamides were tested both *in vitro* and *in vivo* against *Mycotuberculosis*, and sulphathiasole in particular, according to Domagk's results[46] was shown to exhibit some activity. They all proved too toxic however for clinical use in tuberculosis, but there is little doubt that the stimulus provided by this partial success heralded the modern era of chemotherapy of tuberculosis.

At this time many laboratories were working on sulphonamide like compounds and attention was focussed on the related sulphones, typified by the parent compound, 4,4'-diaminodiphenylsulphone (11) (Dapsone). Buttle and Rist[47,48] in 1939 demonstrated that this agent was effective both *in vitro* and *in vivo* against the hitherto

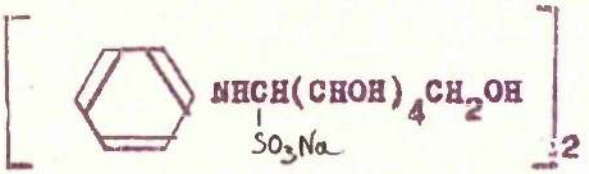


(11)

impregnable tuberculosis bacillus. Dapsone proved too toxic for clinical use as trials were still in progress when other more effective agents were discovered, but it is the most effective antileprotic known.

The manufacturing laboratories attempted to produce less toxic and more effective derivatives of Dapsone. A number of compounds of limited clinical value were formed by substitution of the amino groups, the more important of which were, Promin[49] (sodium p,p'-diaminodiphenylsulphone-N,N'-didextrose sulphonate (111), and

sulphetrone[50] tetrasodium 4,4-bis ( $\gamma$-phenylpropylamino)-diphenyl-sulphone-$\alpha,\gamma,\alpha,\gamma$-tetrasulphonate (IV). These are said to act by conversion *in vivo* to the parent Dapsone and are less toxic but less active[51]. The most effective of the sulphones Promisole reported in 1944[52] was formed by substitution of a thiazole ring in place of one of the phenyl groups of the parent compound giving 4,2' -diaminophenyl-5-thiazolylsulphone[53] (V), this was used clinically for a short time.

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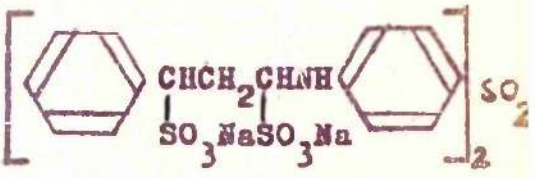


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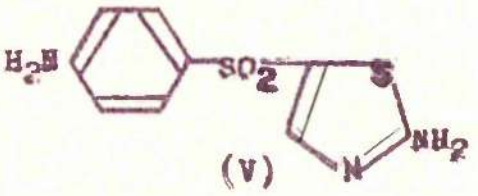
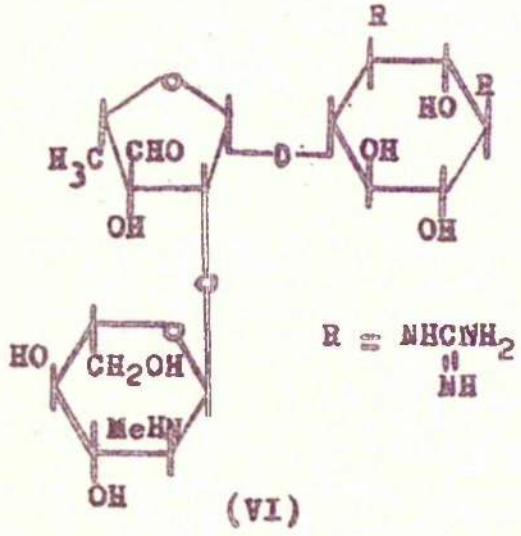



(V)

At this time another series of researches came to fruition. In 1940 the National Tuberculosis Association (U.S.A.) had financed studies by S.A. Waksman of Rutgers to obtain by special procedures, soil organisms antagonistic to the bacillus[54]. After a screening programme for antimicrobial metabolites, amongst the class *actinomycetes* (which has since become a model), Waksman and his colleagues in 1944[55] obtained a crude concentrate from the culture filtrates of *Streptomyces griseus* which was effective *in vitro* against a variety of organisms including *Myco. tuberculosis*. Its low toxicity[56-59] indicated that it would be effective against typhoid, brucellosis and

tuberculosis. These early hopes were borne out in the clinical trials by Feldman[60] and also by the British Medical Research Council[67]. Thus streptomycin became the first relatively non-toxic effective anti-tuberculous chemotherapeutic agent. The details of the chemistry, biosynthesis and mode of action of streptomycin (VI) will be given elsewhere together with the related antibiotics Neomycin, Paromomycin and Kanamycin.

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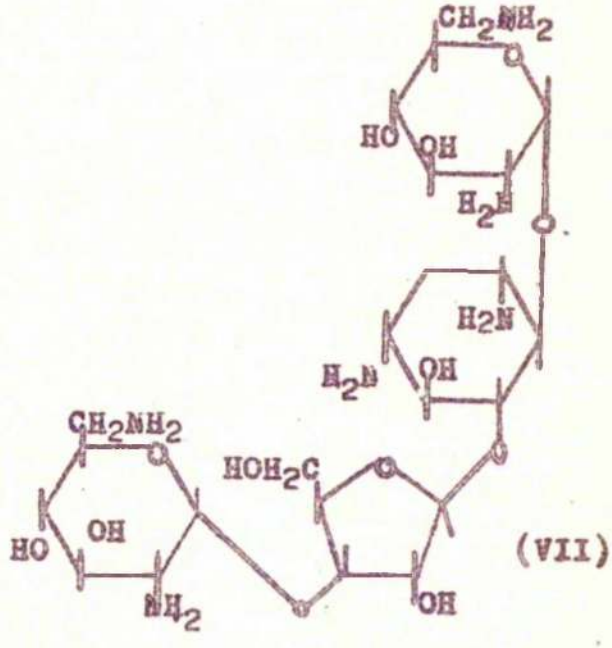
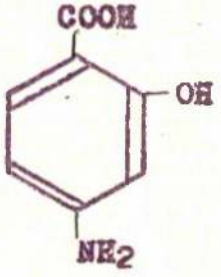



(VII)

A second antibiotic derived from this screening programme was neomycin (VII). It was found active <u>in vitro</u> and <u>in vivo</u> against mycobacteria[61] However its severe toxicity has precluded its clinical use in tuberculotherapy but on account of its close chemical similarity to streptomycin it is of interest.

Also in 1944 as the culmination of six years' work commencing with the observation by Bernheim[62] that the oxygen consumption of tubercle bacilli was increased by salicylates and benzoates, it was announced that the synthetic compound p-aminosalicylic acid (P.A.S.) (VIII),

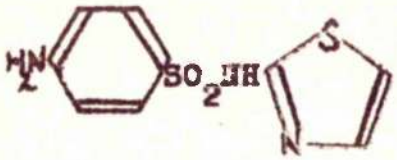
was effective against human tuberculosis. This was the best of a number of benzoic acid substitution products tested by Lehmann[63].




(VIII)

P.A.S. was not long used in monotherapy of tuberculosis as it was found to be only weakly active and resistant strains emerged fairly rapidly. The optimum dose was not determined but initially 10 to 15 grammes per day was found effective and practical[64], in pulmonary disease . It has an established place as a major drug in tuberculotherapy its principal value being as a companion drug to other agents such as streptomycin[65,66,67] whose action it appears to prolong and enhance. The drug is only mildly toxic - the fact that 20 g. may be consumed daily is so indicative; the sodium salt is better tolerated than the free acid; the most common toxic disturbance is digestive upset. More serious rare side effects which include hypokalaemia[68], goitrogenic effects[69], severe allergic reaction[70], and liver damage eg. jaundice occur[71].

The discovery of the effective but toxic thiosemicarbazone antituberculars by Domagk and his associates in 1946[72] stemmed from his investigations of the limited <u>in vivo</u> tuberculostatic activity of the sulphonamides. He had found that sulphathiazole (IX) and the related sulphathiadiazoles (X) were most active[46,73], and



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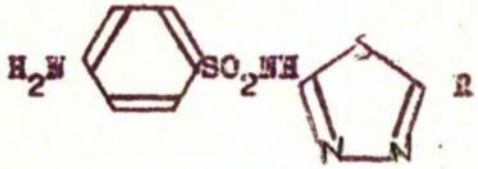

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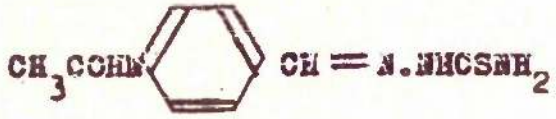


(X1)

he believed that the greater activity of these sulpha compounds was a function of the thiazole ring. Further German investigators (Behnisch) responsive to this argument, found that the open chain arrangement of the nitrogen and sulphur atoms was equally or more effective[74]. A number of compounds were produced some of which were very active but also highly toxic. Domagk found that less toxic compounds were less active bacteriologically whilst more active compounds seemed more toxic[75]. Amithiazone (Tb.I, Tibione) (X1) was the best of the compounds investigated, this being used clinically especially in Germany in doses of about 200 mg daily, but its toxicity which included gastro intestinal effects, and severe liver damage, caused the decline in its use[8].

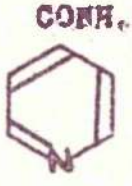
This line of research culminated in 1951-52 with the simultaneous announcement by three laboratories of the most effective antitubercular to date, Isonicotinic acid hydrazide (isoniazid I.N.A.H.)[77,78,79](X11). Fox in attempting to prepare isonicotinaldehyde thiosemicarbazone (X111) used I.N.A.H. as an intermediate and found[80] that this intermediate

first described in 1912[81] was very active against Myco. tuberculosis


(XII)


(XIII)

Fox[82] has reviewed the structure antitubercular activity relationships of I.N.A.H. and related compounds. Many attempts have been made to modify the structure of I.N.A.H. improving on its action, but none of the derivatives has become an accepted drug. For a time iproniazid[83] (XIV) was shown to be therapeutically valuable but later proved too toxic for routine use[84].




(XIV)


(XV)

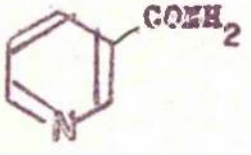
An interesting compound prepared by condensing I.N.A.H. through the aldehyde group of streptomycin is streptohydrazid[85] (XV). This was shown to be effective and well tolerated in a brief study on a small number of patients[86].

The therapeutic efficiency of I.N.A.H. has been well documented (McDermott[87], Crofton[88], Middlebrook[89], Ferrebee[90], M.R.C.[91]) both alone and as a companion drug with P.A.S. or streptomycin. It is the most effective of the major drugs by itself, the dose being about 300 mg. daily[87] though, due to development of resistant strains,

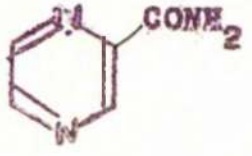
monotherapy is no longer recommened.

The most commonly observed toxic effect is peripheral neuritis which is more frequent in high doses[92]. Various observers after noting that these were the symptoms of $B_6$ (pyridoxine) deficiency have administered this vitamin as an adjunct to I.N.A.H. therapy. McDermott[87] however suggests that although the reaction between I.N.A.H. and $B_6$ to give a hydrazone is easily reversible it results in antimicrobial deactivation.

Fox[93] has written that one of the pathways of investigation which led to the discovery of I.N.A.H. was an investigation of the antitubercular activity of the vitamin nicotinamide (XVI) first observed in 1945[94,95]. These studies led Kushner and his



(XVI)



(XVIa)

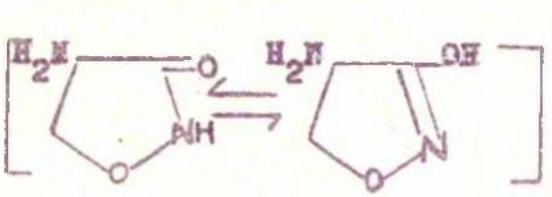
coworkers to prepare the pyrazine analogue[96] (XVIa) which was found fairly active. This drug used alone is of limited value, but it is of great effect in combination with I.N.A.H. or streptomycin. McDermott[87] states that it is probable that pyrizinamide containing regimens are most active of all used and cause no gastro intestinal upsets. Unfortunately about 3% of patients suffer from hepatitis and jaundice and 1% are fatal. For this reason it is only employed

at a dose level of 20 mg./Kg. in cases resistant to the main drugs.

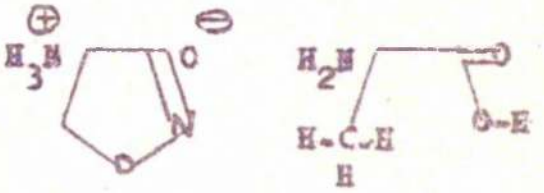
The year 1951 also witnessed the discovery of a second antibiotic antitubercular viomycin which was later found to be a group of substances. This group consist of metabolites of a number of actinomycetes, Streptomyces puniceus[97], S. floridae[98], and S. vinaceus [99] The chemistry of this complex has yet to be fully elucidated but it probably contains three components Vinactins A,B and C[100], which are strongly basic and possibly cyclic polypeptides. Viomycin is largely Vinactin A[101]. It occurs as a polyacidic base $C_{17-18}H_{31-33}N_9O_8$ marketed as the crystalline sulphate, very soluble in water. Hydrolysis products yield several amino acids together with a guanidine positive (Sakaguchi) and creatinine group[102]. It is stable at pH 6 for a week in aqueous solution in which form it is administered as intramuscular injection. It has only moderate activity in tuberculosis, and is less active than streptomycin[103]. Use is limited to cases where organisms are resistant to the major drugs, careful attention being paid to toxic signs[104]. Toxicity includes renal effects, impairment of vestibular function and deafness[103].

A third antitubercular antibiotic cycloserine (XVII) introduced in 1955 has found limited use in cases resistant to the major drugs. Derived from Streptomyces orchidaceous[105] as well as other streptomyces species. Kuehl and his associates[106], identified it as

D-4 amino-3-isoazolidinone. Synthesis of cycloserine and several of its analogues has been accomplished[106,108].



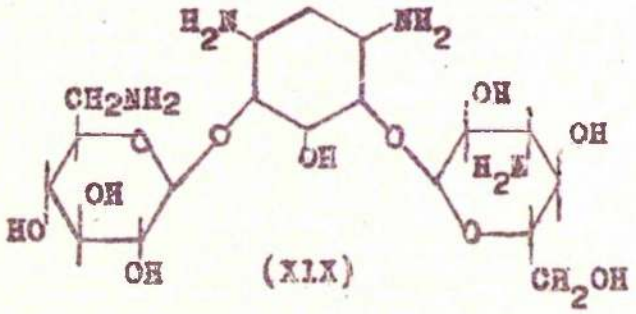
(XVII)



(XVIII)

Soluble in water, stable in alkali but very unstable in neutral or acid solution[105]. Cycloserine is usually used as a companion drug with I.N.A.H. in doses of 250 mg. twice daily[87]. Unfortunately it is neurotoxic if this dosage is exceeded, the toxicity manifesting itself in epileptiform seizures. Nearly all clinical reports have mentioned psychotic effects in a percentage of patients[109,110]. The margin of safety is low and toxic effects are frequent. If a lower dose were feasable or some means of preventing seizures available this drug would join the major three[87] as it penetrates most tuberculous lesions with facility. It has also been used in leprosy[111]. The mode of action of cycloserine is said to depend on its similarity to D-alanine (XVIII). The apparent paradox in the action of cycloserine - it is inactive in mouse or guinea pig tuberculosis and active in human tuberculosis may be explained by D-alanine antagonism in vivo. D-Alanine is not found in human serum whilst it appears in mice and guinea pig sera[113].

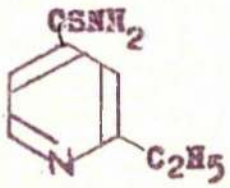
Kanamycin A (XIX) was introduced in 1958[114] as the result of a large Japanese screening programme for antitubercular antibiotics. It is derived from Streptomyces kanamyceticus and has been shown to be a trisaccharide[115,116] related to neomycin and streptomycin.




(XIX)

It is supplied as the water soluble sulphate for intramuscular injection. In tuberculotherapy it is less effective than streptomycin and ototoxicity limits its use except for short periods in surgical intervention[117,118]. Apparently continuation of kanamycin therapy beyond a cumulative dose of 30 - 50 g.[119] causes irreversible[120] auditory and vestibular damage in a sizable minority[121]. It is also nephrotoxic. The recommended dosage is 3 g. weekly divided into three intramuscular injections each of 1 gramme. The factor having most influence on the efficiency of kanamycin is the type of lesion - the older fibrotic type being resistant[122]. Cross resistance occurs with viomycin and neomycin but streptomycin resistant strains are said to be susceptible to kanamycin, though the reverse is not true[123,124]. McDermott[87] condemns the use of neomycin and kanamycin in human tuberculosis on grounds of severe toxicity.

The introduction of ∝-ethyl thioisonicotinamide (XX) (Ethionamide) as a tuberculostat is a further outgrowth of the work on nicotinamide (p19) Thioisonicotinamide (XX1)

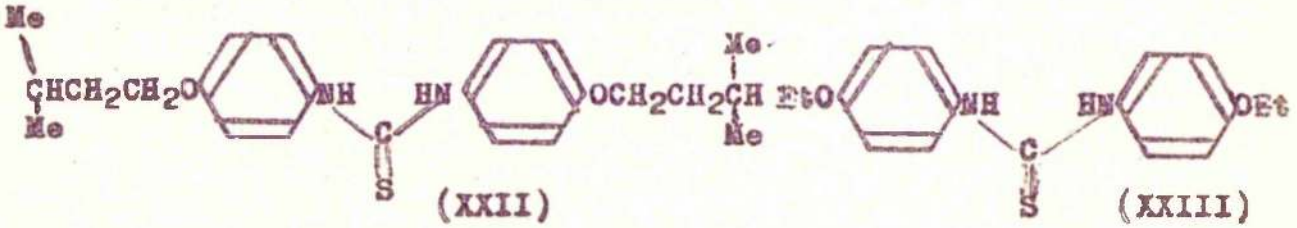



(XX)


(XX1)

was first prepared and on testing was found to be several times more active than nicotinamide in experimental tuberculosis[126]. It was, however too toxic for human use causing nausea. Various 2-alkyl derivatives were then examined and the 2-ethyl derivative (XX) was found even more active in animals[127]. Introduced clinically it causes nausea and vomiting in 40% of cases[128]. Attempts to use enteric coated tablets have been of doubtful success[129]. Its use with cycloserine[130] or pyrazinamide[131] has been recommended against strains resistant to standard drugs. Conflicting reports[129,132] as to its clinical efficiency reflect the difficulties in testing new antituberculous agents adequately. Oral dosage of 0.5 g. twice daily appears to be optimal[133,134]. Suppositories have also been used[135] with equal effect. Liver impairment has also been reported a rare side effect[132,136,137]. The gastro-intestinal side effects are said to be reduced by simultaneous Vitamin PP (choline phosphate) administration[138,139].

A recent synthetic tuberculostat introduced about 1960 is 4,4'-diisoamyloxythiocarbanilide (XXII) the discovery of which stems from work begun in 1941 by Mayer[140] investigating the anti-tubercular activity of certain antifungals. Thiourea and its derivatives were thus found to exhibit antitubercular activity[140,141], but due to goitrogenic action were of no clinical value.

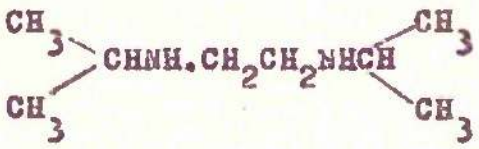


(XXII) (XXIII)

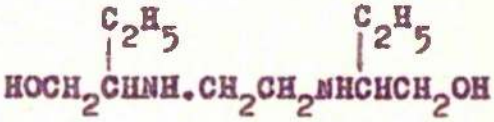
It was only in 1952 that Mayer[142] and Buu-Hoi[143] independently found that diarylthioureas are devoid of this drawback and a series of these were prepared and tested in experimental tuberculosis. The best of these was the 4,4'-diethoxy compound[144](XXIII). Buu-Hoi report this as being of value in leprosy[145] and having been used clinically in the Soviet Union in tuberculosis[146]. However the 4,4'-isoamyl derivative was found to be more active in experimental tuberculosis[147-149] Various recent clinical reports indicate that at a dose of 2-6 grammes p day orally or 100 mg/Kg this drug compares favourably with P.A.S. and ethionamide[150-152]. Toxic disturbances are chiefly intolerance, nausea and vomiting. It is too early to assess this drug adequately but it would appear to be a useful addition to the arsenal of new antituberculous agents active against resistant strains. Some strains have been observed cross-

resistant with thiosemicarbazones and ethionamide[153].

A random screening programme for tuberculostats found N,N'-diisopropylethylene diamine (XXIV) protected mice from an otherwise fatal dose of tuberculosis. Synthesis of numerous related compounds did not improve this activity until hydroxyl functions were introduced[154]. The most promising of this series was found to be Dextro-2,2'-(ethylenediimino)-di-1-butanol dihydrochloride (Ethambutol)(XXV)[155](1961). This has an efficiency index tolerance/potency similar to that of I.N.A.H. orally and is superior to streptomycin by the parenteral route in mice[154,156] and guinea pigs[157]. It appears to have an effect only in proliferating Mycobacteria[158]. The levo-isomer shows no antituberculous activity[155].



(XXIV)



(XXV)

When tested with I.N.A.H. in guinea pigs the activity of both drugs was enhanced[159]. It is also compatible with streptomycin[154]. Clinical trials are in progress and one recent report gives details of the combined use of ethambutol and 4,4'-diisoamyloxythiocarbanilide in advanced drug resistant tuberculosis which gave improvement. Reversible sight effects were observed[160,161], in some patients the dose used being 1 to ? g. daily. Tablets of 500 mgm. are supplied

25 mg/Kg. being recommended[161].

The above listed drugs represent the agents utilised by the clinician in the chemotherapy of tuberculosis. As has been noted whilst reviewing the individual compounds, two drug combination therapy is to be preferred whenever possible. This enhances the effect of each drug, delaying the onset of Mycobacterial resistance to either. Brief mention is here made of some routine regimens extensive reviews having been written in the clinical journals[87,162,163]

The three standard drugs are always tried first, the second line drugs being reserved for cases resistant to this therapy. The most useful treatment is probably P.A.S. (12g.) and I.N.A.H. (300mg) daily as these drugs require no injection and can be used for out-patients where hospitalisation is impractical.

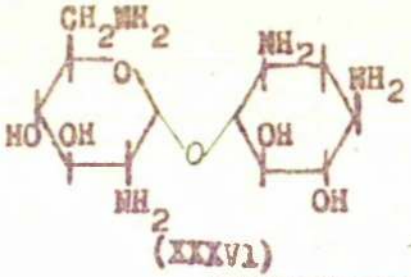
Streptomycin (1g.) daily in combination with I.N.A.H. (300 mg.) is probably more effective and is a well recognised regimen as also is a similar treatment with streptomycin ( 3 x 1g.)weekly. The disadvantage of these being that the parenteral route is required. Treatment with P.A.S. (20g.) and streptomycin (1g.) daily is not such an effective regimen but has given good results[164].

A recent M.R.C. report[165] suggests that I.N.A.H. (2 x 200mg.) P.A.S. (2 x 5g.) supplemented by streptomycin (1g.) daily for the first six weeks gives better results than I.N.A.H. with P.A.S. without streptomycin. This report also emphasises the need for

continuing therapy over 2 years to prevent relapse, a topic reviewed by Fox recently[166]. In older patients streptomycin regimens must be used with care as they are more susceptible to ototoxicity[167,168].

In the event of resistance to these major drugs - and this is an ever increasing problem[169], resort must be given to the newer more toxic drugs. These should always be administered in hospital where vigilance quickly reveals any toxic manifestations. The best secondary drug combinations have not been established[170]. According to Crofton[10,171] preliminary evidence suggests that a combination of at least three minor drugs is better than two. At present a combination of daily ethionamide plus pyrazinamide plus cycloserine may be best[171].

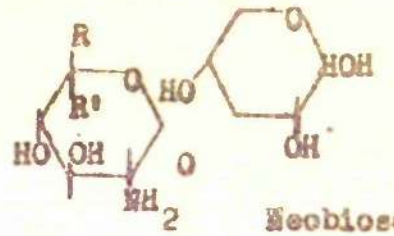
## Neomycin Degradation (Figure I)



(XXXVI)

Neomycin B or C

MeOH
HCl



Neobiosaminide

B. R = H, R' = $CH_2NH_2$; C. R = $CH_2NH_2$, R' = H

dil. HCl

Neamine

$C_{12}H_{26}N_4O_6$

HCl

N,N'N",N"-Tetra-acetyl

Methyl neobiosaminides B or C

$C_{12}H_{24}N_2O_8$

dil HCl

N,N'-Dibenzoyl

2-Deoxy-streptamine

$C_6H_{14}N_2O_3$

Neosamine C

$C_6H_{14}N_2O_4$

Neosamine B or C

$C_6H_{14}N_2O_4$

D-ribose

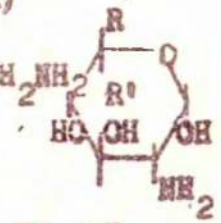
$C_5H_9O_5$

(XXVI)

(XXVII)

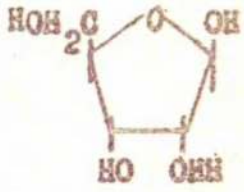
(XXXIII)

B. R = H, R' = $CH_2NH_2$



(XXXII)

C. R = $CH_2NH_2$, R' = H.



(Furanose)

## THE CHEMISTRY OF THE AMINOGLYCOSIDE ANTIBIOTICS.

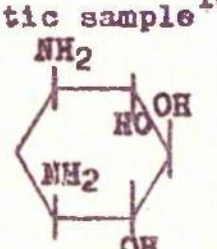
### Neomycin Complex.

The antibiotic neomycin secured from the culture filtrates of actinomycete Streptomyces fradiae was introduced by Waksman and Lechevalier in 1949[61]. It was shown to be effective both in vitro and in vivo against the tubercle bacillus, but its severe auditory and renal toxicity limited its clinical use. McDermott[87] condemns the use of neomycin in tuberculosis.

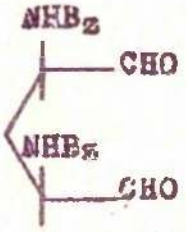
Investigation of the chemistry of neomycin soon revealed that the antibacterial activity of the antibiotic was not due to a single discrete entity but to a group of closely related substances termed the "neomycin complex". New antibiotics have been reported which subsequent investigations have shown to be similar to or identical with the original neomycins B and C. These include aminosidin[172], catenulin[173], dextromycin[174], framycetin[175], hydroxymycin[176], paromomycins I[177] and II[178], and Zygomycins $A_1$ and $A_2$[179]. The confusion reigning here was caused by the difficulty of separation from mixtures of isomers of varying proportions. Proper characterisation was only recently achieved by the use of improved methods for the separation of individual components, followed by physical and chemical comparison of their breakdown products which allowed assigment of structures (Figure 1).

Early workers[180] described the separation of neomycin A hydrochlorides from culture filtrates utilising counter-current distribution techniques.

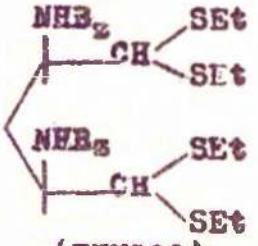
Degradation of this compound with 6 N hydrochloric acid at 140°C gave the dihydrochloride of an optically inactive diacidic base $C_6H_{14}N_2O_3.2HCl$. This was identified as 1,3-diamino-4,5,6-trihydroxycyclohexane (XXVI) by mercaptolysis of the periodate oxidation product, a dialdehyde (XXVII), followed by hydrogenolysis to meso-1,3-dibenzamidopentane (XXIX), which was identical with an authentic synthetic sample[181].



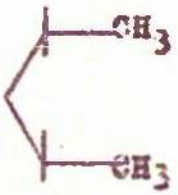
(XXVI)



(XXVII)



(XXVIII)



(XXIX)

The neomycin A was later found to be identical with a degradation product, designated neamine, from acid hydrolyses of neomycins B and C[182]. The two products were compared (mixed melting points, infrared and papergrams) in proof of identity[183].

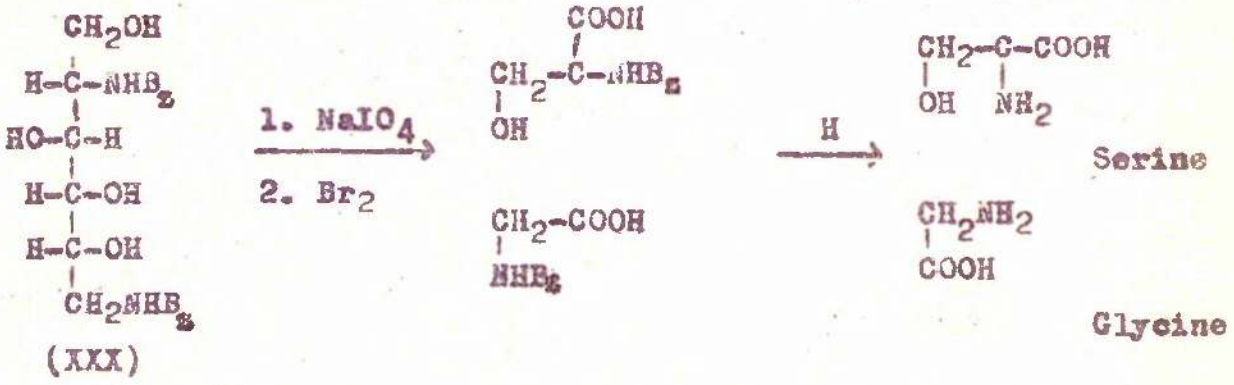
Dutcher and his associates[184] first described the methanolysis of neomycins B and C into two components separated chromatographically on methanol/alumina in equimolar[185] amounts. The first, later identified as neamine, -a non-reducing diamine, and the second differing for neomycins B and C, the isomeric methyl neobiosaminides B and C, the acetyl derivatives of which analysed as $C_{11}H_{16}O_6N_2(OCH_3)(COCH_3)_3$. These fragments were further degraded by vigorous hydrochloric acid hydrolysis to give the dihydrochlorides of $C_6H_{14}O_3N_2.2HCl$, non-identical but isomeric for the two neomycins.

The remainder of these disaccharide fragments appeared to be accounted for by a pentose which degraded to furfural on acid hydrolysis[184].

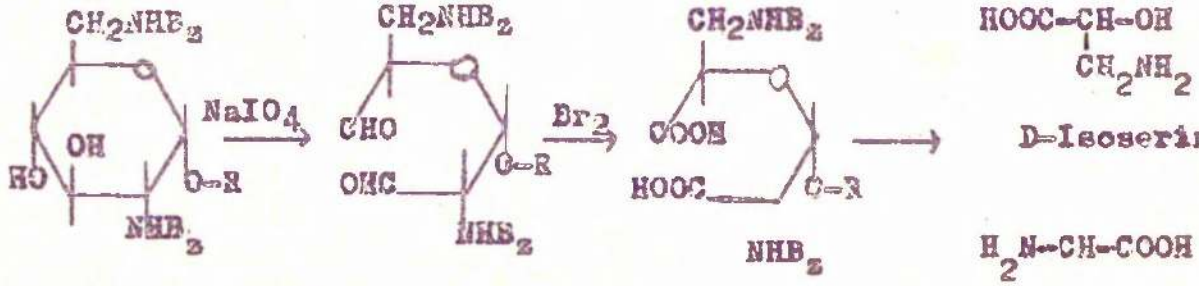
Rinehart and his colleagues who have been responsible for the later work in elucidating the neomycin chemistry, obtained the formulas of $C_{12}H_{24}N_2O_8$ for methylneobiosaminide C[186], from analysis of the base, base monohydrate, and its N,N'-dibenzoyl- and N,N'-di-(p-nitrobenzoyl) derivatives. Isolation of the pentose moiety inferred from furfural formation was complicated by the stability of the glycosidic bond in neobiosamine. The pentose was destroyed under conditions sufficiently vigorous to cleave the disaccharide linkage. This situation is well documented for glycosides of the 2-amino sugars[187,188], where it is suggested that preferred protonation of the amino group and consequent shielding by the positively charged ammonium group of the glycosidic bond from proton attack, resists hydrolysis. This difficulty is most readily circumvented by conversion of the basic amino groups to neutral amide groups when the glycosidic linkage is easily cleaved. In this case[186] the N,N'-dibenzoylneobiosaminides B and C were hydrolysed with dilute hydrochloric acid, the hydrolysate being chromatographed to isolate the neutral pentose sugar which was identified by colour tests, papergram $R_F$ values, and the osazone as D-ribose[189]. The fact that methyl neobiosaminide on mild hydrolysis (HCl) gave neobiosamine, a single spot on papergrams was indicative of a diaminohexosidopentose, which would be resistant as above to acid hydrolysis rather than a

pentosido-diaminohexose which would readily hydrolyse into its two components.

These workers next[190] identified the diaminohexose of neobiosaminide C as 2,6-diamino-2,6-dideoxy-D-glucose (termed neosamine C) (XXXII). This was achieved by the above hydrolysis, followed by sodium borohydride reduction of the neosamine C to N,N'-dibenzoylneosaminol C (XXX), and periodate oxidation studies. This derivative consumed 2 mols of periodate producing no formaldehyde. The periodate oxidation product oxidised with bromine water, followed by hydrolysis gave glycine and serine identified by papergram $R_F$ comparison.




Periodate oxidation of methylbenzoylneobiosaminide C (XXXIb), followed by oxidation woth bromine water and hydrolysis gave D-isoserine from C-4, C-5, and C-6 of dibenzoylneosamine C (XXXIa). These data assign the same stereochemistry as D-glucosamine at C-2 and C-5[191].




(XXXI) a R H
b R D-Ribose

The identity of neosamine C was unequivocally established as 2,6-diamino-2,6-dideoxy-$\alpha$-D-glucose[192] by comparison with a synthetic sample prepared as in Figure 11, from methyl N-acetyl-D-glucosaminide via the 6-O-tosyl derivative, which on heating with methanolic ammonia gave the 6-amino derivative. Hydrolysis with hydrochloric acid removed the methyl glucosidic and N-acetyl groups. Comparison of the physical properties ($[\alpha]_D$, $R_F$ and melting points) of the N-acetyl derivatives of the natural and synthetic products established their identity which was confirmed by the super-imposable nuclear magnetic resonance spectra, a method which is very sensitive to stereochemical differences in the carbohydrate molecule.

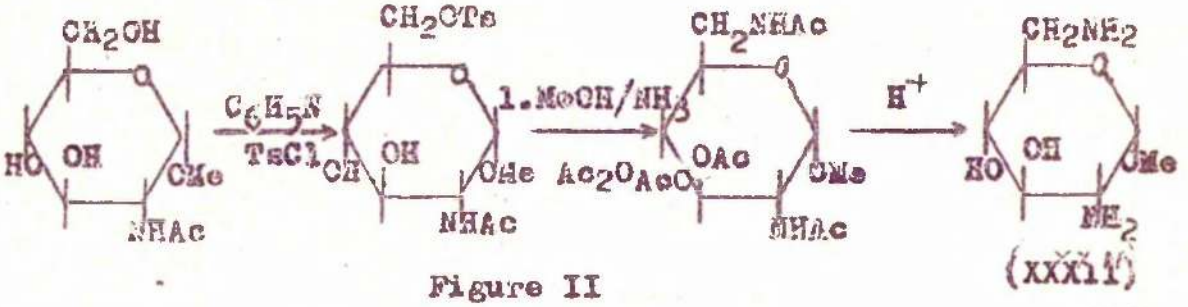
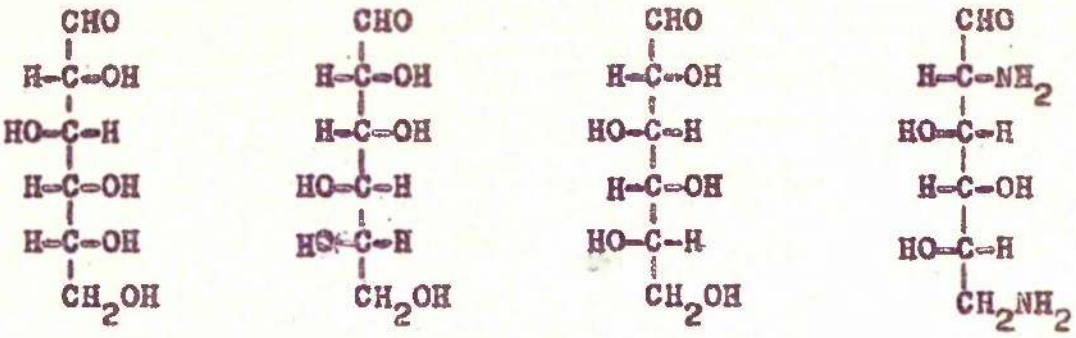



Figure II

A second synthesis via D-glucosamine[193] nitrile has confirmed this structure.

Molecular rotation calculations suggest (from Hudson's rules) an $\alpha$-D glycosidic linkage between neosamine C and D-ribose, further proof of this being an infrared band at 844 $cm^{-1}$ in the spectrum of N,N'-dibenzoylneobiosaminide C attributed to C-H deformation[191].

This paper also described the hydrolysis of methyl neobiosaminide B to give neosamine B dihydrochloride, isomeric with the neomycin C derivative, this being the only portion of the neomycin B molecule differing from C is therefore responsible for chemical and biological differences between the two antibiotics. These are the first two natural diaminohexoses to be described. The full stereochemistry of neosamine B is still in doubt, but the idose configuration (XXXIII) has been proposed based on the following observations[194,195]. First periodate - permanganate oxidation of both N,N'-(bis-2,4-dinitrophenyl)-neosaminol B and of N-(2,4-dinitrophenyl)-D-glucosamine gave L-serine indicating that the stereochemistry of C-2 is identical. Secondly periodate oxidation of methyl neobiosaminide B and 6-amino-6-deoxy-glucose, followed by N-dinitrophenylation and hydrolysis, gives L-isoserinealdehyde-DNP and D-isoserinealdehyde DNP respectively, which assigns the L-configuration to C-5. Thirdly the lack of periodate uptake of N,N'-dinitrophenyl derivatives of neosamine B and methyl-neobiosaminide B under mild conditions suggests that the hydroxyls of C-3 and C-4 are trans to one another. Rinehart in recent work with quinoxalines confirms C-3 stereochemistry eliminating talose[196]. This leaves a choice between L-idose and L-mannose, the idose being favoured on biogenetic grounds differing from D-glucose only by inversion at C-5 whereas L-mannose differs in C-3, C-4, and C-5[194].



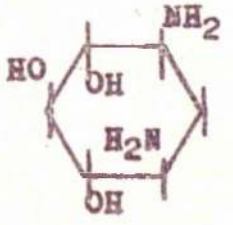
D-glucose L-mannose L-idose neosamine B (XXXIII)

The position of the ribose linkage in neobiosamine B was determined by periodate oxidation studies and confirmed by methylation experiments. Methyl neobiosaminide B subjected to periodate oxidation and followed by hydrolysis released ribose indicating that neosamine B is not linked to C-4 or C-5 of ribose, in which case ribose would have been degraded by periodate. Periodate oxidation of neobiosaminol B, obtained by borohydride reduction of neobiosamine B used 4 moles of periodate liberating 1.5 mole formaldehyde, thus establishing linkage at C-3 of ribose rather than C-2[195].

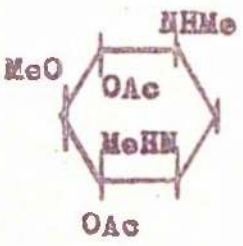
N-Acetylmethylneobiosaminide B was methylated with methyl iodide and barium oxide when hydrolysis with dilute hydrochloric acid gave 2,4-O,O-dimethyl-D-ribose as established by comparison with an authentic sample. Thus since C-2 and C-4 hydroxyls were free for methylation and the C-3 hydroxyl must have been bound in glycosidic linkage and the C-5 in the pyranose ring in neobiosamine[195].

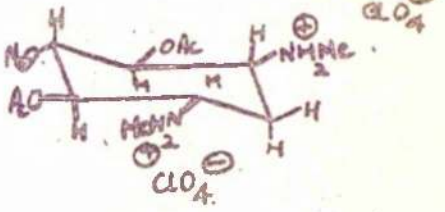
Neamine, the fragment common to both neomycins B and C, which is released on methanolysis, was early found to give meso-1,3-diamino-4,5,6-trihydroxy cyclohexane in 83% yield on hydrolysis with hydrobromic acid. The evidence for this structure termed 2-deoxystreptamine[197]

(because of its relationship with streptamine) (q.v.) has already been cited (page 29). These workers expected an all trans stereochemistry on biogenetic grounds[181]. Rinehart and his associates[198] gave further evidence towards the probability of this assumption by comparison of the behaviour of cis- and trans-2-benzamidocyclohexanol with the N,N'-dibensoyl derivatives of 2-deoxystreptamine and streptamine. The trans isomer and the two streptamine derivatives fail to undergo N---> O benzoyl migration whilst the cis isomer does. Streptamine has on synthetic evidence been assigned the trans configuration[199]. Lemieux and Cushley[197] confirmed this trans stereochemistry by a study of the nuclear magnetic resonance spectra of 2-deoxystreptamine (XXXIV) and its 5-O-methyl-1, 3-di-N-methyl-4, 6-di-O-acetyldihydrogen perchlorate derivative (XXXV) The spectrum of deoxystreptamine in deuterium oxide required the two amino groups to be equatorially orientated. The spectrum provides no information about the configuratio at other centres because the small chemical shift between signals for 4-, 5-, and 6- hydrogens give a bunched peak. Substitution of the hydroxyl hydrogens at those positions by 4- and 6-O-acetyl and 5-O-methyl achieved a substantial chemical shift between the signals for these neighbouring hydrogens. The spectrum indicated that the 4- and 6-hydrogens were axial and coupled with two neighbouring hydrogens.




(XXXIV)



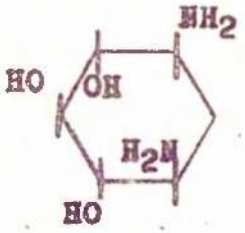




(XXXV)

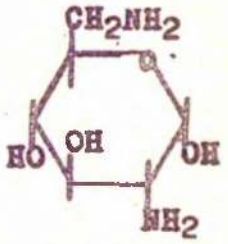
Hydrolysis of N,N',N'',N''' tetra-acetyl neamine for 10 hours followed by cellulose column chromatography gave a number of fractions, one of which was neosamine C (2,6-diamino-2,6-dideoxy-α-D-glucose)(XXXII as already described, a second 2-deoxystreptamine, some unchanged starting product also coming through. Conclusive evidence for the identity of the hydrolysate as neosamine C was provided by N-acetylation of the product to N,N'-diacetyl neosamine C which was identical with an authentic sample 198.

N,N',N'',N''' Tetra acetyl neamine

10 hours 6NHCl

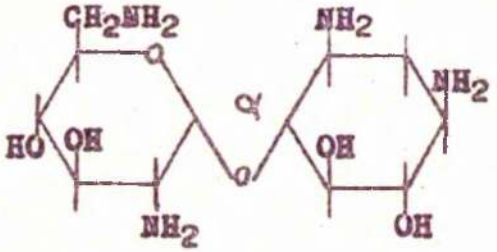


(XXXVI)



(XXXII)

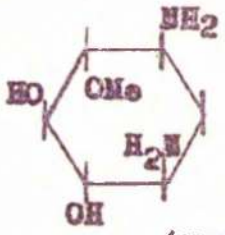
Periodate oxidation studies on N,N',N'',N'''-tetra-acetyl and tetra-benzoyl-neamine established a C-4 rather than a C-5 glycosidic linkage in deoxystreptamine and a pyranose structure for neosamine C in neamine. Molecular rotation observations indicated an α-glycosidic linkage allowing structure (XXXVI$_2$) to be written for neamine[198].



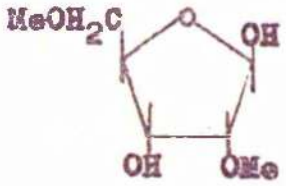
(XXXVI$_2$)

The remaining features of the chemistry of the neomycins, only recently elucidated were the position of attachment of neosamine C to ribose, the stereochemistry of the neosamine B -D-ribose bond and the position, ring form and stereochemistry of the linkage of ribose to neamine.

Neamine is attached through the C-5 hydroxyl of the deoxystreptamine moiety to neobiosamine B and C[200], as evidenced by the isolation of mono-O-methyl deoxystreptamine (XXXVII) from the hydrolysate of poly-O-methylhexa-N-acetylneomycins B and C. This same hydrolysate gave a 2,5-di-O-methyl-D-ribose (XXXVIII) fragment separated on a cellulose column, thus confirming that neosamine of neobiosamine B or C is attached glycosidically to C-3 of D-ribofuranose in neomycins[201], whereas in the degraded molecules of the neobiosamines it assumes the pyranose form[195].



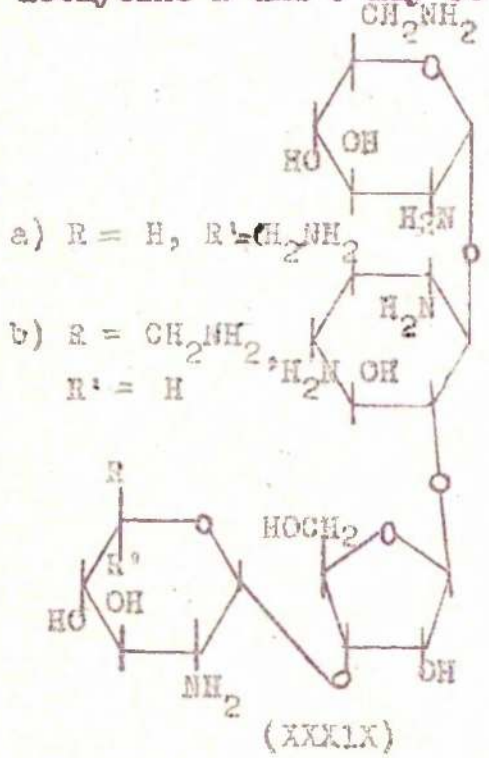

(XXXVII)



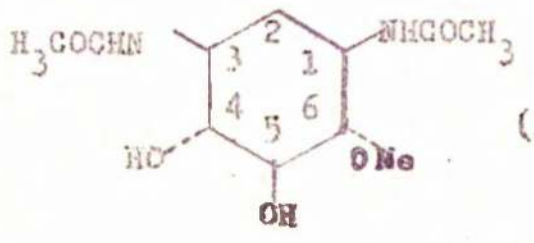

(XXXVIII)

Nuclear magnetic resonance study[202] of hexa-N-acetyl neomycins B and C allowed the assignment of $\beta$ -ribofuranose linkage between ribose and neamine from comparison with known $\alpha$ - and $\beta$ -ribofuranoses.

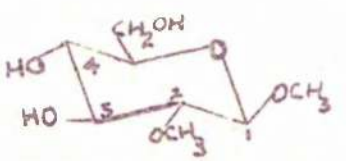
Moreover this study also allowed the assignment of an α linkage between neosamine B-D-ribose in neobiosamine B, and also confirmed the α-linkages previously assigned by rotation to neosamine C in neamine and neobiosamine C. From these data the gross structure of neomycins B and C may be written (XXXIX)a and b respectively.



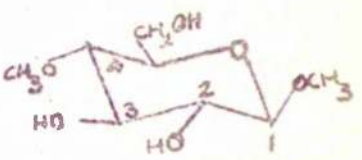

(XXXIX)



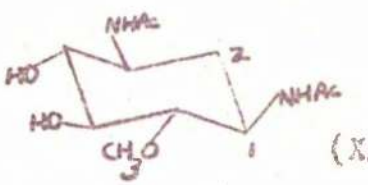
(XL)



(XLI)



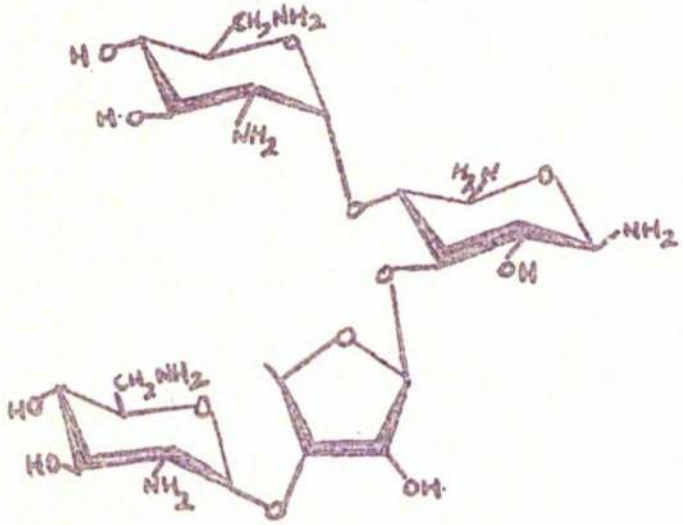
(XLII)



(XLIII)

The final uncertainty in the stereochemistry of neomycin C, the absolute configuration of the unsymmetrically substituted 2-deoxy-streptamine portion has been resolved by Nuclear magnetic resonance studies as reported[197] (p. 35) Simultaneous studies by Rinehart[203] and his colleagues confirmed this conclusion by optical rotation results. The method of Reeves[204] was adopted where the change in optical rotation $\Delta [M]$ cupra B was measured when cuprammonium hydroxide solution (Cupra B) is substituted for water.

N,N'-Diacetyl-6-methyldeoxystreptamine (XL) gives a high positive increment similar to that observed with methyl-2-O-methyl-β-D-glucoside (XLI) but opposite in sign to that of the 4-O-methyl β-D-glucoside (XLII). Thus the adjacent hydroxyl groups in 6-O-methyl-2-deoxystreptamine are related as those at C-3 and C-4 of the glucopyranose ring and the conformation may be written (XLIII). It follows that the final stereochemistry of neomycin C may be written (XLIVa). The configuration of neomycin B still awaits final proof of the idose configuration for neosamine B.



(XLIV)

(XLV)

(a) R = Neobiosamine C

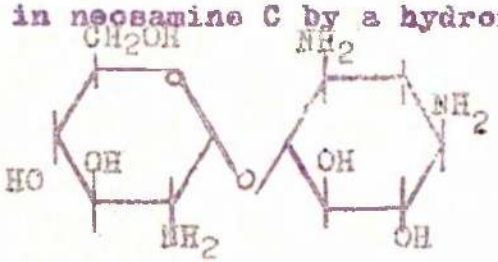
(b) R = Neobiosamine B

Neomycins $LP_1$ and $LP_2$ are minor constituents of the complex found to be monoacetylated. Their relationship to neomycins B and C was established[205] by acetylation and comparison with hexa-N-acetyl-neomycins B and C. Penta-N-acetyl-neomycin $LP_1$ being identical with hexa-acetyl-neomycin C and penta-N-acetyl-neomycin $LP_2$ identical with hexa-acetyl-neomycin B. Acid hydrolysis of neomycin $LP_2$ gave

neamine, 2-deoxystreptamine, D-ribose and neosamine B. An additional product of mild hydrolysis being mono-*N*-acetyl-neamine. The position of the *N*-acetyl group on the amino group of 2-deoxystreptamine adjacent to the glycosidic linkage joining neosamine C to 2-deoxystreptamine was established by *N*-methylation and periodate oxidation studies. Structures (XLVa) and (XLVb) may be assigned to neomycins $LP_1$ and $LP_2$ respectively on the basis of this degradation.

## Paromomycins

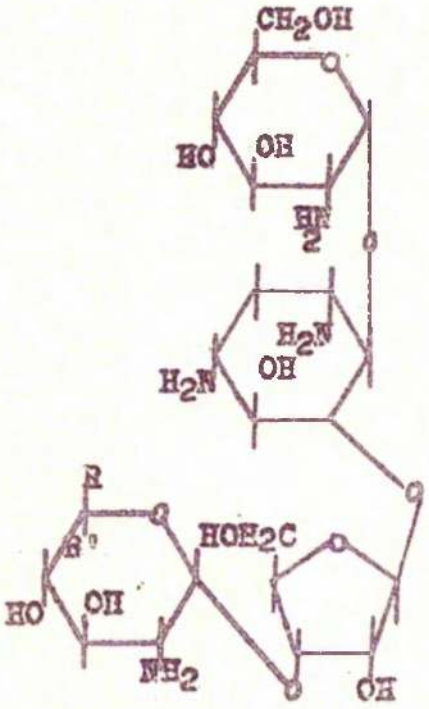
These antibiotics were obtained from the culture filtrates of *S.rimosus* and have very similar structures to neomycin B. Haskell and his colleagues[206] showed the presence of paromamine (XLVI) which is similar to neamine differing only in the replacement of the 6-amino group in neosamine C by a hydroxyl .




(XLVI)

The D-glucosamine portion was isolated as the hydrochloride by hydrochloric acid treatment and was identical with an authentic sample. Acid hydrolysis of paromomycin gave $\alpha$- and $\beta$-paromobiozamine also obtained as the methyl glycosides. Dilute acid hydrolysis of the methylparomobiosaminide-*N*,*N*'-dibenzoyl derivative followed by carbon chromatography yielded D-ribose[207]. Stronger acid conditions in the

treatment of the base resulted in the breakdown of the pentose but a diaminohexose paromose was obtained[207], which gave the identical crystalline N,N'-diacetyl derivative in comparison with neosamine B[209]. Haskell showed that paromose is 2,6-diamino-2,6-dideoxy-L-idose and assigned a gross structure (XLVIIA) for paromomycin I[208,177] Rinehart[201], has suggested a possible stereochemical structure (XLIV) based on the evidence of nearly superimposable N.M.R. spectra of neomycin B and paromomycin (1)[202] differing only in the obvious lack in paromomycin of the 6-amino group of neosamine C in paromamine. Rinehart[178,209] also mentions that a paromomycin (11) (XLV11b) has been isolated from commercial samples of paromomycin differing only in the paromose moiety. Paromomycin (1) has paromose identical with neosamine B whereas paromomycin (11) has paromose identical with neosamine C.




D glucosamine

2-deoxystreptamine

} Paromamine or Pseudoneamine

(XLVII) a. R = H, R' = $CH_2NH_2$

b. R = $CH_2NH_2$, R' = H

## Hydroxymycin.

Hydroxymycin introduced in France during 1958 was obtained from the cultures of Streptomyces sporogenes[176,210]. Hydrolysis or methanolysis gave a neamine like moiety which degradative studies indicated was composed of D-glucosamine and 2-deoxystreptamine. It was termed pseudoneamine and is identical with paromamine (XLVI).

## Zygomycin.

The zygomycin A complex obtained from Streptomyces pulveraceous in Japan(1960) has been found to resemble paromomycin. The scheme of investigation is shown in Figure 111[179,211,212].

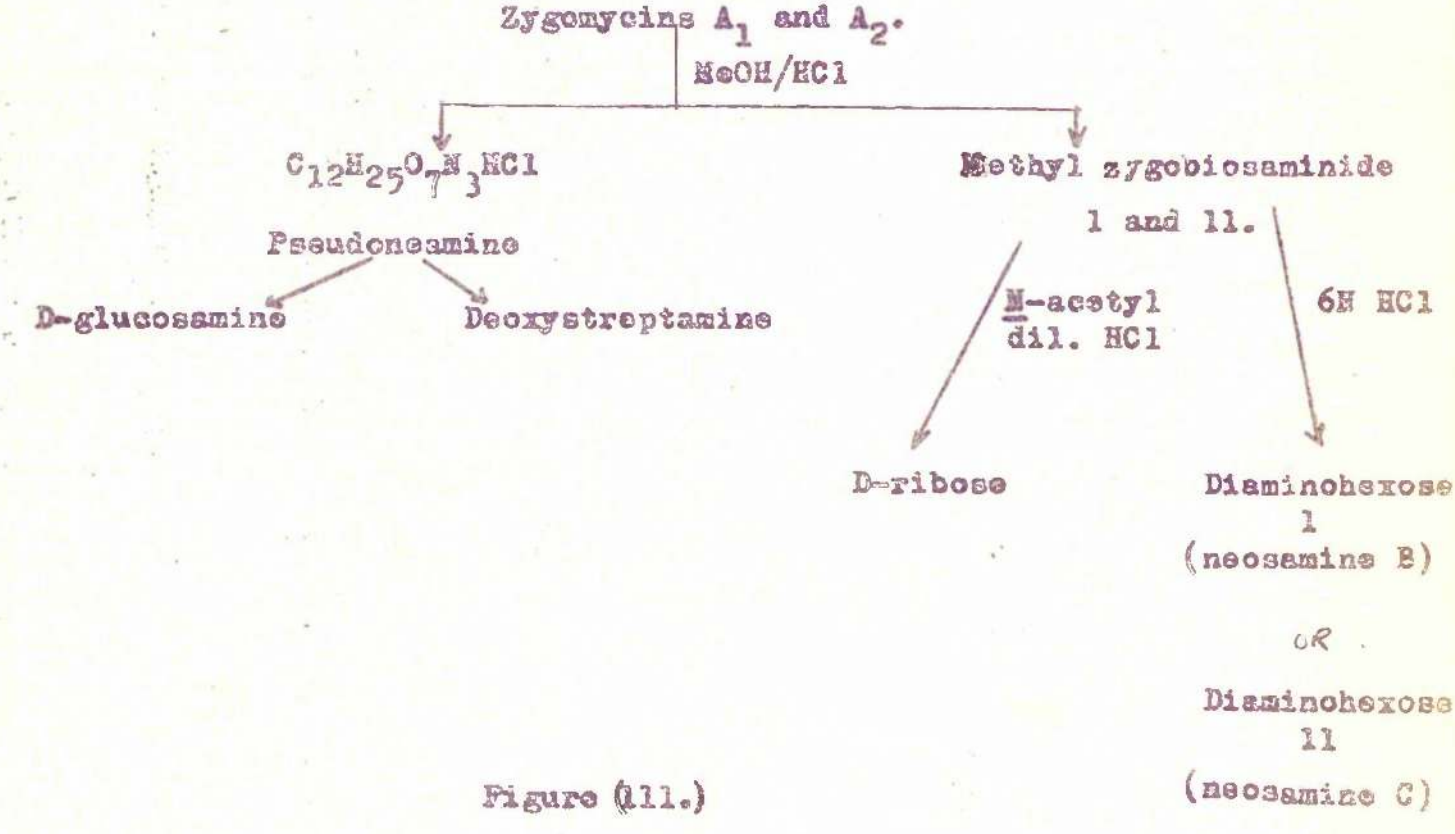



Figure (111.)

Pseudonesamine one of the products of hydrolysis was found by degradation to give D-glucosamine and 2-deoxystreptamine and was identical with the like derivative of hydroxymycin and paromomycin. Degradation of the second hydrolysis product methylzygobiosaminide (1)and(11)by further acid hydrolysis, after column chromatography gave D-ribose and two different diaminohexoses named(1)and(11) respectively. Diaminohexose(1)proved identical on comparison with neosamine B (melting point, infrared spectrum, X-ray diffraction and chromatograms)[179]. Diaminohexose(11)separated by these same methods was proven identical with neosamine C[192] and the synthetic sample already described (p.32 ), the nuclear magnetic resonance spectra being superimposable confirmed this finding.

The gross structures of zygomycins $A_1$ and $A_2$ were assigned by Horii[217]. Rinehart[209] has suggested tentatively that from the similarities of paromomycin 1 and 11 and zygomycins $A_1$ and $A_2$ they may be identical with (XLV11a) and (XLV11b). Horii has recently confirmed this finding by methylation studies[213].

The Japanese make an interesting comment on the lack of toxicity of zygomycin compared to neomycin B attributing this to the lack of a 6-amino group in pseudonesamine[179].

Recently a comparison of various members of the neomycin complex by Schillings and Schaffner[214], including purification by column chromatography N-acetylation, papergram and infrared studies showed that various members of the group obtained from different sources

were identical. These were hydroxymycin, paromomycin and aminosidin. Horii in a similar examination of members of this group including zygomycins $A_1$ and $A_2$ found these antibiotics almost identical, differing only in the ratio of the two isomeric components[215].

## BIOGENESIS OF NEOMYCINS.

Although much work remains to be done in this field of study, Sebek[216] showed that $^{14}C$ - glucose added to growing culture was readily incorporated into neomycin. Rinehart and his colleagues[194], noting the similarity in stereochemistry, of neosamines B and C, and 2-deoxystreptamine moieties suggest the following biogenetic pathway which would be amenable to tracer studies Fig. IV.

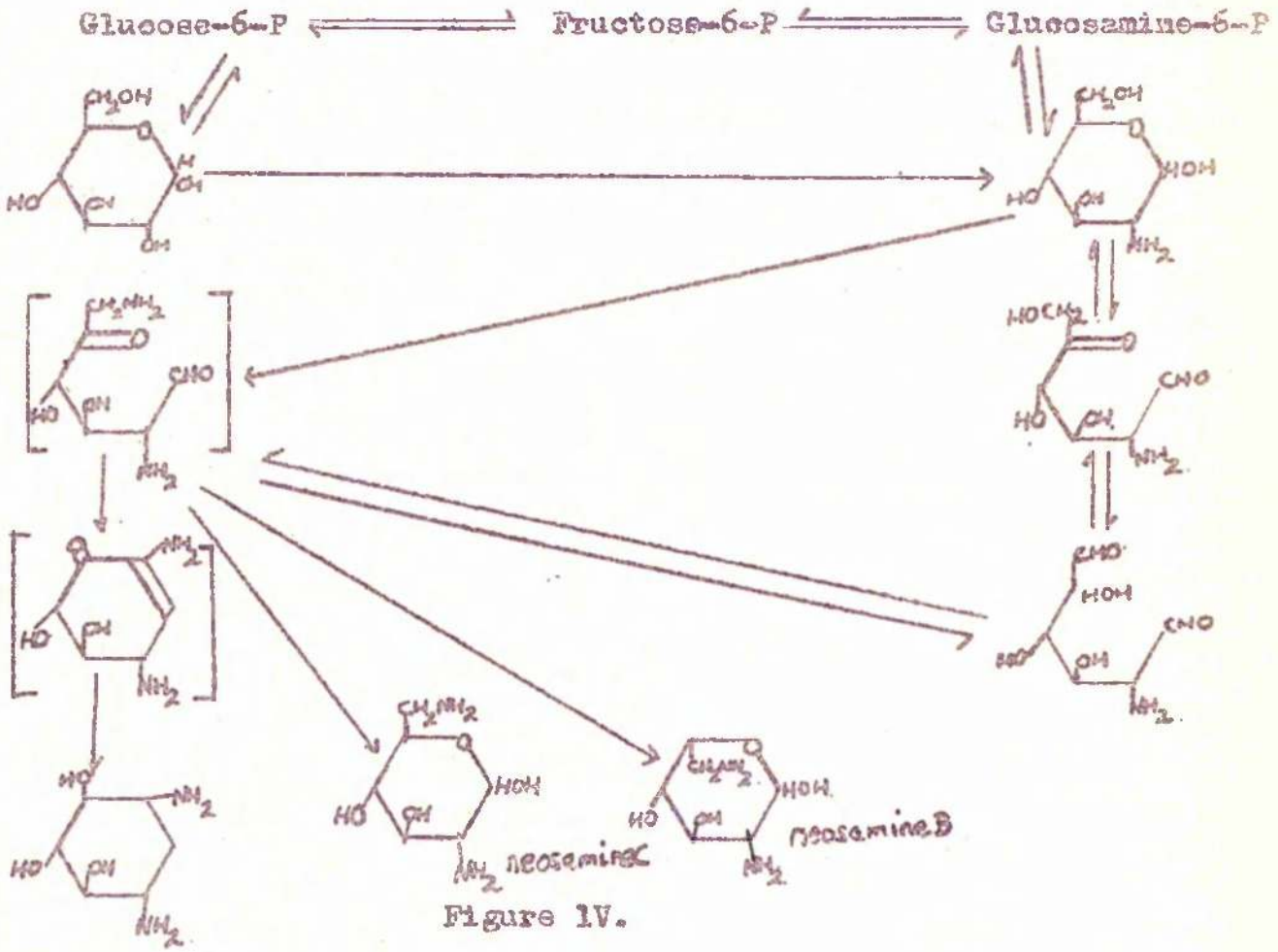



Figure IV.

Recent evidence of this nature supports this scheme.

[1 - $^{14}C$]-D-Glucose and [6 - $^{14}C$]-D-Glucose are good precursors for neosamines B and C and deoxystreptamine. Ribose of reasonable activity was also obtained. [1 - $^{14}C$]-D-Glucosamine is a much better precursor for the neosamines and 2-deoxystreptamine but much worse for ribose. The fact that different levels of activity were given with neosamine B and $C_2$ the latter with a level equivalent to the deoxystreptamine level suggests that the fragments are formed separately before junction[196].

## Kanamycin Degradation (Figure V)

Kanamycin

4N HCl

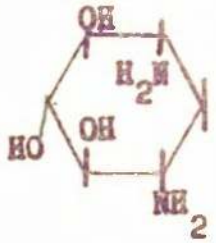
concentrate

EtOH

Filtrate

2-Deoxy-streptamine

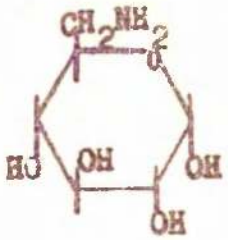
$C_6H_{14}N_2O_3$



(XXVI)

6-glucosamine

$C_6H_{13}O_5$

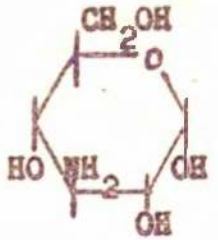


(XLVIII)

3-glucosamine

(Kanosamines)

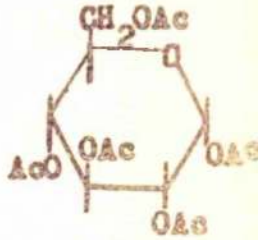
$C_6H_{13}O_5$



(XLIXa)

$HNO_2$

$Ac_2O$



α-D-glucose

penta-acetate

Kanamycin

dilute acid

chromatography

Disaccharide slow

strong acid

2-Deoxystreptamine

6-glucosamine

Deoxystreptamine - Kanosaminides

strong acid

2-Deoxystreptamine

3-glucosamine

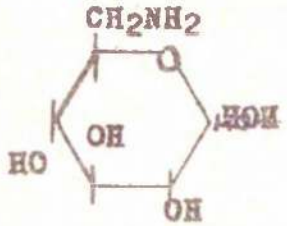
## Kanamycin.

This antibiotic, which is closely related to neomycin, was introduced by Umezawa and his colleagues (1957-8)[114]. The structure of kanamycin was quickly determined by these workers and by an American team who have given a concise review of this work[217] as summarised in Figure V.

Kanamycin is best obtained from the culture filtrates of Streptomyces kanamyceticus as the crystalline water-soluble sulphate. The free base was formulated as $C_{18}H_{36}N_4O_{11}$ from analysis of the N-acetyl-, per acetyl- and various Schiff base derivatives. The presence of four primary amino groups was indicated by Van Slyke nitrogen determinations, and formation of the tetra-N-acetyl derivative[217,218]. The base gave positive Molisch, ninhydrin, and Elson-Morgan tests, being negative to reducing sugars and Sakaguchi tests.

Hydrolysis with boiling 4 N hydrochloric acid, followed by papergram study showed three ninhydrin positive spots; the two faster moving spots also gave reducing sugar tests. Evaporation of the hydrolysate to small volume and addition of ethanol yielded 2-deoxystreptamine dihydrochloride. This was characterised as the free base and its pentaacetyl- and N-acetyl-derivatives. The dihydrobromide was identical with the corresponding salt of 1,3-diamino-4,5,6-trihydroxy cyclohexane (XXVI) isolated from neomycin[218,219].

Chromatography of the ethanol filtrate on cellulose powder[220] or on Dowex-50 cation exchange resin[221], separated the remaining two amino-sugar degradation products. The slower running substances obtained as the hydrochloride, $C_6H_{13}NO_5.HCl$, $[\alpha]_D$ +23°, was characterised as the N-acetyl-and pentaacetyl-derivative. Nuclear magnetic resonance spectrum studies of this latter derivative indicated a straight chain aldose with diaxial arrangement for the C-1 and C-2 hydrogens. Absence of axial acetyl groups from the signal suggested a D-gluco-configuration; this was confirmed by nitrous acid deamination of the tetra-O-acetyl-derivative which yielded D-glucose. This information suggested that this fragment must be 6-amino-6-deoxy-D-glucose (6-glucosamine, XLVIII), a conclusion verified by comparison with an authentic synthetic sample, which caused no melting point depression and had an identical infrared spectrum[221].




(XLVIII)

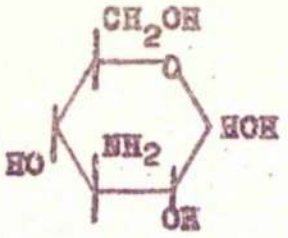
(XLVIII)

The third fragment from the acid hydrolysis of kanamycin, the fastest moving on papergrams was isolated in the form of the hydrochloride and as the N-acetate. Full acetylation gave a penta-acetate characterised as a monoaminohexose $C_6H_{13}NO_5$[222,220], termed kanosamine. N-Acetylkanosamine treated with dilute periodate consumed 1 mole rapidly, forming 1 mole of formic acid, thus establishing an aldohexose structure. Excess neutral periodate gave 2.8 moles of formic acid

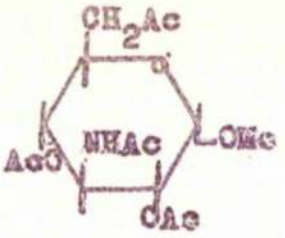
with 0.7 mole formaldehyde after consumption of 6.2 moles periodate. These values are very similar to those obtained in parallel experiments with 2-acetamido-2-deoxy-D-glucose, establishing the presence in kanosamine of only one terminal primary hydroxyl. Kanosamine is thus a straight chain aldohexosamine[220].

N-Acetylkanamycin oxidised with weak periodate (pH 2.5) utilised 2 moles of periodate forming one mole of formic acid, no formaldehyde or ammonia being produced. Hydrolysis of the oxidation mixture studied on papergrams showed the presence of 2-deoxystreptamine and kanosamine, but 6-amino-6-deoxy-D-glucose was destroyed. Periodate survival indicated the presence of 3-amino-3-deoxyglucose linked glycosidically to 2-deoxystreptamine.

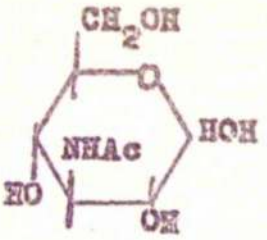
Nitrous acid deamination of crude O-acetylated kanosamine followed by re-acetylation yielded α-D-glucose pentaacetate which could have been derived from 3-amino-3-deoxyglucose or 3-amino-3-deoxyallose. The glucose configuration was confirmed in kanosamine by comparison of the methyl glycoside tetra-acetate (XLIXb) with an authentic synthetic specimen[115] when the infrared spectra were identical and the mixed melting point caused no depression. Kanosamine (XLIXa) has now been synthesised by the cyanohydrin[223] and nitromethane[224] methods. Comparison of natural and synthetic N-acetyl derivatives (XLIXc) (mixed melting point, X-ray diffraction, $R_F$ values, and mutarotation) confirmed their identity.



(XLIXa)



(XLIXb)

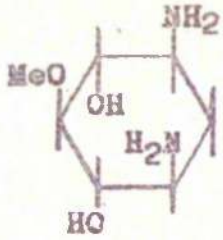


(XLIXc)

Kanamycin base subjected to mild acid hydrolysis followed by papergram analysis gave six spots, four of which were accounted for by unchanged kanamycin and the three fragments described above. The remaining two spots having $R_F$ intermediate between kanamycin and 2-deoxystreptamine were eluted separately on cellulose powder and further hydrolysed[222]. The faster moving 2-deoxystreptamine-kanosamide, m.pt. 240-2°[225], gave, on hydrolysis, 2-deoxystreptamine and kanosamine, whilst the slower moving (α-D-6-amino-6-deoxyglucopyranosyl)-deoxystreptamine[226] yielded 2-deoxystreptamine and 6-glucosamine results which suggested that both hexosamines are glycosidically linked to 2-deoxystreptamine[222]. This latter fragment is a mild bacteriostat *in vitro*[226].

Kanamycin base in acidic periodate solution (pH 4.5) rapidly consumed 6 moles of periodate. Paper chromatography of this reaction mixture indicated deoxystreptamine but no 6-glucosamine. The survival of 2-deoxystreptamine under these conditions suggests substitution at positions 4 and 6 of this moiety[226]. Further support for this conclusion was gained by exhaustive methylation of N-acetylkanamycin followed by acid hydrolysis when 1,3-diamino-4,6-dihydroxy-5-methoxycyclohexane (L)[227] was released indicating that positions 4 and 6

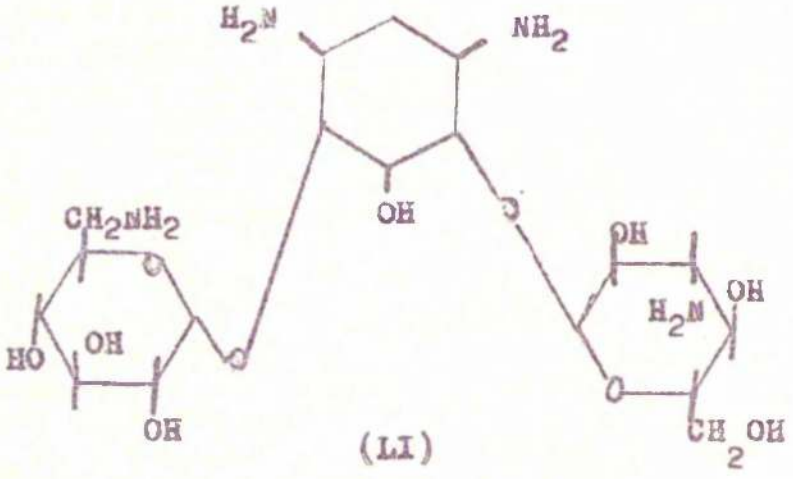
were not free for methylation.




(L)

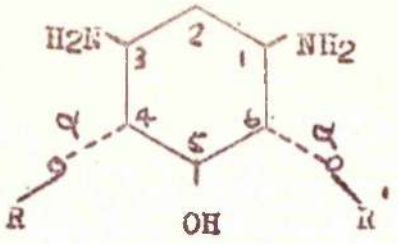
Also released in the methylation hydrolysate recovered by cellulose column chromatography are 3-acetamido-3-deoxy-2,4,6,-tri-O-methyl-D-glucose and 6-amino-6-deoxy-2,3,4-tri-O-methyl-D-glucose[228], indicating C-1 glycosides.

The glycosidic links between aminohexoses and 2-deoxystreptamine are believed to be  because of the presence in the infrared spectra of kanamycin base of bands at 838 cm.$^{-1}$ and 823 cm.$^{-1}$ assigned to $\alpha$-glycosidic bonds in comparison with infrared studies[115]. These data allow a gross structural formula (LI) to be written for kanamycin.




(LI)

The final uncertainty in the stereochemistry of kanamycin, the absolute configuration of the unsymmetrically substituted 2-deoxystreptamine portions of the antibiotic was resolved as detailed under neomycin (p. 38) by nuclear magnetic resonance[197] and rotation[203] studies Rinehart and Hichens give (LII) as the structure.




R = 6-Glucosamine

R' = 3-Glucosamine

(LII)

## Kanamycin B.

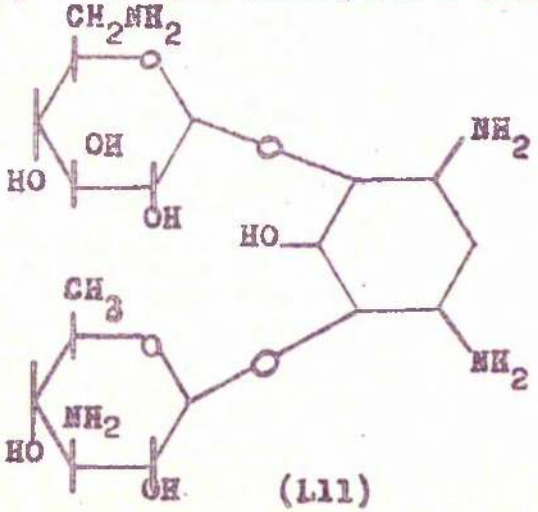
Paper chromatography of early kanamycin preparations revealed the presence of a second antibiotic, termed kanamycin B, separated from the main product by means of water-saturated n-butanol containing 2% toluene sulphonic acid. The infrared spectra were similar[229]. Isolation was first achieved by countercurrent distribution of the salicylidene derivative of the crude antibiotic and chromatography on weakly basic Amberlite resin. Later workers separated the mixture by chromatography on Dowex-1 basic resin crystallising kanamycin B from dimethylformamide. The base $C_{10}H_{32}N_4O_{10}$[230], melting point above 170° (decomp.) $[\alpha]_D$ + 114° [230], + 135[228], was water-soluble and gave positive Molisch, Elson-Morgan, and ninhydrin colour tests but negative reducing sugar and Sakaguchi. A polyacetylated derivative has been prepared and from this N-acetyl kanamycin B was obtained by de-O-acetylation ($[\alpha]_D$ + 150° m.p. 220-225° (decomp.). The latter on hydrolysis, gave kanosamine, deoxystreptamine and an unidentified ninhydrin positive spot but no 6-glucosamine[228]. This spot is now known to be a diaminohexose[203b]. The Japanese[230] report that kanamycin B is less active against most organisms than kanamycin A but more active against Mycobacteria.

## Kanamycin C.

Partial hydrolysis studies by Japanese workers on the recently isolated antibiotics by paper chromatography and ion exchange resin treatment[231] have shown it to contain pseudoneamine (paromamine) 4-(2-amino-2-deoxy-D-glucosyl)-2-deoxystreptamine[203b,231], itself an antibiotically active fragment[232]. Thus in kanamycin C,6-glucosamine is replaced by 2-glucosamine. The antibiotic is less active than kanamycin A on Mycobacteria.

## Deoxykanamycin.

Deoxykanamycin has been prepared recently by partial synthesis from kanamycin A[233]. The amino groups were first protected by forming the carbobenzoxy derivative after which treatment with toluene-p-sulphonyl chloride selectively tosylated the C-6 primary hydroxyl of kanosamine. Refluxing with sodium iodide gave the iodo compound which reduced over Raney-nickel to give the deoxykanosamine. Hydrogenation with palladium-charcoal in acetic acid gave the tetra-N-acetyl deoxykanamycin. The free base is also reported[3](L111). It is reported equipotent with kanamycin A except with Mycobacteria.




(L11)

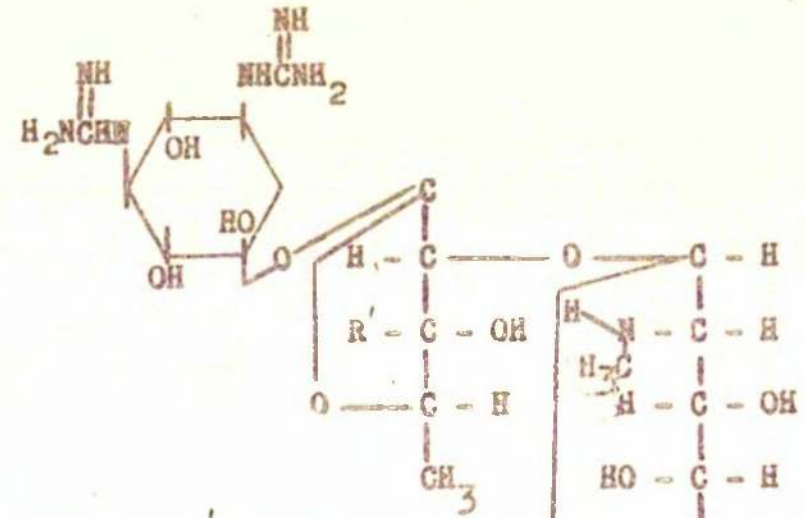
## The Chemistry of the Streptomycin Group.

Following the discovery of the activity of streptomycin against various organisms, including Mycobacterium tuberculosis by Waksman and his associates in 1944[55], a number of laboratories were involved in detailed chemical investigation of the antibiotic culminating in the designation of streptomycin as structure (LIIIa). This work has been reviewed extensively elsewhere[234,235,236] and is briefly cited here.

Streptomycin is provided commercially almost exclusively as the sulphate of the triacidic base $(C_{21}H_{39}O_{12}N_7)_2 3H_2SO_4$. Other salts which were of importance are the trihydrochloride and the crystalline calcium chloride double salt $2(C_{21}H_{39}O_{12}N_7 \cdot 3HCl)CaCl_2$. Crystalline salts such as the reineckate and helianthate were used by early workers, for characterisation[237,238,239].

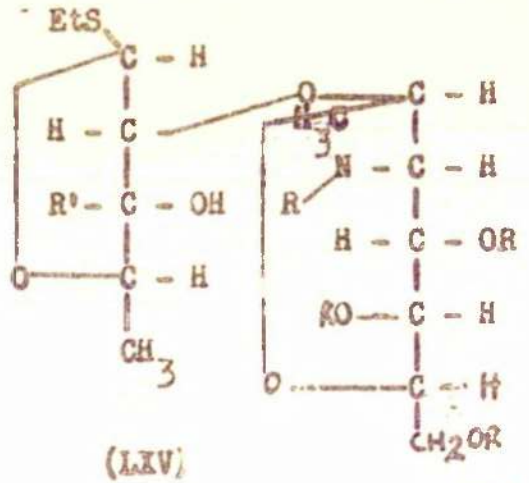
The base $[\alpha]_D$ $-78^\circ$ is soluble in water but insoluble in almost all organic solvents. The sulphate in contrast to the hydrochloride, is insoluble in methanol, both salts being water soluble. The dry powder hydrochloride is stable for six hours at $110^\circ$ in vacuo and relatively stable between pH 2 to 9 at $25^\circ$.

Streptomycin(VI)is composed of three monosaccharide fragments streptidine (a), streptose (b) and N-methyl-L-glucosamine (c) joined together by glycosidic bonds. Streptidine is a diguanido-inositol, streptose a unique branched chain sugar with two aldehyde groups, and N-methyl-L-glucosamine is a 2-aminohexose. The disaccharide formed by streptose and N-methyl-L-glucosamine was termed streptobiosamine.



(LIII) a. $R' = CHO$

b. $R' = CH_2OH$

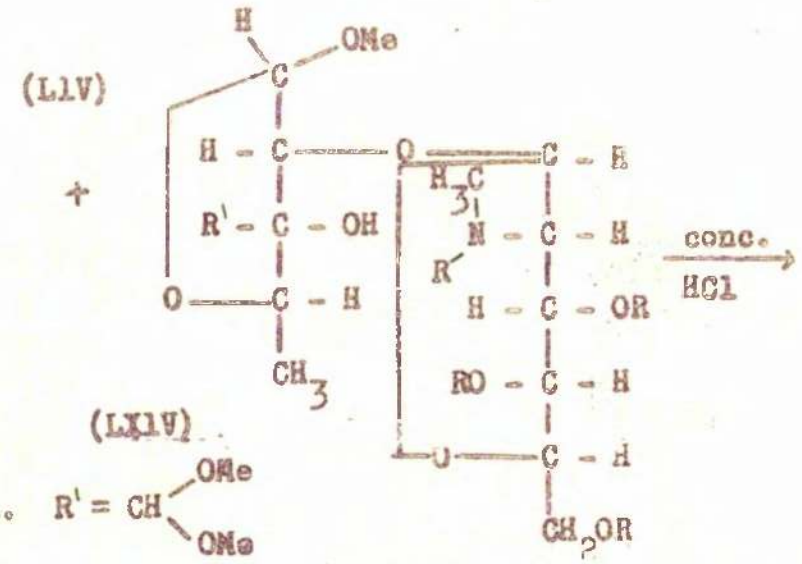


(LXV)

a. $R' = CH(SEt)_2$ $R = H$

b. $R' = CH_2OH$ $R = H$

c. $R' = CH(SEt)_2$ $R = Ac$

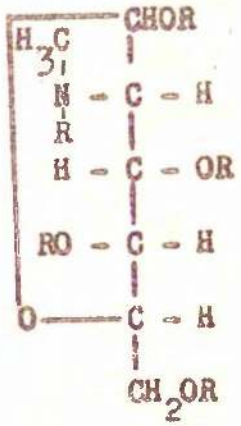


(LIV) +

(LXIV)

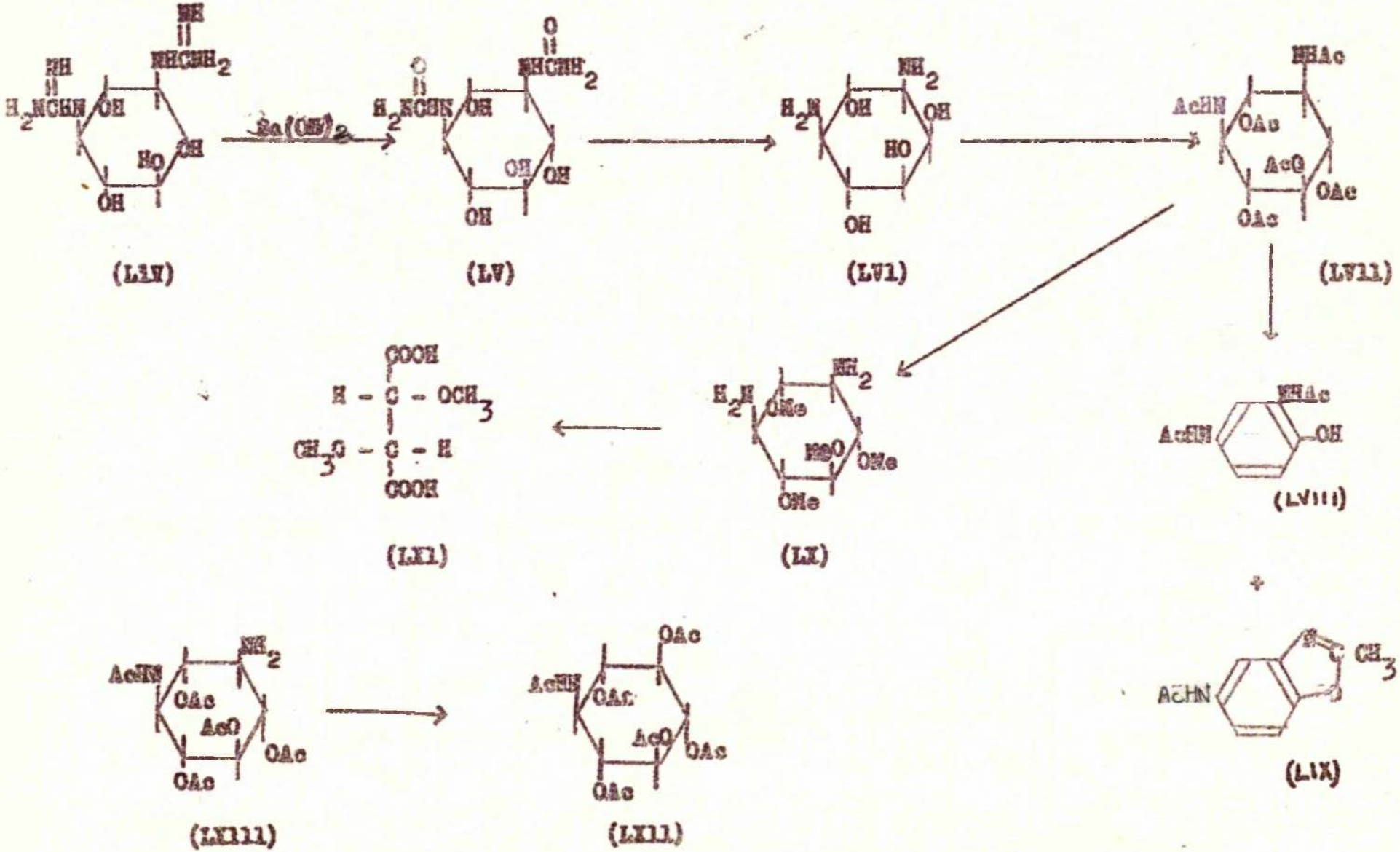
a. $R' = CH(OMe)_2$

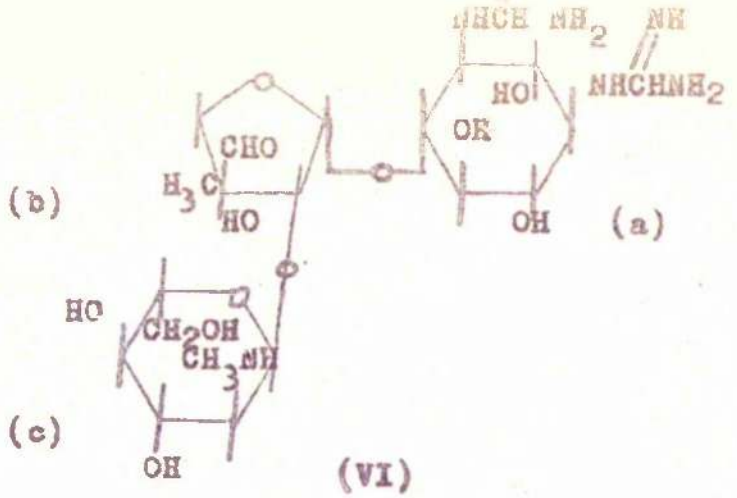
b. $R' = CH_2OH$



(LXVI)

a. $R = H$ b. $R = Ac$


Ba(OH)2
(LIV)
(LV)
(LVI)
(LVII)
(LVIII)
(LIX)
(LX)
(LXI)
(LXII)
(LXIII)




(VI)

Qualitative tests for active groups within the molecule gave evidence of reactive carbonyl[241,242,243], C-methyl (Kuhn-Roth)[244,245,246], N-methylamino[241,244] and a positive Sakaguchi test for guanidine groups[247].

Mild acid hydrolysis or methanolysis is sufficient to cleave the streptidine - streptobiosamine glycosidic linkage, the action with N-sulphuric acid giving crystalline streptidine sulphate in good yield[248]; this moiety was identified as $C_6H_{18}O_4N_6$ by analysis of salts including the picrate[249], which also confirmed the presence of two basic groups. Guanidino groups were detected by a positive Sakaguchi[248,249] test, and by titration with aqueous permanganate, when two mols of guanidine per mole were released. Thus all six nitrogens were accounted for in two basic guanidine groups. Acetylation of streptidine gave an octaacetyl derivative, four of the acetyl groups being attached to nitrogen and four to oxygen atoms. A carbocyclic structure with four hydroxyl and two guanidino groups was therefore suspected[250].

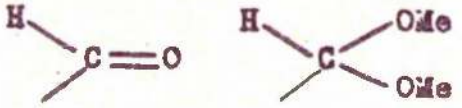
The main degradative work on streptidine followed alkaline hydrolysis and pyrolysis by Folkers and his co-workers[250]. Mild treatment with barium hydroxide released two molesof ammonia and gave streptursea $C_8H_{16}N_4O_6$ (LV). More vigorous alkaline treatment released

a further 2 moles of ammonia and 2 moles of carbon dioxide to give a base $C_6H_{14}N_2O_4$ streptamine (LVI) in which the guanidino groups were degraded to primary amino.

Thermal treatment of hexa-acetylstreptamine (LVII) gave high yields of 2,4-diacetamidophenol (LVIII) and 5-acetamido-2-methyl-benzoxazole (LIX), thus indicating that the amino group were in the 1,3-position.

Streptamine is optically inactive and therefore must be a meso form. The brilliant synthesis of streptamine[251] and streptidine[252] from D-glucosamine by Wolfrom and his colleagues proved that they were indeed 1,3-diamino inositol derivatives. The known configuration of D-glucosamine established C-3, C-4 and C-5 as all trans and C-1 and C-6 were deduced as being trans[251b]. This deduction verified by oxidative degradation of O-tetramethylstreptamine (LX) to D,L-dimethoxysuccinic (LXI) acid, identified as its diamide[253,254] showing that the C-5 hydroxyl was orientated trans with respect to the C-4 and C-6 hydroxyl. This left the configuration at C-2 to be established. This was proven unequivocally by preparation of D,L-myo-inosamine hexa-acetate (LXII) in which the C-2 configuration is known, an identical compound being obtained from the mono-N-acetyl streptamine (LXIII) by deamination[254] with nitrous acid. Thus the all trans configuration of streptamine was rigorously established and streptidine is shown to be 1,3-diguanidino-scyllo-inositol (LIV).

Streptobiosamine was first isolated as the isomeric methyl glycosides obtained on methanolysis of streptomycin, and removal of streptidine by selective precipitation[241,244]. The carbonyl absorption was also extinguished by this action and as analysis showed the presence of three methyoxyl groups a dimethyl acetal was suspected.




The analytical figures are consistent with the equation[244].

$$C_{21}H_{39}N_7O_{12}\cdot 3HCl + 3CH_3OH \longrightarrow C_8H_{18}N_6O_4\cdot 2HCl + C_{13}H_{22}NO_7(OCH_3)_3HCl.H_2O$$

and the fragment was designated methyl streptobiosaminide dimethyl acetal hydrochloride[241](LXIV).

The hydrogenation of streptomycin with a platinum catalyst[256,257,258] or Raney nickel[246] yielded the biologically active dihydro-derivative (LIIIb) in which carbonyl reactions were absent. Dihydrostreptomycin also forms isomeric methyl glycosides[241,244], only one methoxyl group being present (LXIVb). These reactions confirm the hemiacetal structure of the streptomycin derivatives.

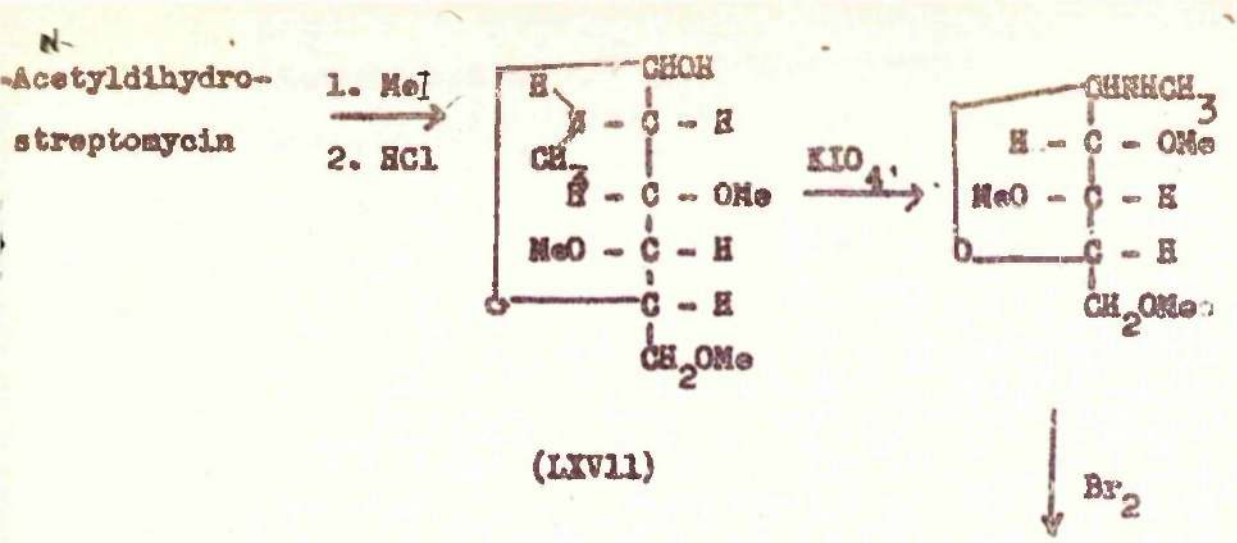
Glycosides from both were amorphous mixtures of $\alpha$- and $\beta$ -, isomers best characterised by formation of acetyl derivatives[244]. Streptomycin gave methyl tetra-acetylstreptobiosaminide dimethyl acetal with three O-acetyl groups and one N-acetyl group whilst dihydrostreptomycin gave penta-acetyl-methyl dihydrostreptobiosaminides with four O-acetyl and one N-acetyl group on differential acetyl determination.

The $\alpha$ and $\beta$ anomers of penta-acetyl dihydro streptobiosaminide are separable by crystallisation. Refluxing with concentrated aqueous alkali gave methylamine indicative of the presence of a methylamino group[244].

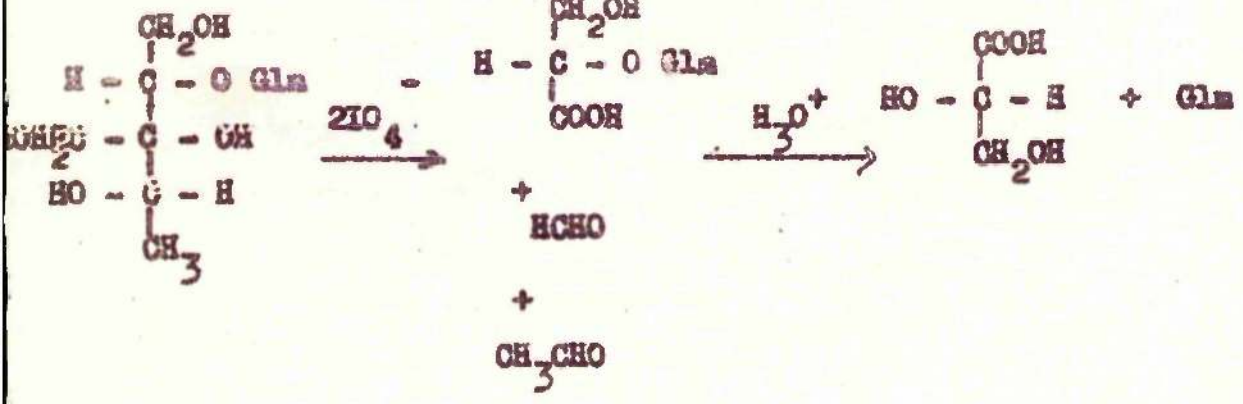
Mercaptolysis, the analogous reaction to methanolysis utilising ethyl mercapton and dry hydrogen chloride resulted in the formation of ethylthiostreptobiosaminide dithioacetal hydrochloride (LXVa) and ethylthiodihydrostreptobiosaminide hydrochloride (LXVb) from streptomycin or methylstreptobiosaminide dimethyl acetal and dihydrostreptomycin or methyldihydrostreptobiosaminide respectively. In these derivatives the methoxyl groups are replaced by ethylmercapto groups[246,259].

Hydrolysis of methylstreptobiosaminide dimethyl acetal with concentrated hydrochloric acid followed by acetylation yielded the pentaacetyl derivative, of a hexosamine[245,260] (LXVIb). This was identified as N-methyl-L-glucosamine (LXVIa) by formation of a phenylosasone and a phenylosatriazole identical with those of L-glucose, by oxidation to a product with the same properties but an equal and opposite rotation to N-methyl-D-glucosaminic acid and finally by synthesis[260,261]. This synthesis commenced from L-arabinose, methylamine and hydrogen cyanide forming the nitrile which gave on lactonisation and reduction N-methyl-L-glucosamine. The crystalline pentaacetyl derivatives of synthetic and natural specimens were identical[260]. Kuhn and Bister have improved the yields in this synthesis[262].

N-Acetyldihydrostreptomycin







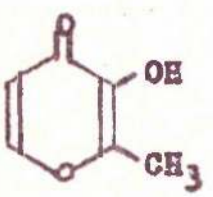

Glm = N-Methyl-L-glucosamine

The configuration about C-2 was established by methylation of D-glucosamine to give N-methyl-D-glucosamine and its penta-acetyl derivative. These compounds were identical but with an equal and opposite rotation and thus C-2 has the configuration for L-glucosamine[260]

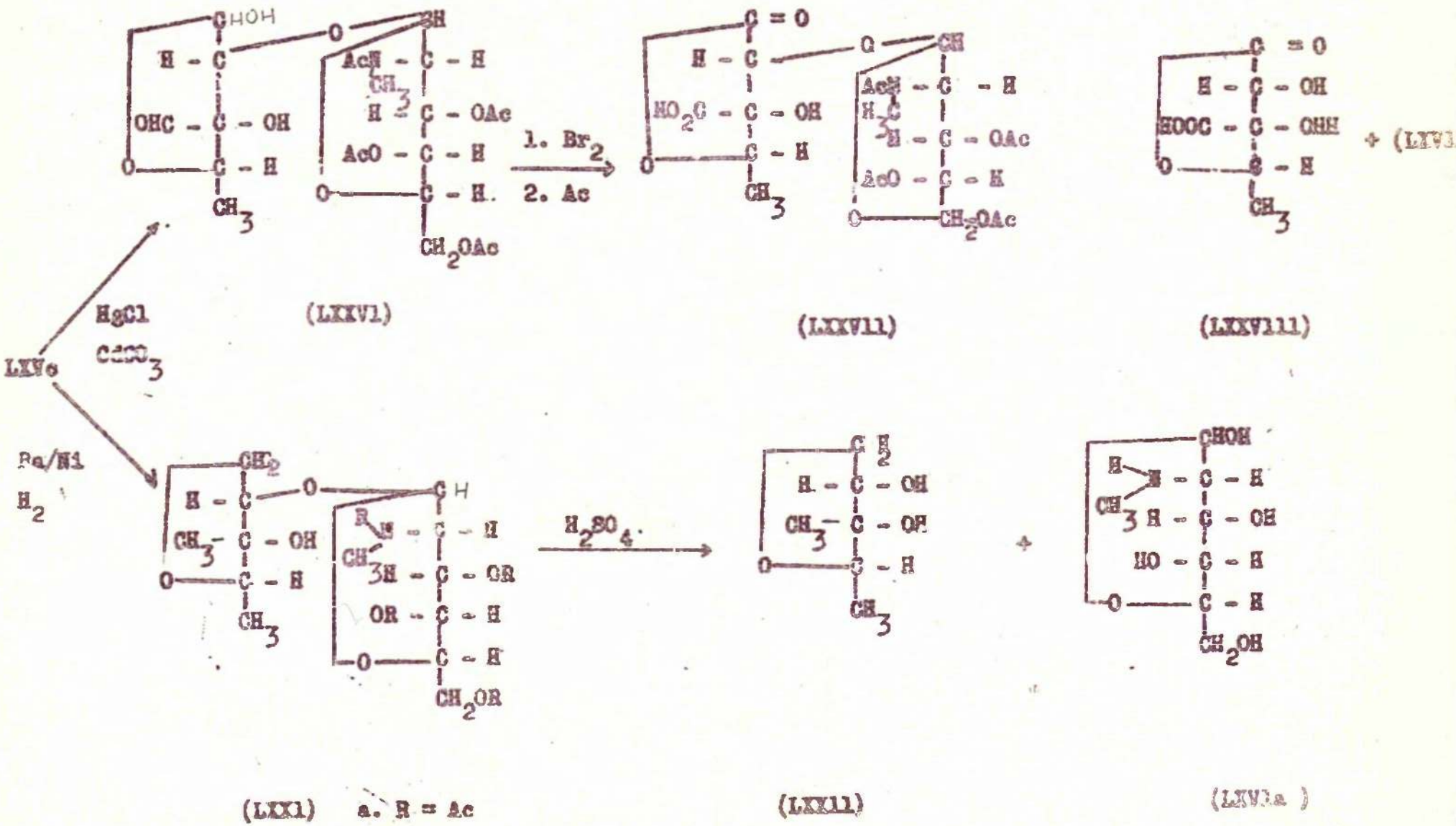
The pyranose ring form of the glucosamine moiety was confirmed by methylation of N-acetyldihydrostreptomycin followed by hydrolysis using 2.5N hydrochloric acid. The product after acetylation and chromatography yielded the crystalline diacetate of a tri-O-methyl-N-methyl-α-L-glucosamine which on further hydrolysis with 2.5 N hydrochloric acid gave the parent tri-O-methyl-N-methyl-L-glucosamine hydrochloride (LXVII). Periodate oxidation of this product (one mole) followed by bromine water oxidation led to the known 2,3,5-tri-O-methyl-L-arabinolactone (LXVIII) which gave a crystalline amide (LXIX) identical with an authentic sample[263].

The rigorous conditions necessary to achieve scission of the glucosaminide bond present in streptobiosamine resulted in the destruction of the streptose moiety. This represented a major problem in structural investigations . However the structure of this fragment has been adduced by a study of degradation products.

Alkaline hydrolysis of streptomycin yielded a substance characterised as maltol (LXX) 161-162° [264]. Dihydrostreptomycin failed to give this product or any of its derivatives.



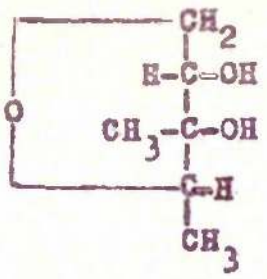

(LXX)


HgCl
CaCO3
LXVe
Ra/Ni
H2
1. Br2
2. Ac
H2SO4
(LXXVI)
(LXXVII)
(LXXVIII)
(LXXI)
a. R = Ac
b. R = H
(LXXII)
(LXVIa)

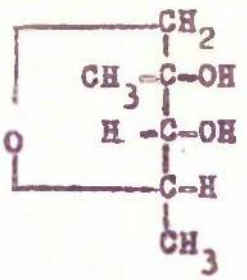
The key product in the elucidation of the structure of the central streptose moiety was the ethyl tetra-acetyl-diethylmercaptal (LXVe), obtained[259,246] above. Two series of degradations involving this compound led to isolation of derivatives of streptose and the proposal of structure (LXXXIV) scheme opp.

The first route involved hydrogenolysis of the mercaptal over a Raney catalyst to give tetra-acetylbisdeoxystreptobiosamine[259,265,266] (LXXIa). This compound exhibited a non-acetylatable hydroxyl group present, presumed tertiary, and observed on infrared analysis and yielding one mole of methane per mole on Zerewitinoff determination. De-O-acetylation, gave N-acetylbisdeoxystreptobiosamine(XVIIb) which on Kuhn-Roth estimation showed the presence of three C-methyl groups, one accounted for by N-acetyl, one present in the parent streptobiosamine and one formed in hydrogenation[266].

Hydrolysis of tetra-acetylbisdeoxystreptobiosamine with 5% $H_2SO_4$ gave N-methyl-L-glucosamine and a new compound bisdeoxystreptose $C_6H_{12}O_3$ in which Kuhn-Roth estimation showed two terminal methyl groups no carbonyl group being present. The formation of a bis-p-nitrobenzoate indicated the presence of two hydroxyl groups. Periodate oxidation resulted in the uptake of one mole proving that the two hydroxyls were adjacent, and examination of periodate oxidation products enabled the choice to be made between two possible structures (LXXIII and LXXIV)[260].

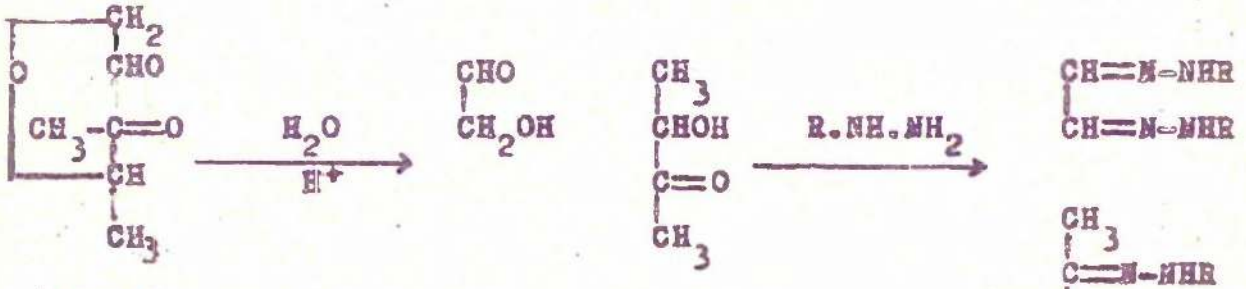


(LXXII)



(LXXIII)

Oxidation of (LXXII) followed by hydrolysis would give on reaction with hydrazine, osazones of biacetyl (LXXIV), whereas (LXXIII) would form osazones of pyruvaldehyde. The biacetyl osazones were obtained. Structure (LXXII) is therefore preferred to (LXXIII) for bisdeoxystreptose. Periodate degradation is shown below.



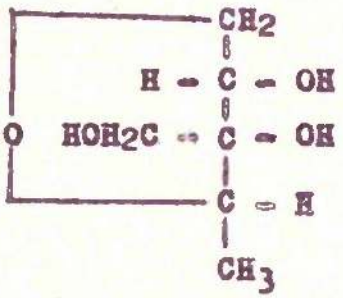
(LXXII)

(LXXIV)

It can be seen that in bisdeoxystreptose, the methyl group adjacent to the tertiary hydroxyl must have been formed in the hydrogenolysis. Proof of this point and therefore the position of the aldehyde group was obtained by study of dihydrodeoxystreptose.

Penta-acetyldihydrodeoxystreptobiosamine formed by mercaptolysis of dihydrostreptomycin, hydrogenolysis of the resulting thioglycosides

and subsequent acetylation[244], yielded dihydrodeoxystreptose (LXXV) on hydrolysis with 5% sulphuric acid. Periodate oxidation of this fragment gave formaldehyde consistent with structure (LXXV)[235].

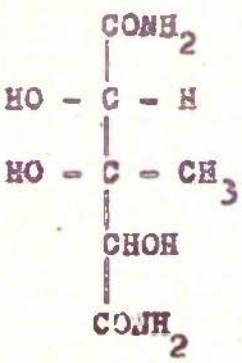


(LXXV)

The second route of degradation of ethyl tetra-acetyl-thiostreptobiosaminide diethyl mercaptal, *vis.* demercaptalation with aqueous mercuric chloride in presence of carbonate buffer, yielded tetra-acetyl-streptobiosamine (LXXVI)[259,267]. This derivative was oxidised with bromine water and then acetylated to give penta-acetyl-biosamine acid monolactone (LXXVII), the free aldehyde having been oxidised to a carboxyl group. Hydrolysis of this compound with 2.5 N hydrochloric acid followed by acetylation gave penta-acetyl-*N*-methyl-L-glucosamine (LXVIb) and the crystalline diacetyl derivative of a substance designated streptosonic acid monolactone (LXXVIII). The nature of streptosonic acid monolactone was revealed by titration, and also by infrared studies in which -COOH and lactone absorption were present. A diamide of this product was prepared with ammonia showing the dibasic nature of this fragment. Kuhn-Roth estimation revealed the presence of a *C*-methyl group, and periodate oxidation indicated three adjacent hydroxyls, two of which were *cis* as shown by boric acid complex formation. Two structures

(LXXIX) and (LXXX) were possible from this data[267].

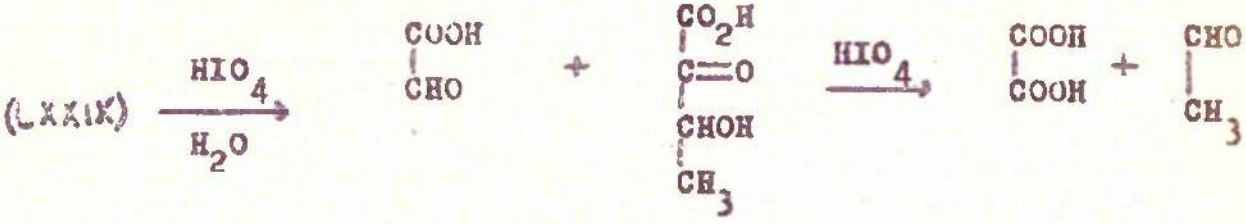
(LXXIX)



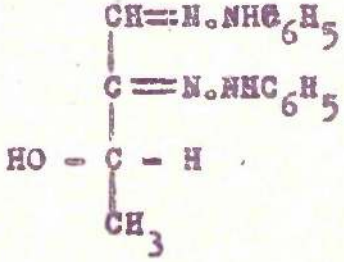
(LXXX)

The choice was again made by periodate oxidation. Structure (LXXX) which would have yielded acetic acid was rejected since no volatile acid was formed.

Periodate oxidation of streptosonic acid monolactone gave glyoxalic acid and oxalic acid confirming structure (LXXIX), the suggested course of reaction being[267]



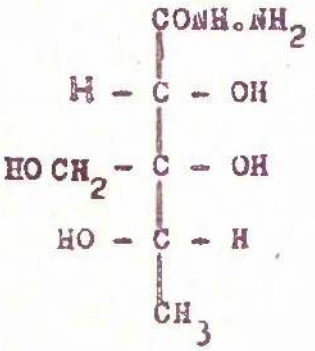
The stereochemistry of streptose was adduced from a number of derivatives. Streptobiosamine hydrochloride reacted with phenylhydrazine to give a crystalline phenylosazone (LXXXI)[268]. Thus streptose is an L-sugar C-4 having the L-configuration.



(LXXXI)

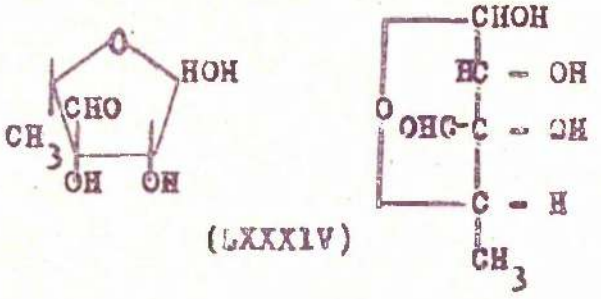
The hydroxyl groups on C-2 and C-3 have the cis configuration as bisdeoxystreptose gives a boric acid complex[266].

Dihydrostreptosonic acid lactone reacted with hydrazine to give dihydrostreptosonic acid hydrazide (LXXXII)[269] $[\alpha]_D$ +23° and application of Hudson's rules indicated that the hydroxyl group at C-2 and (thus C-3) lies to the right



(LXXXII)

A confirmation of this point was achieved by Wolfrom and De Walt[270] who degraded N-acetyltetrahydrostreptobiosamine (LXXXIII) to L-glyceric acid. Thus streptose is 3-C-formyl-L-lyxomethylose (LXXXIV).



(LXXXIV)

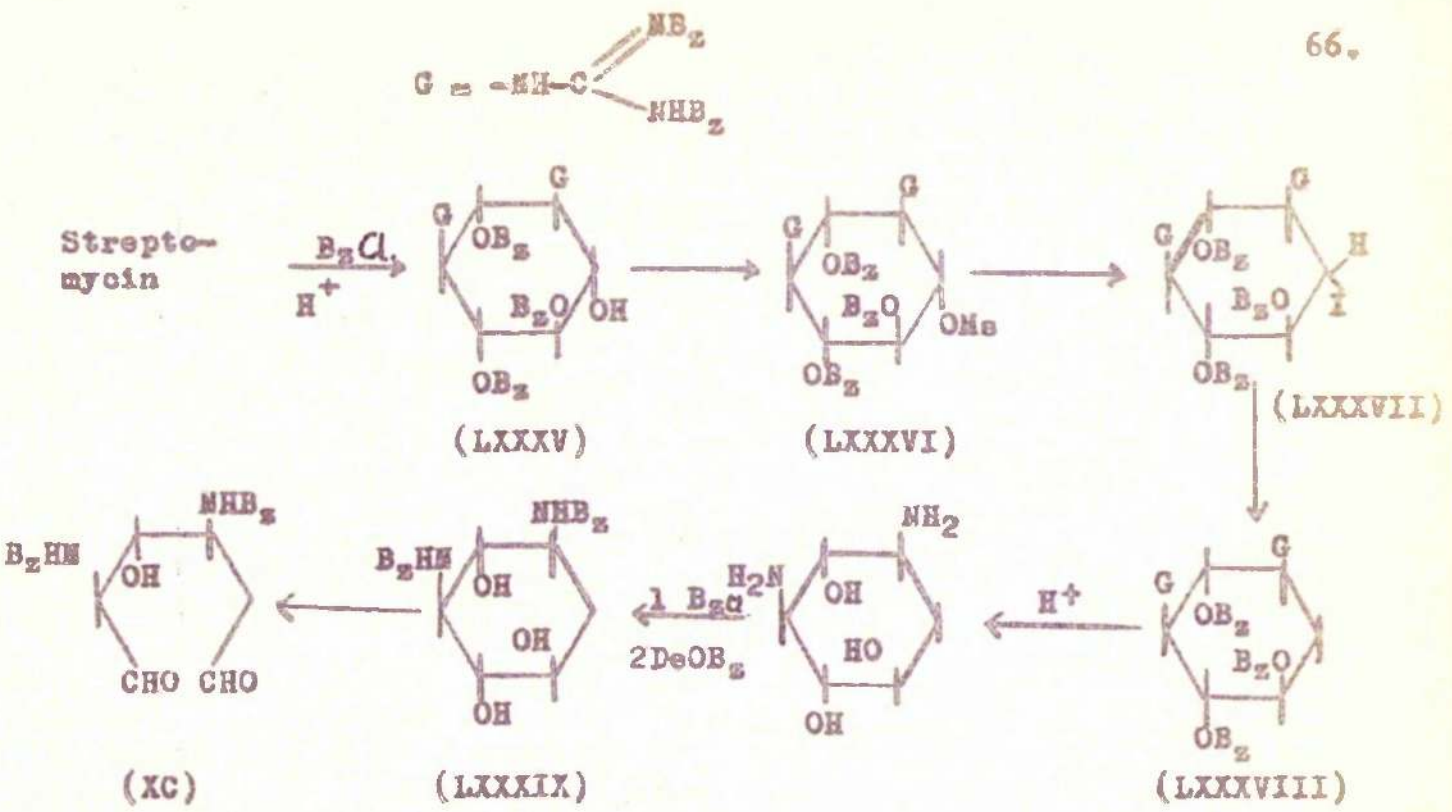
On the basis of this formula for streptose, Wolfrom and his co-workers have suggested a plausible mechanism for the alkaline degradation of streptomycin to maltol[234].

Linkage of N-methyl-L-glucosamine to streptose was through C-1 as evidenced by negative Fehlings reaction of N-acetyl-bisdeoxystreptobiosamine[259] in which both streptose aldehyde groups are reduced. Thus C-1 of glucosamine is concerned in the glycosidic linkage.

The hydroxyl on C-2 of streptose must be linked to N-methyl-L-glucosamine as the C-3 hydroxyl is tertiary[266]. This presumption was confirmed by periodate oxidation of methyl N-acetyldihydrostreptobiosaminide and methyl N-acetyl-α'-L-streptobiosaminide dimethyl acetal; the former consumed one mole of periodate more rapidly, consistent with an α'-glycol, forming formaldehyde[271].

Proof of the linkage of streptidine to streptobiosamine through C-1 of streptose was adduced from dihydrodeoxystreptose (LXXV) in which periodate oxidation released formaldehyde[266], thus the aldehyde in dihydrostreptose is on C-1 and forms the glycosidic linkage.

The point of linkage of streptobiosamine or its dihydro-derivative to streptidine has just been finally established. That either the C-4 or C-6 hydroxyl both adjacent and trans to guanidino groups is involved was proven by periodate oxidation of the N,N'-dibenzoyl-4-deoxystreptamine[272]. The latter was obtained by benzoylation of streptomycin, hydrolysis to heptabenzoylstreptidine (LXXXV), formation of the mesyl derivative (LXXXVI) through the iodo derivative (LXXXVII). The oxidation results in the uptake of one mole and formation of α, γ-dibenzamido-β-hydroxyadipaldehyde (LXL).




Wolfrom and his co-workers[273] utilising benzoylated derivatives of streptomycin, streptidine and methyldihydrostreptobiosaminide for optical rotation experiments suggest that the streptidine - streptose linkage is $\beta$ L- and the hexosamine - streptose linkage is $\alpha$ -L.

Thus the formula (Lllla) given for streptomycin and dihydrostreptomycin was elucidated.

The absolute configuration of streptidine in streptomycin was recently established by Dyer and Todd[426]. Streptobiosamine is attached to C-4 of streptidine. (The ring numbering introduced by Rinehart is followed)[203](see also page 38).

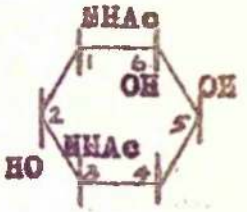
N,N'-Dibenzoyl-4-deoxystreptamine (LXXXIX) was converted to the N,N'-diacetyl derivative (LXXXIXb) by acid hydrolysis, acetylation and de-O-acetylation. The optical rotation of this latter compound was measured in water $[\alpha]_D^{25}$ +5° and cupremmonium B -970° to give the $[\Lambda]_M$ cupra B -2400° calculated by the method

of Reeves[204]. This large negative value is similar to that of -2075° obtained for the 2,3-glycol complex of D-glucosides such as methyl 4-O-methyl-D-glucoside (XLII) rather than the positive value of 2150° given by methyl 2-O-methyl-D-glucoside (XLI) which forms the 3,4-glycol complex with cuprammonium B. This proves that N,N'-diacetyl-4-deoxystreptamine forms a 5,6-glycol cuprammonium complex with a clockwise 60° angle and not a 4,5-glycol complex which would be formed if streptobiosamine were attached to C-6 of streptidine in streptomycin when, with the above reactions, a 6-deoxystreptamine derivative would be obtained. The absolute configuration of N,N'-diacetyl 4-deoxystreptamine is thus (LXXXIXc) from which follows the absolute configuration of streptomycin (except for the streptose ring) (LIIIc).

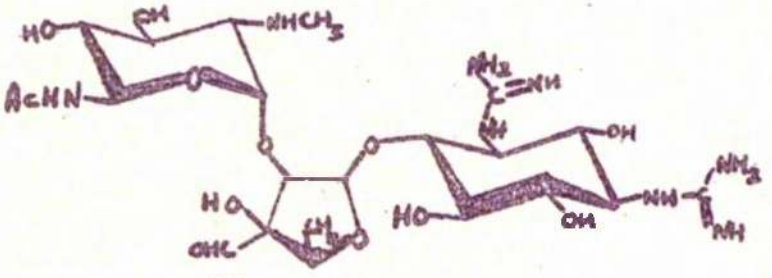
(LXXXIXc)

(XLII)

(XLI)



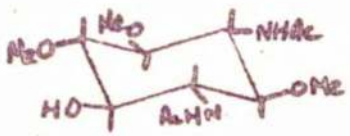
(LXXXIXb)



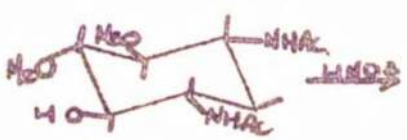
(LIIIc)

The assignment is in agreement with the work of Tatsuoka and Horii[427] which was based on the fact that N,N'-diacetyl-2,5,6-tri-O-methylstreptamine (LXb) prepared by deguanidation, exhaustive methylation and hydrolysis of streptomycin has the same sign of rotation as N,N'-diacetyl-5,6-di-O-methyl-2-deoxystreptamine (XLIIIb)

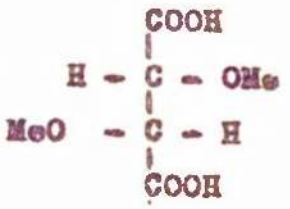
derived from zygomycin (see page 43). The oxidation of this latter derivative with nitric acid gave di-O-methyl-Ds tartaric acid (LXI) permitting the assignment of the R configuration to C-4.



(LXb)



(XLIIIb)



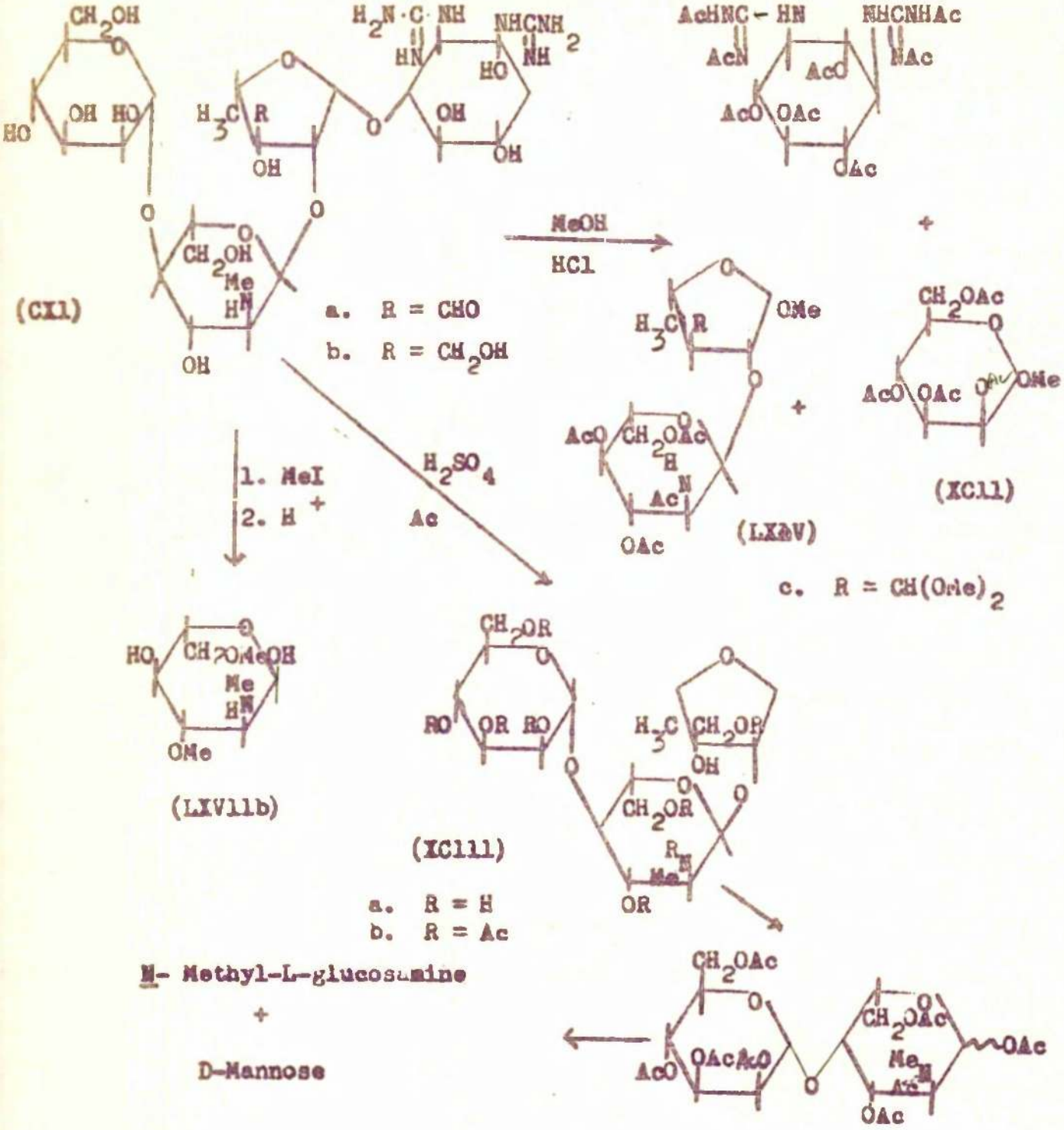
(LXI)

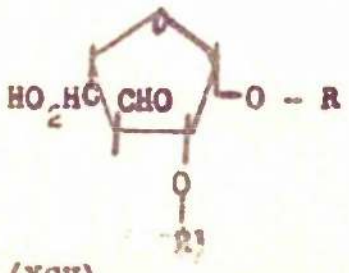
Mannosidostreptomycin (XCI). This antibiotic first termed streptomycin B was separated from culture concentrates by alumina chromatography and counter-current distribution techniques[274]. It was first crystallised as the reineckate. As it is less active biologically than streptomycin (one fifth) it is not a desirable contaminant and its presence constitutes a major problem in streptomycin production.

Methanolysis of mannosidostreptomycin gave, after acetylation methyl tetraacetyl streptobiosaminide dimethylacetal hydrochloride, and α methyl tetraacetyl-mannopyranoside (XCII)[276], identical with authentic samples. Octaacetylstreptidine was also obtained[277]. The analogous mercaptolysis gave streptidine hepta-acetate, β-ethylthio-tetraacetyl-streptobiosaminide dithioacetal and the isomeric thioethyl-tetra-acetyl-mannosides identical with synthetic samples[276,277].

Dihydromannosidostreptomycin (XCIb) was prepared by catalytic hydrogenation when maltol-formation and carbonyl activity observed in mannosidostreptomycin was extinguished. Methanolysis of dihydro-mannosidostreptomycin followed by acetylation gave α and β-methyl-

# Degradation of Mannosidostreptomycin



(XCV)

R = Streptidine

$R^1$ = N-Methyl-L-glucosamine

tetra-acetyl-D-mannopyranosides (XCII). Hydrolysis of dihydromannosidostreptomycin with N sulphuric acid gave dihydromannosidostreptobiosamine (XCIIIa) which on acetylation gave a nona-acetyl derivative (XCIIIb). Differential acetyl determination showed eight O-acetyl and one N-acetyl present in this compound; hence the mannose is linked to a hydroxyl group of streptobiosamine.

Acetolysis of nona-acetyldihydromannosidostreptobiosamine (XCIIIb) gave a disaccharide composed of D-mannose and N-methyl-L-glucosamine (XCIV) which were released on methanolysis thus proving that D-mannose is attached to the glucosamine moiety in mannosidostreptomycin.

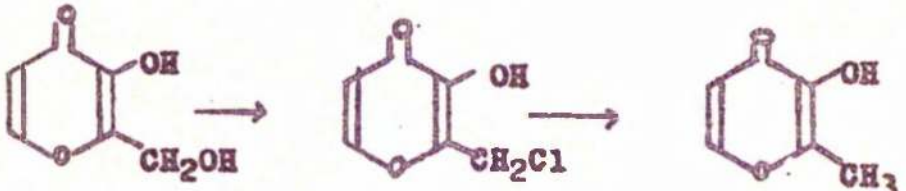
The linkage of D-mannose was shown by methylation to be at C-4 in N-methyl-L-glucosamine when 3,6-di-O-methyl-N-methyl-L-glucosamine (LXVII) was isolated and identified as its acetate by comparison with the synthetic D-enantiomorph[263].

That the trisaccharide is attached to position C-4 of the streptidin as in streptomycin was shown by the preparation of heptabenzoylstreptidin identical with that from benzoylated streptomycin[278]. The streptomycin portion of mannosidostreptomycin was shown to be identical by enzymatic cleavage of the mannose - streptomycin[279] linkage.

Hydroxystreptomycin (XCV). This antibiotic was obtained from the culture filtrates of Streptomyces griseocarneous[280] and since it differs from streptomycin by one additional hydroxyl group, was named hydroxystreptomycin.

Catalytic hydrogenation gave a dihydroxystreptomycin which after methanolysis and acetylation gave a disaccharide hexa-acetate and octa-acetylstreptidine. Dihydrostreptomycin under these conditions gives the

penta-acetate (Methyl penta-acetyldihydrostreptobiosaminide). Penta-acetyl-N-methyl-L-glucosamine was obtained from hydroxystreptomycin by strong acid hydrolysis and acetylation indicating that the extra hydroxyl group is attached to the streptose moiety[280]. Alkaline degradation provided further information on this point when a pyrone was obtained[281]. (XCVI)

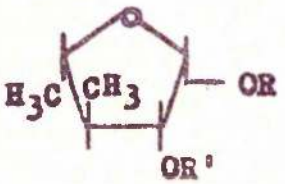



(XCVI) (LXX)

This pyrone was converted by way of the chloro derivative to maltol (LXX), the product of alkaline degradation of streptomycin[282].

Hydroxystreptomycin is not as active as streptomycin but appears to be more stable to acid.

Dihydrodeoxystreptomycin (XCVII). This antibiotic is produced from streptomycin by hydrogenation with sodium amalgam catalyst[283]. Breakdown by methanolysis and investigation of the products indicated that the change had been wrought in the streptose moiety[284].




(XCVII)

R = streptidine

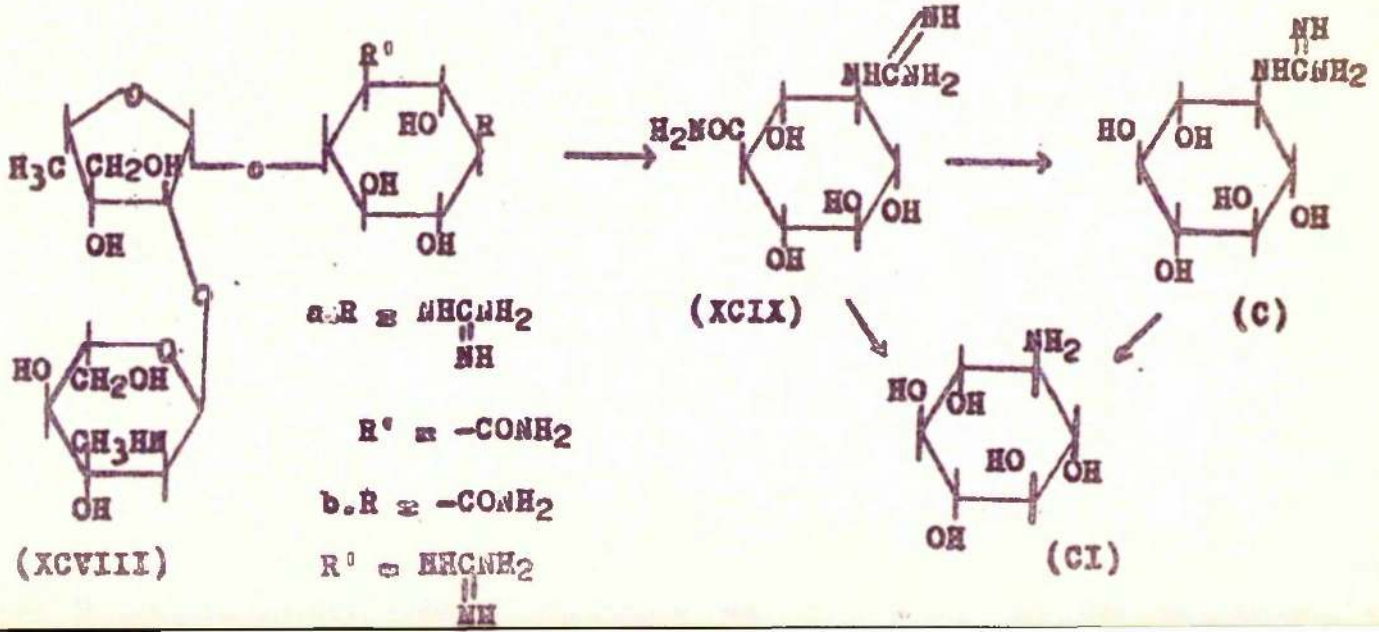
R' = N-methyl-L-glucosamine

A number of patents for similar hydrogenated derivatives have been applied for[285].

## BLUENSOMYCIN.

A new member of the streptomycin group bluensomycin (XCVIIIa or b) was obtained from the culture filtrates of *Streptomyces bluensis* in 1962[286]. It was purified by carbon column chromatography, and the hydrochloride, sulphate and *p*-toluenesulphate prepared[287]. The dihydrochloride $C_{21}H_{39-41}N_5O_{14}$ gave a positive Sakaguchi reaction indicative of guanidino groups.

Methanolysis of bluensomycin split the molecule into two parts[288] which were separated by carbon column chromatography. Elution with water gave a strong base designated bluensidine (XCIX); subsequent elution with acetone gave a larger fragment, $C_{14}H_{26}O_8N$, identical with methyldihydrostreptobiosaminide hydrochloride as evidenced by formation of a penta-acetyl derivative. Hydrolysis with concentrated acid followed by acetylation gave *N*-methyl-L-glucosamine penta-acetate, identical with authentic samples[289]. Mercaptolysis followed by acetylation gave ethyl thiodihydrostreptobiosaminide and served to confirm the structure of this fragment.



Bluensidine hydrochloride $C_8H_{16}N_4O_6.HCl$, the second fragment of methanolysis m.p. 190 - 194° $[\alpha]_D$, + 0.5 to 1.5° was shown to have a positive Sakaguchi reaction and hence a guanidino group. Infrared analysis showed carbonyl absorbtion and hydroxyl or amino nitrogen bands. Vigorous hydrolysis with barium hydroxide released 2 moles of carbon dioxide and 3 moles of ammonia to yield a crystalline optically active base, $C_6H_{13}NO_5$ (CI). This was shown to have one amino group whose hexa-acetyl and N-acetyl derivatives were identical by comparison with the like derivatives of scyllo - inosamine. Acid hydrolysis of bluensidine gave one mole each of carbon dioxide and ammonia plus an optically inactive base, $C_7H_{15}N_3O_5.HCl$ (C), which gave a positive Sakaguchi reaction. Strong alkaline treatment of this base again resulted in scyllo - inosamine (CI) with release of one mole of carbon dioxide and 2 moles of ammonia. It is thus identified as 1-deoxy-1-guanidino scyllo - inositol[288]. The degradation of bluensidine to this base with elimination of one mole of carbon dioxide and ammonia together with infrared carbonyl absorbtion suggests the presence of a primary carbamoyl group. The guanidino and carbonyl group could not be present as 1,4-substituents since the compounds are optically active. The choice between a 1,2- and a 1,3- arrangement was determined by periodate oxidation. Two moles of periodate were consumed for the release of one mole of formic acid hence bluensidine is 1-deoxy-1-guanidino-3-O-carbamoyl-scyllo - inositol (XCIX). The configuration was similar to dihydrostreptomycin on the basis of rotation evidence[288].

This antibiotic, the first in which streptidine has been replaced, by a different, though biogenetically related base, is cross resistant[286] with and less active [290] than streptomycin and kanamycin.

Glebomycin isolated from a new Streptomyces species in Japan[291] appears from preliminary investigations to be identical with[286] bluensomycin.

## BIOSYNTHESIS OF STREPTOMYCIN.

This branch of investigation is of increasing interest, though many of the biogenetic pathways are speculative; improved isotopic tracer techniques aid the elucidation of these routes in Streptomyces griseus, selected strains[292] of which are streptomycin producers of high yield.

Early investigators in the search for optimal media for streptomycin production found empirically that the inclusion of certain nutrients led to improved yields. Hockenhull[275] in an extensive review (1960) attempts to rationalise these results.

Glucose has always been the sugar of choice as a carbon source in media, though other selected monosaccharides such as galactose and xylose allow growth[293]. The production of streptomycin is less efficient from polysaccharides such as starch and is dependent on the organisms ability to hydrolyse the glycosidic linkages. Sucrose and raffinose are thus not utilised by Streptomyces griseus.

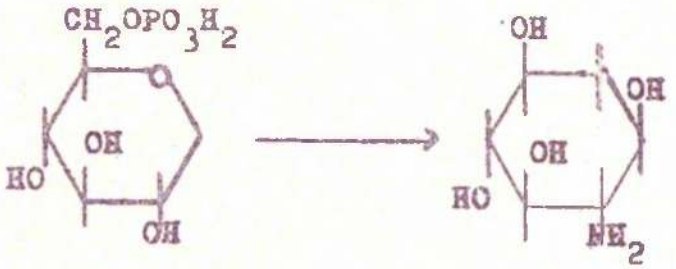
The nitrogen source is also important. An early study of Dulaney[294] showed that simple nitrogen sources such as amino acids gave low streptomycin production. Charge over to complex sources brought immediate improvements. Hockenhull suggested two reasons for this observation[275]. First the supply of ammonia nitrogen from a complex medium is gradual and the rate of utilisation kept the pH between 7 and 8 the optimal for streptomycin fermentations. Secondly the supply of nitrogen was slow enough to limit protein of new cell formation so that carbohydrate was routed into streptomycin production

in mature cells.

Isotope Experiments Final proof of the role of a nutrient as a precursor in streptomycin formation is obtained only by incorporation of the isotopically labelled substance into whole or part of the antibiotic.

Radio-active $^{14}C$ carbon dioxide, glucose, acetate and glycine have been utilised in tracer studies[275,295]. The carbon of the streptidine guanidino groups may be derived from atmospheric carbon dioxide. That arginine has a role in transference of guanidino or urea groups is also suspected from this study with $^{14}C$ carbon dioxide.[296]

Hunter and Hockenhull[247] showed that $^{14}C$ glucose was uniformly distributed throughout the three fragments of the molecule except in the guanidine groups but that from glycine also appeared in N-methyl-glucosamine[298]. Hockenhull[275,297] suggests that the streptidine might arise from ring closure of a glucose derivative, such as glucosamine-6-phosphate (C11) to give scyllo-inosamine (C1).




(C11) (C1)

Recent studies suggesting myoinositol as a streptidine precursor are based on the decrease of $^{14}C$ glucose appearing in streptomycin when inositol is added to the medium[295,299]. Myoinositol occurs in corn steep liquor, soya bean meal and distillers solubles, well known nutrients in antibiotic production.

The formation of N-methyl-L-glucosamine has been studied utilising C-1 and C-6 labelled D- glucose when the C-1 and C-6 of the initial D-glucose was found as C-1 and C-6 of L-glucosamine. Rationalisation of this result led the authors to suggest inversion of all asymmetric C atoms of D-glucose by multiple epimerisations[300].

Hockenhull suggests that scylloinositol as formed above may undergo ring opening to form a hexose derivative of enantiomorphic configuration[275]. He also suggests that the N-methyl group is derived from the moulds methylating pool by way of methionine, a thesis supported by the fact that methionine has been shown to supply the methyl group in the biosynthesis of D-cladinose and desosamine, two branch chain sugars from erythromycin[301]. The methyl group may also be used in streptose formation[295].

The biogenesis of streptose remains to be explained. Hough and Jones reviewing the formation of monosaccharides suggest a scheme[302], involving an aldol condensation of tartaraldehyde with acetaldehyde (Figure VI ).

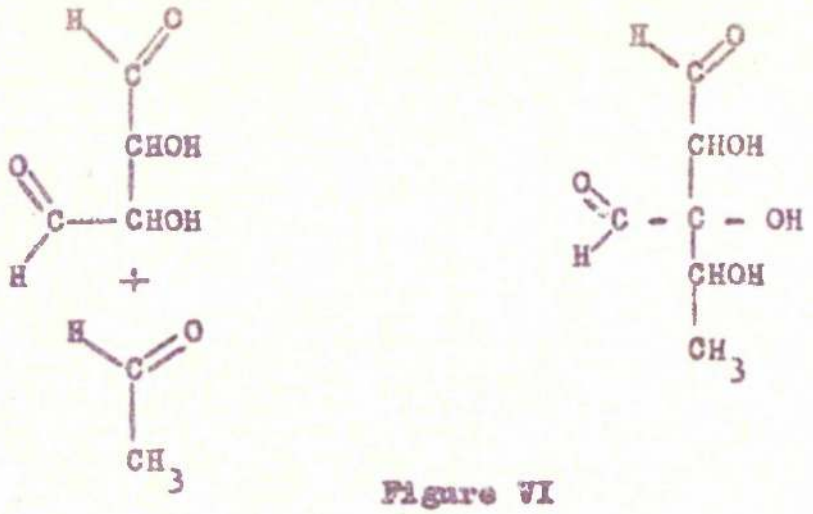


Figure VI

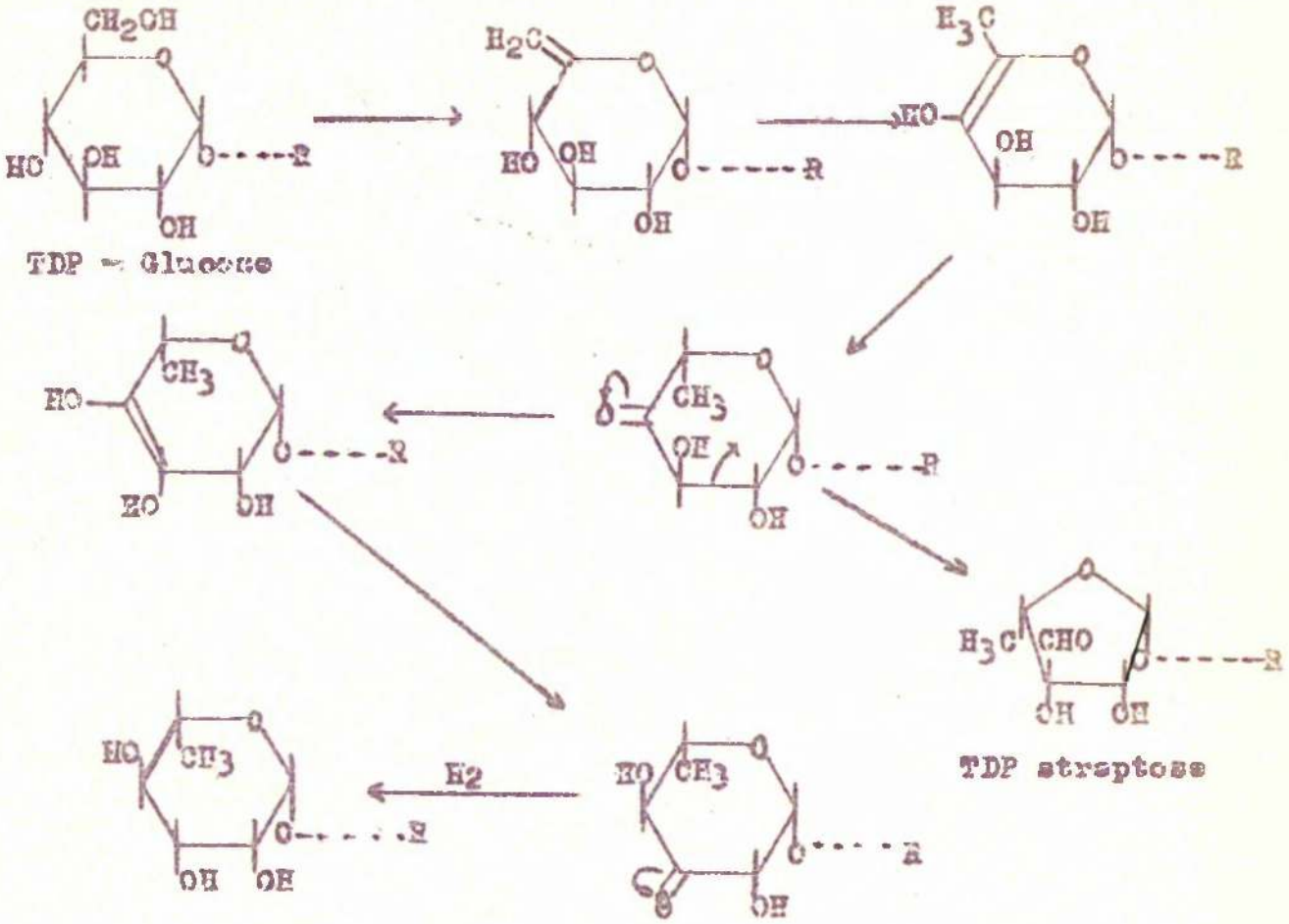
Abraham and Newton[303] propose that a pinacol - pinacolone rearrangement resulting in extrusion of an aldehyde as discussed by Woodward[304] for magnamycin biosynthesis may be involved (Figure VII). A ring cleavage of this nature is supported by

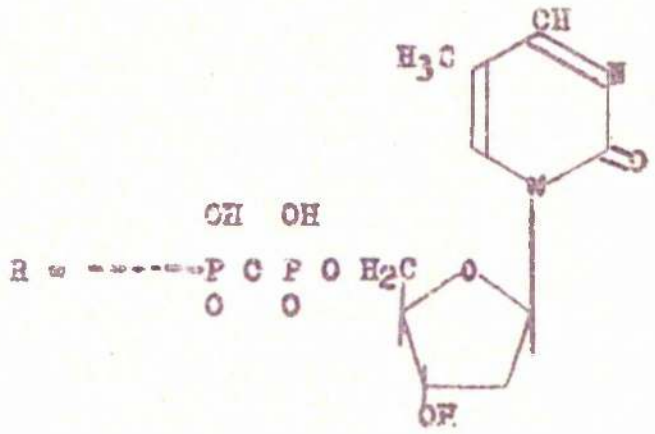
Figure VII

Baddiley who in the course of examining thymidine diphosphate (TDP) nucleotides of S. griseus suggested an enzymatic route of formation from α-D-glucose-1-phosphate. TDP-glucose is converted enzymatically to TDP-rhamnose, one dehydration and one reduction effecting three stereochemical inversions. Two of the intermediates may be precursors of streptose (Figure VIII)[305].

Possible streptose formation route - (Baddiley)

Fig. (VIII)



The role of phosphate in streptomycin synthesis has been considered by Hockenhull[275]. The inorganic phosphate levels should be kept low, an adequate supply of glucose and oxygen should be provided facilitating formation of the phosphorylated carbohydrate intermediates. These intermediates may be shunted from cell formation to streptomycin synthesis by nitrogen levels which will not lead to high-protein (cell wall) synthesis. Work with radioactive phosphorus is in progress which may clarify its function.

The role of mannosidostreptomycin is undecided, whether it is a precursor of streptomycin derived from polysaccharides[306] or a further elaboration from the streptomycin molecule is arguable. The latter view is considered more likely by recent workers[275,295].

Mode of Action of the Aminoglycoside Antibiotics.

Although almost twenty years have passed since the introduction of streptomycin the mode or modes of action of this antibiotic and its structural relatives as an antibacterial or antimycobacterial agent are as yet unknown. Research workers in these complex studies have sought to differentiate between the primary and secondary biological effects and to identify the site of metabolic interference ultimately responsible for growth inhibition and cell death[307].

Umbreit and his colleagues embarked on a systematic study of streptomycin mostly on Escherischia coli[308]. He found that the drug is selectively toxic to bacteria because of a double permeability barrier in animal cells. The amount of streptomycin penetrating from the blood stream into tissue is small as a result of the cell wall

barrier. The host cell is further protected by a permeability barrier at the surface of the mitochondria. These workers believed that streptomycin is a powerful inhibitor of the enzyme system involved in the oxidation of pyruvate and oxidation to citrate in the Krebs cycle[309]. However, streptomycin did not reduce citrate formation[310], though Umbreit proposed a second condensation product of pyruvate and oxalacetate[311] Hahn considers this hypothesis unlikely[307] - a view supported by later experiments by Katagari[312] in which no inhibition of the oxalacetate - pyruvate system was demonstrated.

Davis and his co-workers in experiments with $^{14}C$ labelled streptomycin and E. coli suggested that the aminoglycoside antibiotics had two possibly interrelated sites of action. Firstly, inhibition of ribosomes, which is presumed to be responsible for the interference with cell-membrane protein synthesis. Secondly, cell-membrane permeability is impaired allowing leakage and osmotic imbalance to occur[313]. That this protein and nucleic acid inhibition also occurs with kanamycin has been demonstrated by Tsukmara[314] using Myco. avium

These effects of streptomycin have also been shown to occur in the protein synthesis of Mycobacterium tuberculosis[315].

Speculations concerning the role of streptomycin as a competitive inhibitor in metabolic routes due to its similarity with cell wall polysaccharides was advanced by Stacey[316]. Interference with inositol metabolism has also been considered likely[295].

## SYNTHETIC WORK

The synthetic approach towards the aminoglycoside antibiotics has achieved only limited success. Biological activity is retained only if the antibiotic is subjected to a minor modification such as the formation of dihydrostreptomycin from streptomycin or kanamycin methane sulphate a new salt of kanamycin.

A number of patents have recently been issued concerning the pantothenates of streptomycin and its reduced derivatives. Streptomycin is treated with pantothenic acid D(+)-N-(α,γ-dihydroxy-β,β-dimethyl butyryl)-β-alanine ( CIII ) to yield a salt[317].

(CIII)

These salts are claimed to be less toxic to the auditory nerve and in one case said to be active against streptomycin-resistant organisms. Joint administration of calcium pantothenate and streptomycin is also said to reduce toxicity[318].

Other salts have been prepared as lower toxicity derivatives, including the methionates of streptomycin and dihydrostreptomycin[319] and the N-methane sulphinates ($-CH_2SO_2Na$) and N-methanesulphonates ($-CH_2SO_3Na$) of streptomycin and neomycin[320]. A number of similar salts have been prepared with kanamycin[321,322] by Japanese workers who have also prepared substituted methanesulphonates which may form a chelate ring by hydrogen bonding. Their results are summarised in

Table 2[323]. These derivatives are said to be less toxic than the sulphate but a question as yet unanswered is whether the therapeutic index is improved. Neomycin methanesulphonates are also described.

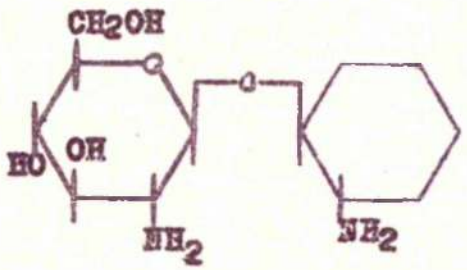
A number of derivatives of dihydrostreptomycin have been prepared by alkaline degradation. Both Stenlake[324] and his colleagues and Polglase[325] found that streptures dihydrostreptobiosaminide possessed low activity. Mital also prepared a number of other derivatives which are listed in Table 3[324].

The structure of streptamine having been shown to be 1,3-diamino scyllo - inositol, a number of workers[326-328] prepared and investigated related inosamines and derivatives. One such series is given in Table 4, but no compound of any positive antibiotic activity was described though some tertiary bases blocked nerve impulses.

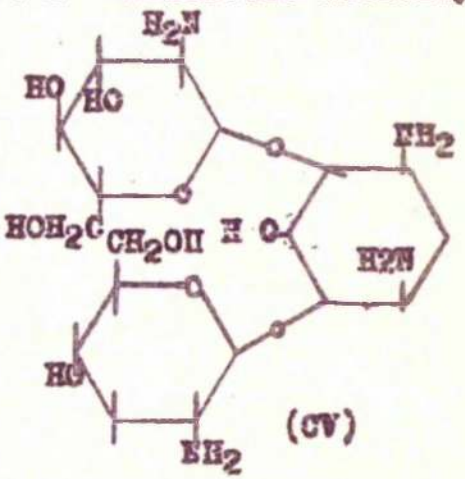
Stenlake and his co-workers[324] synthesised a series of streptamine and streptidine glycosides using the acetylated derivatives of streptidine or streptamine with one free hydroxyl and the glycosyl halides. The compounds are ennumerated in Table 5, none showed any appreciable activity against Mycobacterium tuberculosis in vitro.

May and his co-workers synthesised a series of substituted phenyl glycosides of D-glucose[329] and D-glucosamine[330] by way of the glycosyl halides (Table 6), but none of these compounds showed biological activity.

The discovery of kanamycin stimulated the Japanese to prepare analogous glycosides from 2-deoxystreptamine and its relatives. trans -2-Amino-cyclohexyl-D-glucosaminides (CIV)[331] and 4,6-di-O-(D-glucopyranosyl)-2-deoxystreptamine (CV)[332] showed no antibiotic activity.



(CIV)



(CV)

A further series of kanamycin related compounds prepared from 6-glucosamine were described (Table 7) by Wickstrom[333] but showed no activity as antibacterials though the piperidino and dibenzylamino derivatives were active against certain fungi.

A thesis describing modifications of neomycin and streptomycin antibiotics and effects on activity[334], the details of which are not yet available, suggests that monoamino, monoguanidinodihydrostreptomycin has about one fifteenth the activity of the parent drug. Reaction of neomycin, kanamycin and neamine with S-methythiourea to form partially and completely guanidinated derivatives gave compounds of considerably lower activity.

TABLE 2.

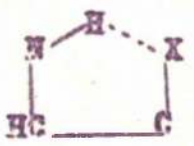
**Derivatives of Kanamycin (K) with reduced toxicity.**

| N-methane sulphonates | | Toxicity |
|---|---|---|
| N,N'-bis(methane sulphonate) | $K(NH_2)_2(NHCH_2SO_3Na)_2$ | Lower |
| N,N',N"N"-tetrakis (methane sulphonate) | $K(NHCH_2SO_3Na)_4$ | Lower |

Substituted N-methane sulphonates

$K(NH_2)_2(NHCH.X.SO_3Na)_2$

| | X | Toxicity of Kanamycin |
|---|---|---|
| I | $CH_2Cl$ | Less |
| II | $CHCl_2$ | Less |
| III | $CH_2Br$ | Same |
| IV | $CH_2OCH_3$ | Slightly less |




Supposed Chelate

TABLE 3

## In Vitro Activity of Some Streptomycin Derivatives on Mycobacterium Tuberculosis

| Name of Derivative | G | $R_1$ | $R_2$ | $R_3$ | Activity |
|---|---|---|---|---|---|
| Streptomycin | $-NH-\overset{NH}{\overset{\|\|}{C}}-NH_2$ | $-CHO$ | H | H | Good |
| N-acetyl streptomycin | $-NH-\overset{NH}{\overset{\|\|}{C}}-NH_2$ | $-CHO$ | $-CH_3CO$ | H | Less |
| N-acetyl trityl dihydrostreptomycin | $-NH-\overset{NH}{\overset{\|\|}{C}}-NH_2$ | $-CH_2OC(C_6H_5)_3$ | $CH_3CO$ | H | Slight |
| Undeca-acetyl streptomycin | $-NH-\overset{NAc}{\overset{\|\|}{C}}-NHAc$ | $-CHO$ | $-CH_3CO$ | $CH_3CO$ | None |
| Dodeca-acetyl dihydrostreptomycin | $-NH-\overset{N-Ac}{\overset{\|\|}{C}}-NHAc$ | $-CH_2OAc$ | $-CH_3CO$ | $CH_3CO$ | None |
| Strepturea-dihydrostreptobiosaminide | $-NH-\overset{O}{\overset{\|\|}{C}}-NH_2$ | $-CH_2OH$ | H | H | very slight |
| Streptamine dihydro streptobiosaminide | $-NH_2$ | $-CH_2OH$ | H | H | None |

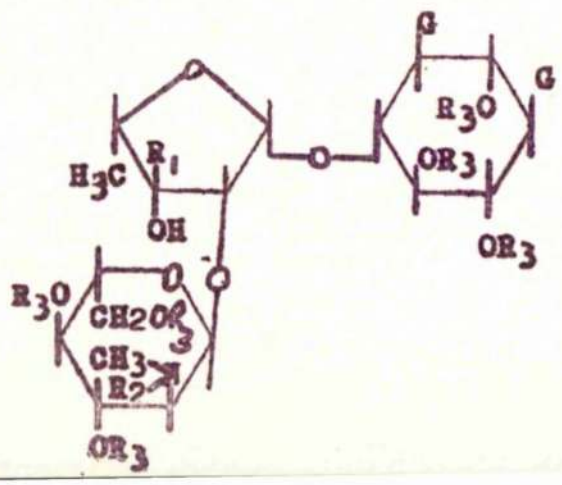


TABLE 4.

Synthetic 2-deoxystreptamine derivatives.

| No. | $R_1$ $R_1$ | $R_2$ | $R_3$ | $R_4$ |
|---|---|---|---|---|
| I | $CH_3CO$ | H | H | H |
| II | $C_6H_5CO$ | H | H | H |
| III | $C_6H_5CH_2$ | H | H | H |
| IV | $C_6H_5CH$ | H | | H |
| V | o-$H_3COC_6H_4CH$ | H | | H |
| VI | $C_6H_5CH$ | $CH_3CO$ | | $CH_3CO$ |
| VII | $CH_3CO$ | $C_6H_5NHCO$ | H | $C_6H_5NHCO$ |
| VIII | p-$H_2NC_6H_4CO$ | p-$H_2NC_6H_4CO$ | H | H |
| IX | $C_6H_5CH_2$ | H | $CH_3$ | H |
| X | $C_6H_5CH_2$ | $C_6H_5NHCO$ | $C_6H_5NHCO$ | H |

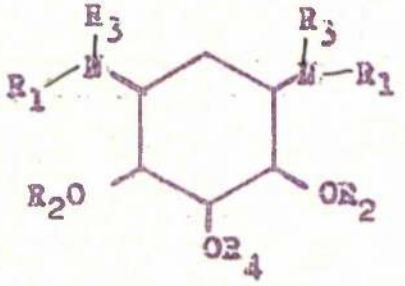
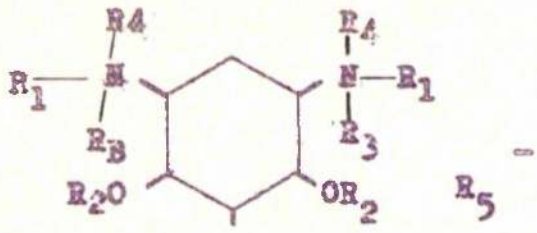
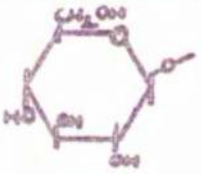
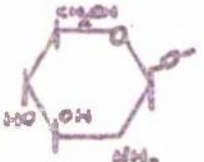
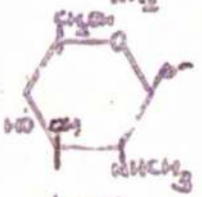
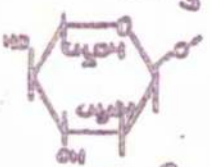
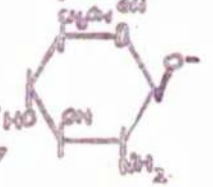


TABLE 4 . (continued)



| No. | $R_1$ | $R_2$ | $R_3$ | $R_4$ | $R_5$ |
|---|---|---|---|---|---|
| X1 | $(CH_3)_2CHCH_2$ | H | H | H | Cl |
| X11 | $CH_3$ | $CH_3CO$ | $CH_3$ | H | Cl |
| X111 | $CH_3$ | H | $CH_3$ | H | Cl |
| X1V | o-$H_3COC_6H_4CH_2$ | H | H | H | Cl |
| XV | $H_2NCNH$ | H | H | H | $SO_4$ |

TABLE 5.

| NAME | $R_1$ | $R_2$ | ACTIVITY |
|---|---|---|---|
| Streptidine-β-D-glucopyranoside | $-NH-C(=NH)-NH_2$ |  | Very slight |
| Streptidine-β-D-glucosaminide | $-NH-C(=NH)-NH_2$ |  | None |
| Streptidine-2-N-methylamino-β-D-glucosaminide | $-NH-C(=NH)-NH_2$ |  | None |
| Streptidine-2-N-methylamino-β-L-glucosaminide | $-NH-C(=NH)-NH_2$ |  | None |
| Streptidine-oxyethyl-β-D-glucopyranoside | $-NH-C(=NH)-NH_2$ |  | None |
| Strepturea-β-D-glucosaminide | $-NH-C(=O)-NH_2$ |  | None |
| Streptamine-β-D-glucosaminide | $NH_2$ | | None |

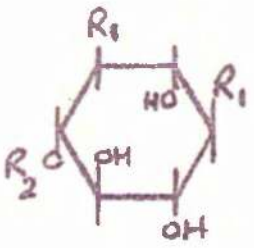
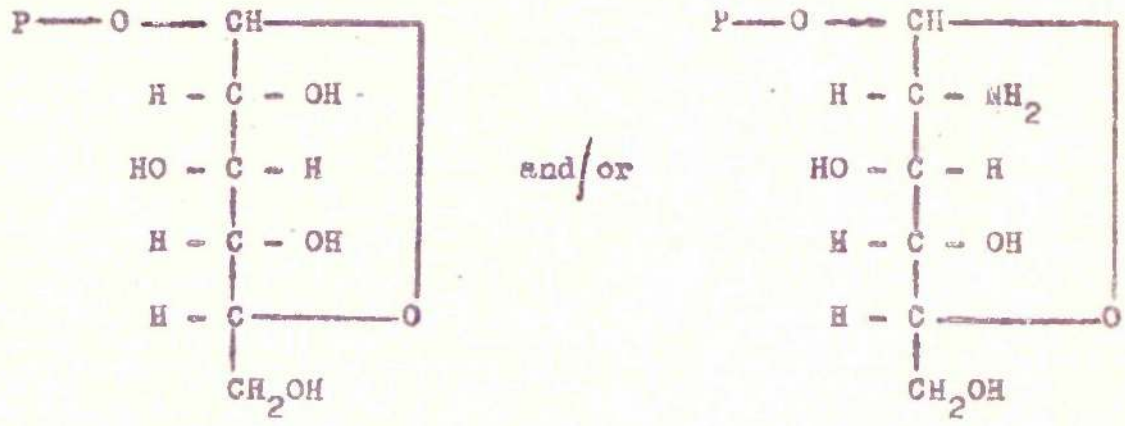


TABLE 6.

## Amino and Guanidino-Phenyl Glucosides



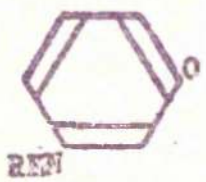
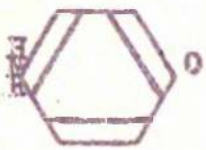
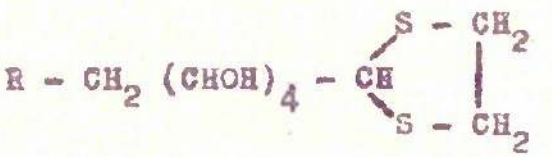
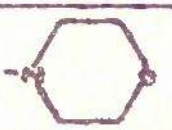
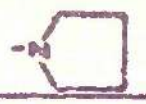
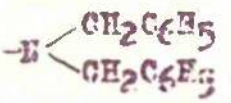

| | R | P | Q | |
|---|---|---|---|---|
| o-Aminophenyl-β-D-glucoside (or glucosaminide) | H |  |  | o-guanidino-phenyl-glucosi or (glucosaminide) |
| m-Aminophenyl-β-D-glucoside (or glucosaminide) | H |  |  | m-guanidino-phenylglucoside or (glucosaminide) |
| p-Aminophenyl-β-D-glucoside (or glucosaminide) | H |  |  | p-guanidino-phenylglucoside or (glucosaminide) |
| 2,4-Diaminophenyl-β-D-glucoside (or glucosaminide) | H |  |  | 2,4-diguanidino phenylglucoside or (glucosaminide) |

TABLE I.

R-substituted derivatives of 6-amino-6-deoxy-D-glucose.



| | R | Antifungal activity |
|---|---|---|
| A. Piperidino |  | Present |
| B. Morpholino |  | None |
| C. Pyrrolidino |  | None |
| D. Dibenzylamino |  | Present |

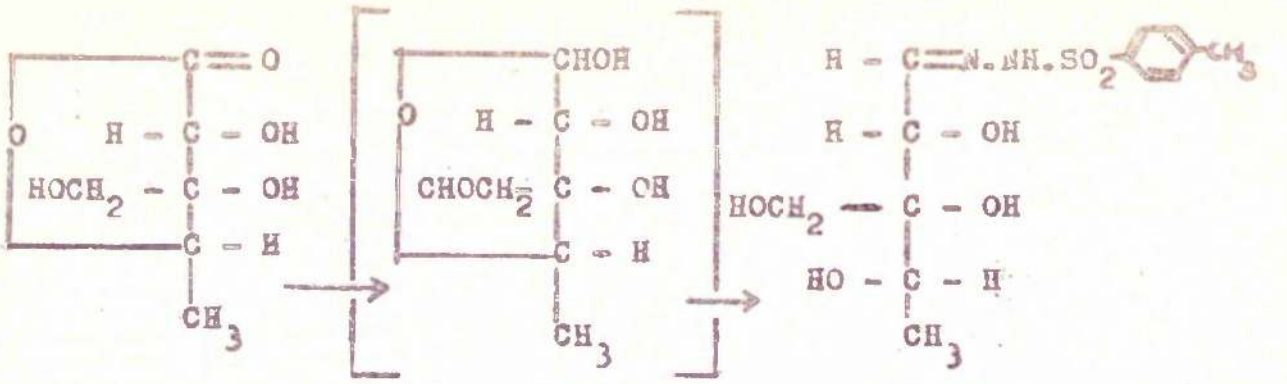
## Discussion

The structure of dihydrostreptomycin has not yet been determined in complete stereochemical detail. Though the point of attachment of streptidine to dihydrostreptose has just been shown to be C-4[426,427] rigorous proof of the nature of the glycosidic linkage and conformation of the nature and ring form of the streptose or dihydrostreptose moiety remains to be obtained.

The breakdown of the molecule into its component parts has been described in detail in the introduction. Streptidine and N-methyl-L-glucosamine, which were isolated in this way, were characterised by comparison with synthetic samples. The central dihydrostreptose moiety, however, was not isolated, though its structure in the molecule was adduced from the study of derivatives.

Isolation of this fragment would be of interest chemically to assign a stereochemical structure, and, biologically, to gain a clearer understanding of the role of this moiety in the action of the antibiotic.

Wang-Yu and his colleagues[335] have described the partial synthesis of L-dihydrostreptose (CVII) by reduction of L-dihydrostreptosonic acid lactone (CVI), with lithium aluminium hydride. The product of this reaction was a syrupy mixture from which a tosyl hydrazone was obtained, the analytical evidence and physical characteristics of which were indicative of dihydrostreptose tosylhydrazone (CVIII).



(CVI) (CVII) (CVIII)

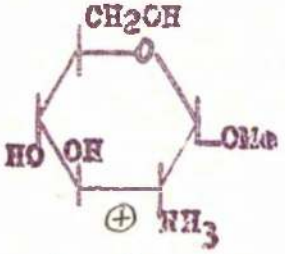
The streptidine - dihydrostreptobiosaminide bond in dihydrostreptomycin is readily cleaved by mild acid hydrolysis or methanolysis to yield a streptidine salt and the salt of the anomeric dihydrostreptobiosamine or their methyl glycosides. A number of methods have been published[244,246,257,258] for the hydrolysis and the separation of the resulting products. None of these methods resulted in the isolation of the $\alpha$- and $\beta$ - anomers of methyldihydrostreptobiosaminide. Methods in which alumina columns were utilised in separation were poor in terms of yield, and hence the methods of Bartz, Controulis, Crooks and Rebstock[258] and Fried and Wintersteiner[257] were tried in this work. Bartz's method included a neutralisation with 10% methanolic sodium hydroxide; the resulting residual sodium chloride and sodium sulphate were somewhat difficult to remove and affected final yields. In all later methanolysis the selective ether precipitation method of Fried and Wintersteiner was adopted.

Early workers attempted strong acid hydrolysis of the disaccharide fragment into its component monosaccharides, dihydrostreptose and N-methyl-L-glucosamine but only the latter fragment was obtained from the molecule intact[234]. Dihydrostreptose appeared to be labile to the

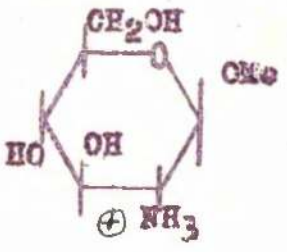
severe acid conditions necessary to achieve scission.

Fragmentation of α-methyl penta-acetyldihydrostreptobiosaminide with acetic anhydride/acetic acid catalysed by sulphuric acid (acetolysis) was thought to have achieved quantitative cleavage, but various attempted methods of separation could not remove the water-soluble sulphoacetic acid from the streptose fragment and only N-methyl-L-glucosamine was isolated[336].

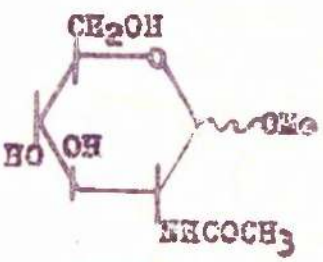
Dihydrostreptomycin is a β - linked glucoside[273] of N-methyl-L-glucosamine with dihydrostreptose - a glucosaminide. The resistance to acid hydrolysis of the D-glucosaminides was studied by Moggridge and Neuberger[187] who suggested that the stability of glucosaminides to acid could be attributed to repulsion by the positively charged basic group while in the cation form; thus the hydrions find difficulty in approaching the glycosidic bond. Evidence in support of this hypothesis came from the study of hydrolysis rates of α - and β - methyl-D-glucosaminides (CIX) (CX) and their acetyl derivatives (CXI) where the methyl-β-glucosaminide hydrochloric was found to have a very much slower rate than that of methyl N-acetyl-β-D-glucosaminide, in which the nitrogen atom carried no charge.



(CIX)



(CX)



(CXI)

Neuberger and Pitt-Rivers[337] compared the rate of acid hydrolysis of the $\alpha$ and $\beta$ -methyl D-glucosaminides and found that the $\alpha$ : $\beta$ ratio was 100:510 emphasising the influence of the positively charged basic group on the glycosidic linkage; apparently a distance effect. Thus the cis $\alpha$ -glycoside in which the glycosidic centre is closer to the amino group is more resistant than the trans $\beta$ -glycoside.

The acid catalysed hydrolysis of methyl-D-glucopyranosides has been studied using isotopically labelled $H_2{}^{18}O$, which showed that cleavage takes place at the glycosyl - oxygen bond. The mechanism of reaction commences with a rapid reversible protonation forming a

$$R\text{—}O\text{—}Me + H_2{}^{18}O \longrightarrow R\text{—}{}^{18}O\text{—}H + H\text{—}O\text{—}Me$$

conjugate acid which undergoes slow unimolecular reaction. Two possible schemes of reaction were postulated[338] (Fig. IX), (A), in which protonation of the glycosidic oxygen occurs to yield a carbonium ion, subsequent reaction with water being rapid, and, (B), in which proton combination occurs with the ring and ring opening takes place between the oxygen and C-1; rapid reaction with water proceeds to release methanol.

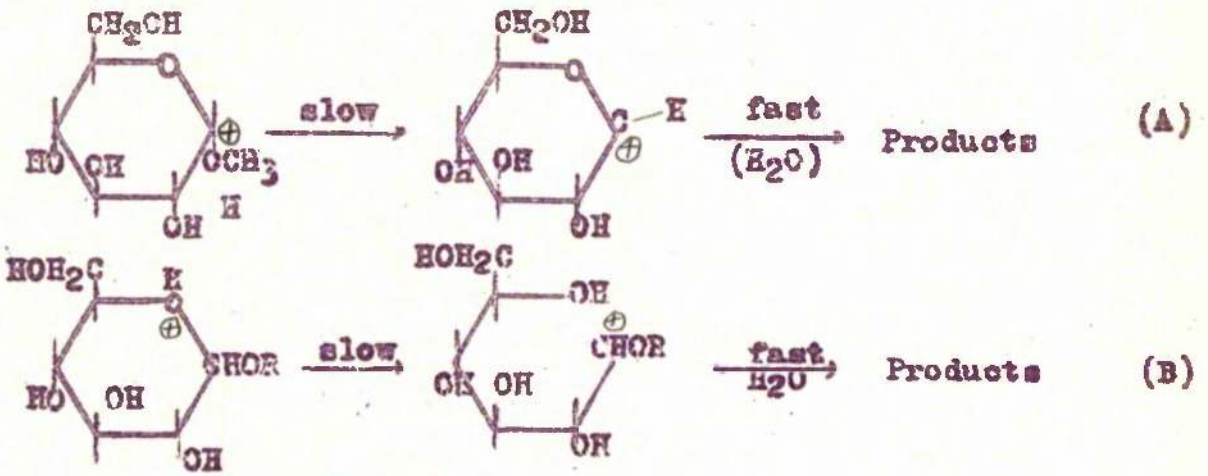
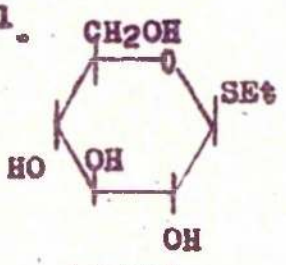
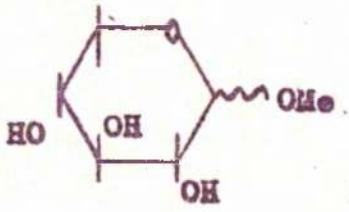



Fig. (IX)

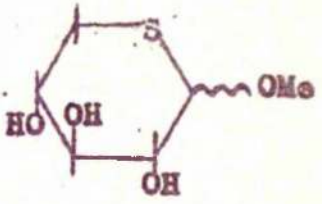
The postulate represented in scheme (A) is regarded as more likely on evidence from comparison of the products of methanolysis of phenyl tetra-O-methyl-α-D-glucopyranoside and tetra-O-methyl-α-D-glucopyranosyl chloride[339]. The latter on methanolysis probably forms a carbonium ion at C-1, and it seems likely that the methanolysis of the glycoside also involves this ion. Supporting evidence also comes from study of the sulphur analogues of the glycosides. 1-Thio-glycosides, e.g. ethyl 1-thio-β-D-glucopyranose, (CXII) have a slower rate of hydrolysis than the corresponding O-glycosides which is attributed to the slower protonation of the glycosidic sulphur compared to that of the more basic oxygen[340]. D-Xylopyranosides (CXIII) on the other hand have a much slower rate of hydrolysis than D-xylothio-pyranosides (CXIV); this is explained on the basis of scheme A by the inductive effect of the sulphur releasing electrons on the exocyclic oxygen[341].




(CXII)




(CXIII)




(CXIV)

Foster and Overend[342], accepting the mechanism of scheme (A) have considered the contribution of different substituents around the glycosidic centre, to the stability of the bond(Fig.X). An increase in the

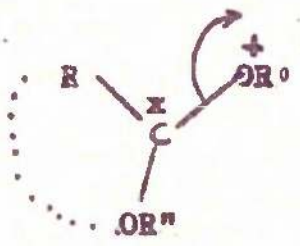



Fig. X

electronegativity of R' will enhance electron transfer x, as evidenced by the known more rapid aqueous acid hydrolysis of arylglycosides compared to alkylglycosides.

The effect of R - R", the pyranose ring and its substituents is exceedingly complex. $\beta$ -Glycosides are generally more rapidly hydrolysed than their $\alpha$-anomers, due to conformational shielding of the glycosidic oxygen from protonation. The preferred conformation of the glycosidic group in $\alpha$-anomers is in most cases the axial position; thus the ring substituents have more influence than with the equatorially orientated $\beta$-anomers. Support for this thesis is given by the study of the gulopyranosides where the $\alpha$-anomer has a preferred equatorial conformation and is more rapidly hydrolysed than the axial $\beta$-anomer.

Various investigators[343] suggest that general conformational resistance, such as caused by the change from chair to half-chair form occurring in the hydrolysis of $\alpha$-methylglucosides at the rate determining step (Fig. XI ) with a consequent increase in non-bonded interactions may affect the rate of hydrolysis. The effect

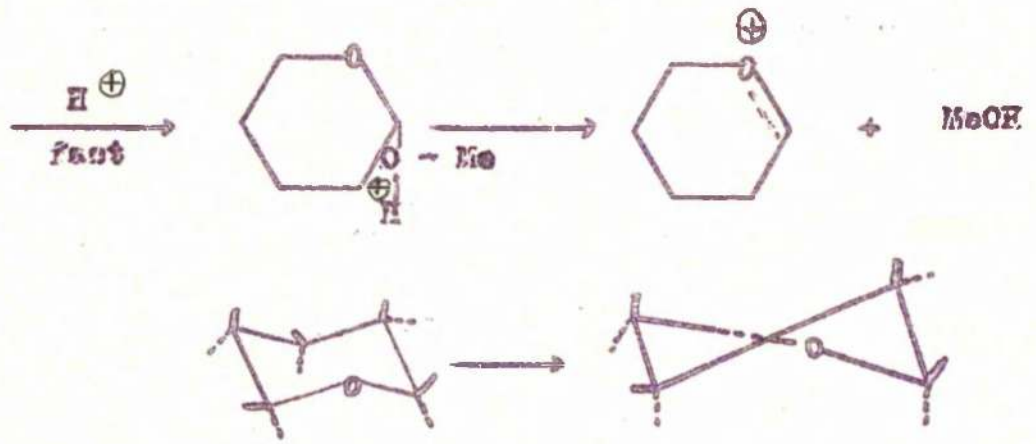



Fig. (XI)

The effects however, are complex and the evidence slight. Shafizadeh[344] commenting on the nature and orientation of substituents at C-2 suggests that correlations between this effect and the conformation of the relevant pyranose ring are doubtful as the same effect is shown by furanosides, and differences in rates of hydrolysis which result in modifications of pyranose conformation are relatively small. 2-Deoxypyranosides are much more labile to acid hydrolysis than the parent sugar. This stabilising effect is probably a function of the more electronegative hydroxyl group. Study of the hydrolysis of 2-amino-2-deoxyglycosides and their N-substituted derivatives by Foster and his co-workers[345] confirmed the findings of Moggridge and Neuberger[187] that two reaction pathways are involved (C) or (D) Fig. (XII)

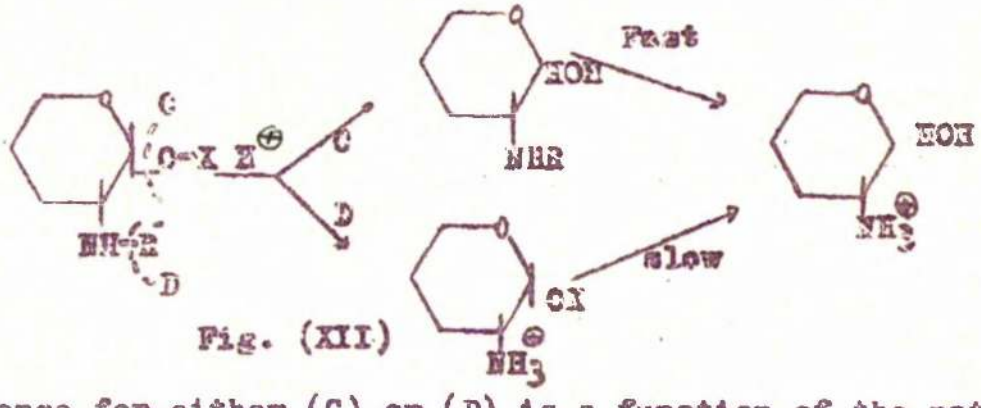



Fig. (XII)

Preference for either (C) or (D) is a function of the nature of the aglycone X and N- substituent R. When R is $-SO_3H$, as in heparin and X is alkyl pathway (D) predominates, when R is acetyl and X alkyl (C) is favoured, D-glucosamine being released. Route (D) is unfavourable as any glucosamine released after the N-substituent has been removed requires severe acid conditions for hydrolysis causing irreversible destruction. Neuberger and his colleagues[346] more

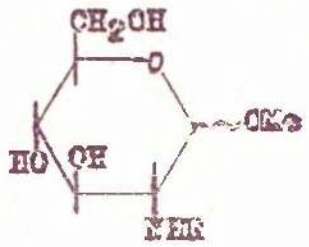
recently considered conditions which would favour cleavage of the glycosidic bond rather than the acetamido link of methyl N-acetyl-glucosaminide. The heat of activation and relative rates of hydrolysis showed that high acid concentrations and high temperatures favour glycosidic cleavage, but that the severity of the conditions is limited by the stability of the amino sugar.

Akiga and Osawa[347] have introduced recent evidence in support of this scheme for acid hydrolysis of methyl N-acylglucosaminides which showed that the rate of hydrolysis decreased in proportion to the increasing dissociation constants of the N-acylating acids. Where the acid amide linkage is stable pathway (C) predominates, while pathway (D) is followed where the amide link is less stable. This evidence agrees with that of Marshall[348] who proposed that the rate of hydrolysis of a methylglucopyranoside may be related in a quantative way to the pK value of the corresponding C-2 substituted acetic acid.

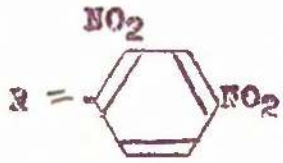
Findings by Wang Yu[349] and his colleagues with methyl N-(2,4-dinitrophenyl)-D-glucosaminide also support Moggridge and Neuberger's thesis that as the C-2 substituent is made less electronegative, with N-substitution, both proton transfer and hydrolysis occur more readily.

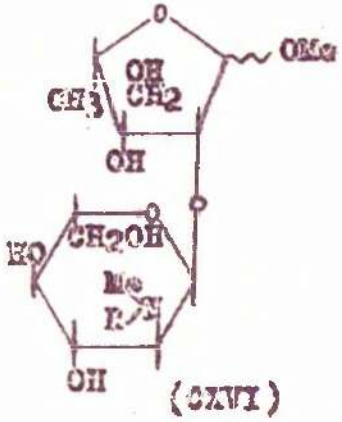
Preparation of N-(2,4-dinitrophenyl)-methyldihydrostreptobiosaminide. (DNP, Methyldihydrostreptobiosaminide).

Wang Yu and his colleagues[350] have prepared α - and β - methyl N-(2,4-dinitrophenyl)-glycosides of D-glucosamine (CXV). These compounds were known to have high $R_F$ values on papergrams[351]. Thus it was considered that the 2,4-dinitrophenyl derivatives of the methyl-dihydrostreptobiosaminides (CXVI) if prepared would be highly coloured fast moving compounds amenable to hydrolytic cleavage and chromatographic study.



(CXV)





(CXVI)

The method of Kent[352] for the preparation of the 2,4-dinitrophenyl derivative of D-glucosamine was modified in this condensation. Difficulty was encountered in the purification of the methyl N-(2,4-dinitrophenyl)-dihydrostreptobiosaminide and very recent papers by Lloyd and his colleagues preparing a number of amino sugar DNP derivatives have reported similar difficulties[353,354]. Sodium chloride and sodium fluoride, produced during the reaction interfered and unreacted 2,4-dinitro-fluorobenzene was difficult to remove. The final product had a high $R_F$ on papergrams but the spot was indeterminate with a long

trail and when the hydrolysis was completed traces of 2,4-dinitrophenol were identifiable from ultraviolet absorption at 280 mμ.

Column chromatographic purification was attempted on this derivative without success. Cellulose powder was used with butanol-acetic acid-water 4:1:5 and butanol-ethanol-water, 4:1:5 as solvents but the product on papergrams exhibited tailing. The method of Rinehart and his colleagues[186a] for the purification of N,N'-bis-(2,4-dinitrophenyl)-neobiosaminide B utilising a silicic acid celite column and ethanol-chloroform-water 1:9:1 as solvent system was also unsuccessful.

A method latterly adopted for the preparation of this derivative entailed use of a basic ion exchange resin, which maintained the slight basicity necessary to effect condensation and also remove in situ, the hydrochloric and hydrofluoric acids released. The product still gave diffuse spots, though the N-DNP-glucosamine produced in this manner was very pure.

These diffuse spots may be due to the presence of a number of isomers. Reports by Lloyd[354] of furanose forms of glucosamine being present after this condensation lends support to this argument.

Hydrolysis of methyl N-(2,4-dinitrophenyl)-dihydrostreptobiosaminide.

In the above studies of hydrolysis rates of methyl aminoacyl-glycosides, the course of hydrolysis was followed by measurement of the free glucosamine produced. This useful criterion was not available in the present study as the methylamino group of N-methyl-L-glucosamine

moiety interfered. Recourse was therefore made to polarimetric measurements, papergrams and Fehlings reducing sugar tests for evidence of hydrolysis. Paper electrophoresis was used in later work.

Preliminary experiments investigating conditions for hydrolysis with hydrochloric acid gave no evidence of reaction below 2N, at this and higher acid concentrations a positive Fehlings, a change in optical rotation and new spots on papergrams indicated probable cleavage.

Various runs with 2.5N hydrochloric acid followed by neutralisation with silver carbonate initially and in later experiments with a 10% chloroformic solution of purified di-n-octylmethylamine[355], showed two particular spots, that were not present before acid treatment. ($R_F$ 0.3 and $R_F$ 0.55 in n-butanol-acetic acid-water solvent.) By scaling up the paper chromatographic separation small amounts of these fractions were obtained in an impure state. The faster moving compound was subjected to micro periodate oxidation which indicated that this fraction could be the methyl glycoside of dihydrostreptose. (Appendix 1).

Cellulose column chromatographic separations were then applied. Extrusion technique. Extrusion column chromatography was investigated on initial experiments for the separation of the products of this hydrolysis. Improvised extrusion columns were set up using 2 cm. bore glass tubing, extrusion being accomplished with

positive pressure. An even pressure was found difficult to achieve, and despite use of silicone treated tubes breakages were frequent. The extruded column containing the material was painted with a thin line of developing reagent (aniline hydrogen phthalate or ammoniacal silver nitrate) to identify bands. The method was abandoned in favour of elution techniques.

The incomplete success of this work with methyl N-(2,4-dinitrophenyl)-dihydrostreptobiosaminide, due to the difficulties associated with the entrainment of dinitrophenol at all stages, led to the consideration of other N-substituted methyldihydrostreptobiosaminides the most obvious being the N-acetyl derivative.

Methyl N-acetyldihydrostreptobiosaminide.

Selective N-acetylation of primary amino sugars was first accomplished with glucosamine in 1898[356]. More elegant methods have been introduced since that time[357], and two of those were applied to the N-acetylation of the secondary methylamino group in methyldihydrostreptobiosaminide without marked success.

Roseman and Ludowieg[358] prepared N-acetyl-D-glucosamine from D-glucosamine hydrochloride by dissolving the sugar in 10% aqueous methanol and stirring together with acetic anhydride and a basic ion-exchange resin. It is not clear whether the resin is added to prevent O-acetylation or to act as an "acid acceptor" in hastening the reaction[357]. Passage of the filtered reaction solution through a

cationic resin removed the unreacted base, the neutral N-acetyl derivative being obtained in near quantitative yield. Application of this method for the preparation of methyl N-acetyldihydrostreptobiosaminide was unrewarding the maximum yield being about 20%.

A second selective acetylation method described by Inoue and his co-workers for the preparation of N-acetyl-D-glucosamine[359] was adapted to methyldihydrostreptobiosaminide. In this method the sugar hydrochloride was dissolved in methanol containing an equivalent amount of sodium, the resulting sodium chloride centrifuged off, and the base treated with 1.5 equivalents of acetic anhydride at 50°C. Chromatograms showed only partial N-acetylation had occured; attempts to separate starting product from the N-acetyl derivative by ion exchange and cellulose column chromatography gave very poor yields.

The reason for these failures is not clearly understood. Initially it was thought that moisture interfered with the latter preparation but when the experiment was repeated under rigorously anhydrous conditions no improvement was observed. The presence of the N-methyl group may exert some bulk or steric effect hindering the facile N-acetylation exhibited by the primary amino compounds.

Rinehart and his colleagues working with neomycins sought, as described in page 30 , to cleave the diaminohexose - pentose disaccharide of the neomycins. This was achieved by formation of the N,N'-dibenzoyl compound followed by hydrolysis[186b]. The diacetamido

derivatives was also prepared by a simple method[186a] which has been applied in the present work successfully to dihydrostreptomycin.

Methyldihydrostreptobiosaminide base prepared by ion exchange treatment of the hydrochloride was placed in methanolic pyridine and treated with 1.5 equivalents of acetic anhydride. The *N*-acetylation was followed by the ninhydrin test for primary and secondary amino groups, the test giving a negative result when *N*-acetylation was complete. Isolation included extraction with chloroform to remove any fully acetylated derivative. Pyridine tended to adhere to the hygroscopic derivative and on occasion had to be removed by passage through a cation-exchange resin, which also removed any starting base.

In early experiments recourse was made to the preparation of fully acetylated derivatives and selective de-*O*-acetylation. This reaction as described by Mital[360] gave good yields in the preparation of *N*-acetyldihydrostreptomycin from dodeca-acetyldihydrostreptomycin with methanolic ammonia as the de-*O*-acetylating agent, but the analagous conversion of methyl penta-acetyldihydrostreptobiosaminide to the methyl *N*-acetyldihydrostreptobiosaminide was somewhat disappointing giving an impure product as seen on papergrams. This reaction has recently been investigated for the de-*O*-acetylation of α-D-glucosamine penta-acetate[361] with various alkaline agents in methanol; uncharacterised artifacts were produced when the de-*O*-acetylation

mixture was examined by paper and thin layer chromatography. Apparently these artifacts are produced in the de-O-acetylation by traces of hydroxyl ion. Any water in the system would yield hydroxyl ions with most acetylating agents and rigorous anhydrous conditions are almost impossible to achieve.

Penta-acetyl-N-methyl-D-glucosamine and N-methyl-N-acetyl-D-glucosamine were prepared as described by Folkers[244]. The yields could not be improved - probably due to the lack of stability (caramellisation) of the glucosamine in water at elevated temperatures when boiled with zinc chloride.

Methyl N-toluene-p-sulphonyldihydrostreptobiosaminide.

This substance was prepared by heating two equivalents of recrystallised toluene-p-sulphonyl chloride with methyldihydrostreptobiosaminide in carefully dried pyridine at 40°C for two days, following the course of the reaction with the extinction of the ninhydrin reaction. The crude product containing starting material, was purified by rapid passage down a cation-exchange column when any basic sugars were removed. Slow passage down this column appeared to cause some de-O-acetylation as shown by papergrams and electrophoresis.

The N-toluene-p-sulphonyl substituent was considered a very strong electronegative group for weakening the glycosidic linkage. It is however not obtained in good yields and the hydrolysis has not yet been investigated.

Hydrolysis of N-Acetyl derivatives.

The hydrolysis of methyl N-acetyldihydrostreptobiosaminide was expected to follow the pattern of hydrolysis of the N-acetyl-D-glucosaminides proposed by Foster and Overend[345]. That partial de-N-acetylation occurred on acid treatment was clear from papergrams in which the ninhydrin test for free >N-H groups originally negative reverted to positive and a spot running parallel with N-methyl-L-glucosamine was visible. Paper chromatography also provided evidence for further products of hydrolysis, two new spots appearing at $R_F$ 0.5 and 0.3 respectively.

Theoretical considerations allow the prediction of the following products in the hydrolysate - dihydrostreptobiosamine, dihydrostreptose N-methyl-L-glucosamine and N-acetyl-N-methyl-L-glucosamine. A cationic exchange column (Dowex 50 $H^+$) proved useful for the separation of these fragments, including the separation of the amino sugars from the neutral dihydrostreptose. The N-acetyl-N-methyl-L-glucosamine also behaves as a neutral sugar but it had a slightly slower rate of migration in the column than dihydrostreptose and a separation was achieved if small fractions were collected. In larger scale work, however, this presented a problem. Paper electrophoresis was useful in the early qualitative work, showing the presence of neutral and amino sugars after hydrolysis.

Dihydrostreptose obtained from the early fractions of the hydrolysate eluted off the column has not previously been described. The Chinese workers[335] prepared the tosylhydrazone (m.p. 136°C) by treatment of a mixture with toluene-p-sulphonylhydrazine. This was obtained in the present work in trace amounts only, (m.p. 140°C), but insufficient was available for analysis. A monoacetate (m.p. 172°C), has however been prepared. A triacetate would be expected from this sugar but as the reaction took place at room temperature perhaps the conditions were too mild for complete acetylation.

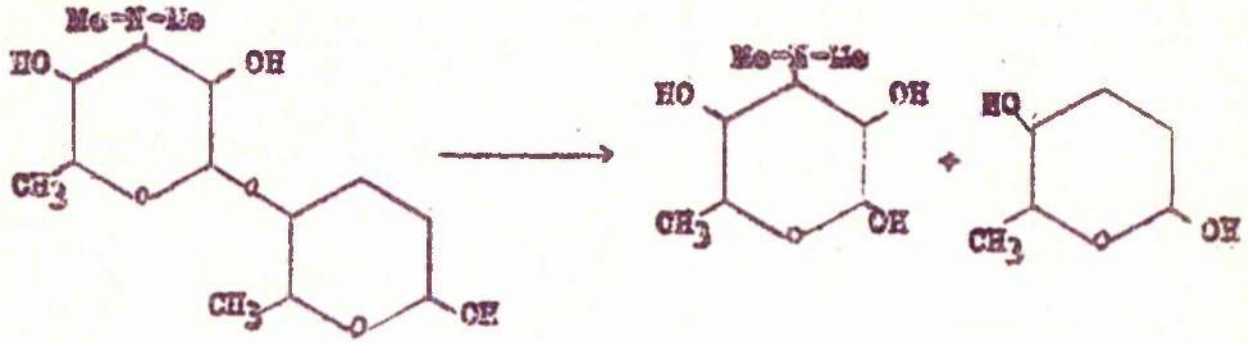
Acid hydrolysis of methyl N-acetylstreptobiosaminide dimethyl acetal.

Methyl N-acetylstreptobiosaminide dimethyl acetal was prepared by a method analagous to that for the dihydro derivative. Attempts to hydrolyse this compound with 2N hydrochloric acid into the components streptose and N-methyl-L-glucosamine caused extensive degradation. Passage of the neutralised hydrolysate down a Dowex 50 $H^+$ cation-exchange resin did not effect separation of any neutral sugars. Paper chromatographic evidence showed the presence of amino sugar in early fractions. Attempted preparation of penta-acetyl-N-methyl-L-glucosamine from these fractions although it did not give a crystalline derivative gave the same $R_F$ as authentic material on papergrams.

Ion Exchange Hydrolysis of Methyldihydrostreptobiosaminide.

As the preparation of pure N-acyl derivatives of methyldihydrostreptobiosaminide was time consuming and the aim of this work was to find a reasonable method for the isolation of dihydrostreptose several other methods were tried but with very little success.

Direct acid hydrolysis of dihydrostreptobiosaminide and its derivatives has been reported as destructive to the streptose moiety[234]. In the degradative study of amicetin an antibiotic from Streptomyces plicatus and S. vinaceous - drappisa, Haskell and his associates reported the scission by a cation-exchange resin (Dowex 50 $H^+$) of the amino glycosidic bond of amicetamine[362](CXVII) to yield amosamine (CXVIII) a 3-amino-sugar and the neutral deoxyhexose amicetose (CXIX).



(CXVII) (CXVIII) (CXIX)

In this case a 3-aminoglycosidic bond is involved and the amino neighbouring group effect is less as the disaccharide is hydrolysed readily with 3N hydrochloric acid.

Painter[363] has examined the use of water soluble non-dialysable polystyrene sulphonic acid for the partial hydrolysis of amino-polysaccharides and, in model experiments, glucosaminides. He states that the polysulphonic molecules are surrounded by a very high localised hydrogen ion concentration, and, since the cation-binding properties of some polyelectrolytes can be clearly demonstrated it is reasonable to suppose that any positively charged-ion would be attracted into these regions of high acidity. Moreover if as a result of this, an electrically neutral fragment were split off from the molecule by hydrolysis, this fragment would then be exposed to much lower average acidity[364]. Basic carbohydrates were hydrolysed in these experiments much faster with polystyrene sulphonic acid than with an equivalent amount of mineral acid.

The water insoluble polystyrene sulphonic acid ion-exchange resins are non-dialysable but are rather unsatisfactory for hydrolysis of polysaccharides, probably owing to the difficulty with which the large molecules penetrate into the resin particles. However, in view of the success of the amicetin work and the relatively small size of the methyldihydrostreptobiosaminide molecule it seemed worth attempting ion-exchange hydrolysis.

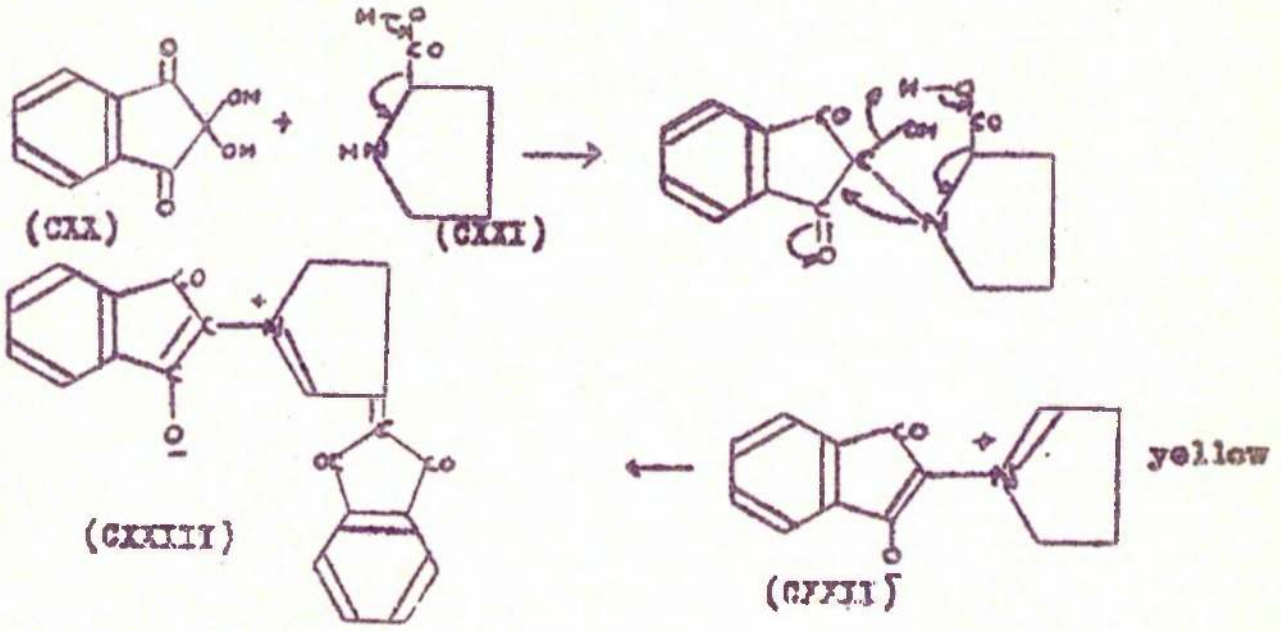
Preliminary work with Dowex 50 $H^+$ form in which the hydrolysis of methyldihydrostreptobiosaminide in water at 50°C was followed by papergram and paper electrophoresis showed that hydrolysis had occurred.

Passage of the hydrolysate down a Dowex column and fraction collection led to the isolation of an electrically neutral fragment $R_F$ 0.3 in small amounts. However, degradation had occurred and although a tosylhydrazone was prepared from repeat experiments on a larger scale, the yield and analysis were poor. The method was therefore discontinued.

A second chemical method examined in the search for facile cleavage of the glucosaminide bond of methyldihydrostreptobiosaminide was deamination to give a neutral sugar which would allow milder hydrolytic conditions to prevail. The secondary amino group, however, is not amenable to nitrous acid deamination and no attempt was made to use this reagent.

Deamination of primary amino sugars with 1,2,3-indane trione hydrate (CXVI), (ninhydrin) yielding pentoses has been reported[365]. D-Glucosamine was degraded to D-arabinose, whilst galactosamine gave lyxose; the method has now been adapted for the identification of amino sugars in polysaccharides[366]. The mechanism of deamination has not been studied but the formation of a pentose would indicate a ring cleavage[367]. Violet or yellow intermediates, which are responsible for the use of ninhydrin as a reagent of detecting sugars on papergrams, are formed during this action; the nature of these chromagens being imperfectly understood. Work with the amino acids has given some information on the structure of these intermediates[368]. Proline (CXXI) which gives both a yellow (CXXII) and a violet colour

(CXXIII) is said to undergo the following reaction with ninhydrin.




N-Methylamino acids are also detected by ninhydrin[369] though no mention is made of the methylamino sugars.

Methyldihydrostreptobiosaminide gave a central yellow spot ringed with violet, or under certain conditions a violet spot only indicating from analogy with amino acids that more than one compound is formed. It was felt that since this ninhydrin reaction occurred with the N-methylamino group, deamination should be attempted paralleling the work with glucosamine[365, 365a]

Equal quantities of methyldihydrostreptobiosaminide and ninhydrin were boiled together for half an hour in slightly acid aqueous media. The resulting dark brown solution gave on evaporation a dark brown syrup. Papergrams indicated that extensive caramellisation had occurred, a spot with $R_F$ similar to glucosamine was present but no

other detectable sugar derivative. Repetition of the experiment with a longer reaction time showed even more degradation with no positive result. The experiment was discontinued in favour of more fruitful methods of degradation.

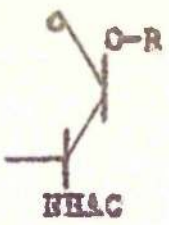
## Enzymatic Hydrolysis

Various chemical methods of glycosidic cleavage of methyldihydrostreptobiosaminide were attempted with limited success. Some attention was therefore directed towards enzymatic hydrolysis.

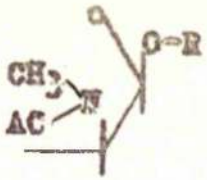
Information concerning streptomycinases is scant. The papers available refer to an $\alpha$-mannosidase, splitting off the mannose portion of mannosidostreptomycin releasing streptomycin[279]. Pramer and associates, however, have found a species of Pseudomonas which releases N-methyl-L-glucosamine from streptomycin[370]. That enzymes capable of cleaving the $\alpha$-L-glucosaminidic linkage occur in the body was obvious from metabolic studies.

After a review of the available hydrolytic enzymes it appeared that the enzymes most likely to achieve scission was N-acetyl-$\beta$-D-glucosaminidase an enzyme fairly specific for N-acetyl-$\beta$-D-glucosaminide bonds, which occur in a number of plant and animal tissues[372].

The N-acetyl-$\beta$-glucosaminide bond is not configurationally equivalent to the acetylated dihydrostreptobiosaminide bond which is




β- D




α- L

However, as the mechanism of enzymatic hydrolysis is unknown it was felt worth attempting preliminary experiments utilising this enzyme.

The enzyme which is similar to chitinases may be obtained from the digestive glands of the snail Helix pomata, emulsins and some micro-organisms including Streptomyces[373], but the richest sources are found in mammalian tissue. The best source was boar epididymis[374] but a more readily available crude source was freeze-dried ram-testes-extract, the enzyme being active after 2 years at 0 - 5°C[375]. Pure enzyme has not been isolated from these sources, but using the crude extract, Leaback and co-workers have collated data concerning the optimal conditions for the enzyme[376].

Freeze-dried ram-testes-extract was obtained and its activity tested using p-nitrophenyl-β-N-acetyl glucosamine as substrate, when the p-nitrophenol was released and estimated spectrophotometrically from the absorption peak at 400 mμ. The enzyme was found to be active and Leabacks' work was thereby confirmed, the recommended optimum pH temperature, time and substrate/enzyme concentration being followed.

Application of this enzymatic technique to methyl N-acetyl-dihydrostreptobiosaminide was difficult as concentrations were at a micromolar level and no spectrophotometric method was available to measure release of aglycone (dihydrostreptose) or N-acetyl-N-methyl-L-glucosamine. Papergrams were resorted to for evidence of hydrolysis.

The papergrams showed no evidence of hydrolysis either at the recommended enzyme concentration, or in repeat experiments using twice this quantity of enzyme.

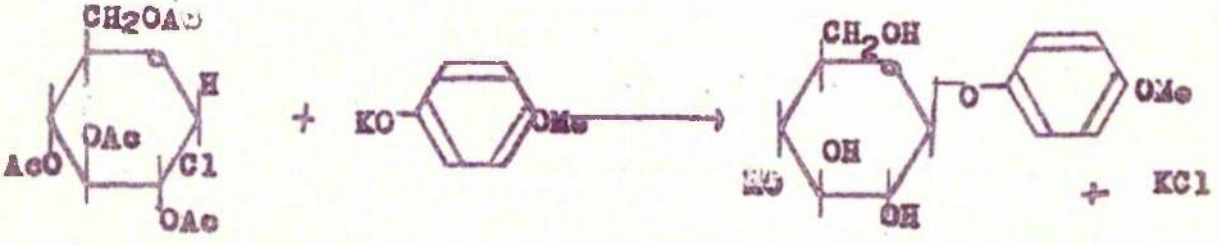
# GLYCOSIDES OF DIHYDROSTREPTOBIOSAMINE

## Introductory

The original aim of this work was to prepare glycosides of dihydrostreptose, the study of which may aid the understanding of structure-action relationships of streptomycin antibiotics. The difficulties encountered in preparing reasonable quantities of this hitherto unknown sugar precluded the preparation of these glycosides, hence, as an interim approach, some new glycosides of dihydrostreptobiosamine were prepared in which the glycoside carbon of dihydrostreptose was involved. The readily available methyl dihydrostreptobiosaminide hydrochloride was used as starting product.

## Methods of Glycoside Synthesis

The first successful glycoside synthesis was described by Michael in 1879 [377]. Tetra-O-acetyl-α-D-glucopyranosyl chloride (CXXIV) was treated with the potassium salts of a phenol yielding a deacetylated phenyl glycoside e.g. methylarbutin (CXXV).

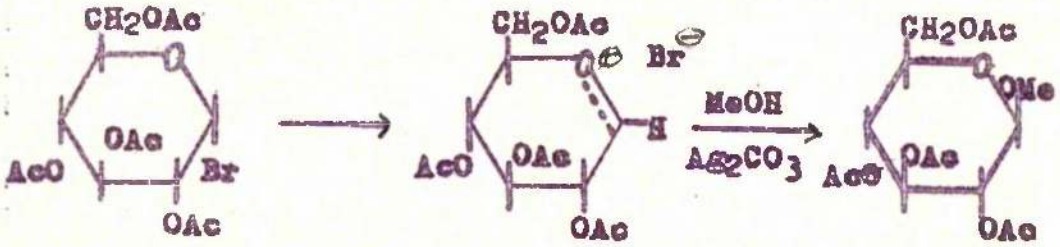



(CXXIV) (CXXV)

This method with modern refinements is of value in the preparation of phenyl glycosides but cannot be used for glycosides of alcohols or disaccharide synthesis.

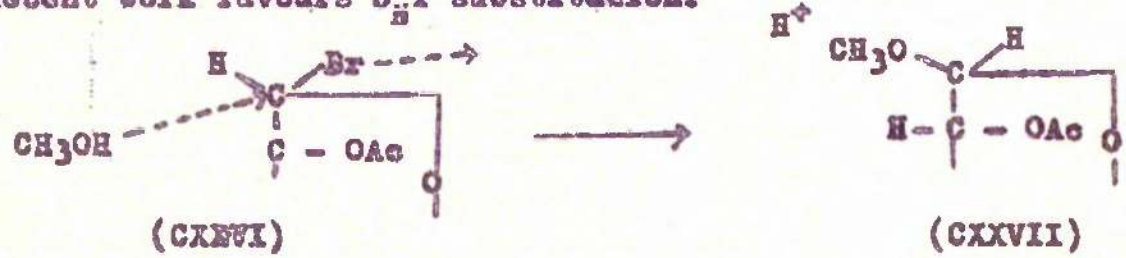
O-Acetylglycosyhalides are key compounds in glycoside synthesis. A method of wide application first described by Koenigs and Knorr[378] (1901) involved the condensation of an acetyl glycosyl halide with a substance containing a free hydroxyl group, generally in the presence of a heavy metal salt or organic base, though glycosides have been obtained in poor yield without this catalyst[379].

The mechanism of the Koenigs-Knorr reaction has been discussed in detail by Pacsu[380] and Frush and Isbell[381] when as in tetra-O-acetyl-α-D-glucopyranosyl bromide (CXXVI), the halogen and the neighbouring




(CXXVI) (CXXVII)

C-2 acetoxy group are cis the halogen is replaced with inversion by a negative group from the environment - (methoxyl in this case) - (CXXVII). Recent work favours $S_N1$ substitution.[339a]

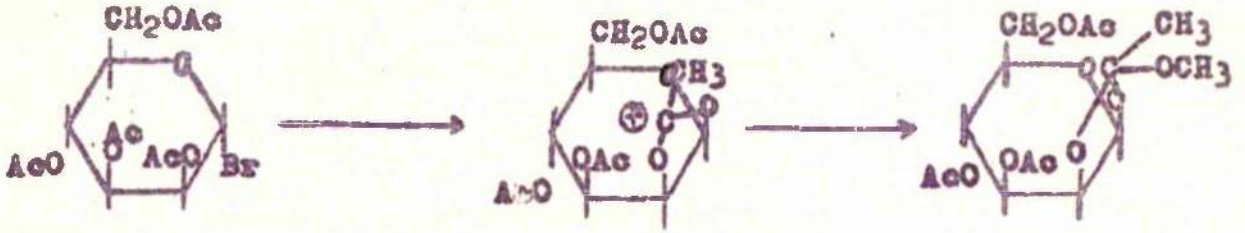



(CXXVI) (CXXVII)

Where silver oxide or carbonate are used, the equilibrium is driven to the right by removal of the bromide ion. The organic base catalyst (generally quinoline) removes the hydrogen ion.

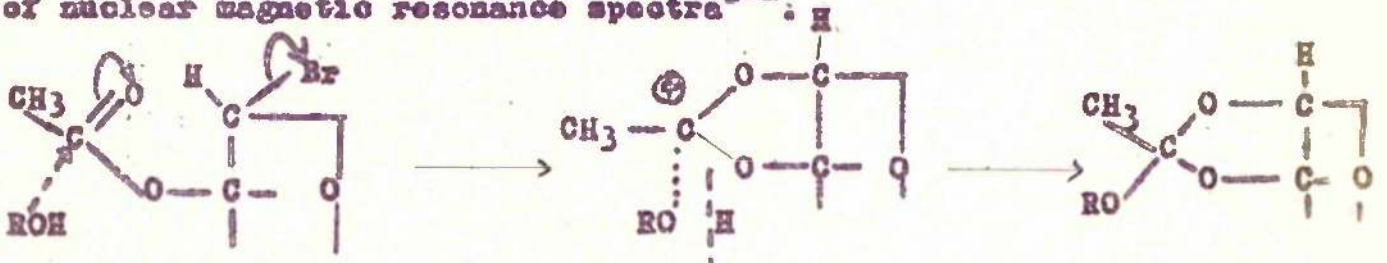
A much more complex system however obtains in the case of trans

orientation of C-2 acetyl and the halogen group of the acetohalogeno sugar as with the tetra-O-acetyl-α-D-mannopyranosyl bromide (CXXVIII).



(CXXVIII) (CXXIX) (CXXX)

As the halogen departs the nucleophilic oxygen of the neighbouring acetyl group attacks the opposite face of C-1 to give an orthoester carbonium ion (CXXIX) which is electron deficient and under the Koenigs-Knorr basic conditions reacts with solvent, in this case methoxyl, to give stable orthoesters[381] (CXXX) which are diastereoisomers for which full stereo structures have now been assigned on the evidence of nuclear magnetic resonance spectra[382].



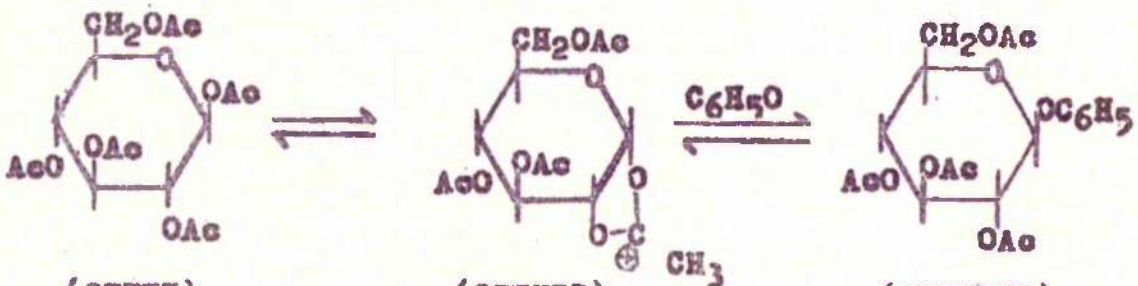
(CXXVIII) (CXXIX) (CXXX)

A competing reaction is the replacement of the halogen without participation of the 2-acetyl group to give the alkyl glycoside with inversion but this is a minor product as the rate controlling step the dissociation of the halogen is speeded up by the neighbouring group effect[381c].

The Koenigs-Knorr synthesis is useful for phenyl and alkyl glycosides as well as in disaccharide synthesis. Anhydrous solvents should be used, as water competes in reacting with the glycosyl halide to give the free acetyl sugar. Its presence is therefore undesirable and "drierite" (finely divided $CaSO_4$) may be incorporated to remove any formed during reaction.

A limitation of the Koenigs-Knorr reaction besides the above orthoester formation is the difficulty of forming $\alpha$- linkages. Most of the glycosyl halides are stable in the $\alpha$-form as predicted on conformational grounds[383] and on Walden inversion the $\beta$-glycosides are obtained. The reaction has been used generally for pyranosides but ethyl $\beta$-galactofuranoside is reported[384].

A second common method of glycoside formation developed by Helferich[385] concerns the replacement of the C-1 acetoxy group of acetylated aldoses with a phenol in the presence of an acid catalyst. Penta-O-acetyl-$\beta$-D-glucopyranose (CXXXI) gave the $\beta$-phenyl glycoside (CXXXIII).

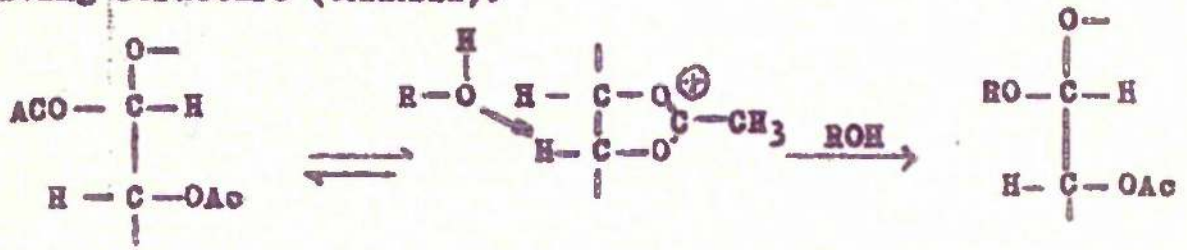



(CXXXI) (CXXXII) (CXXXIII)

The steric result depends on the reaction conditions. By proper choice of kind and amount of catalyst, temperature and reaction time

the reaction can be made to yield either of the anomeric phenyl glycosides as the main product. Zinc chloride catalyst gives predominantly the $\beta$ -anomer whilst p-toluenesulphonic acid yields the $\alpha$-glycosides[385].

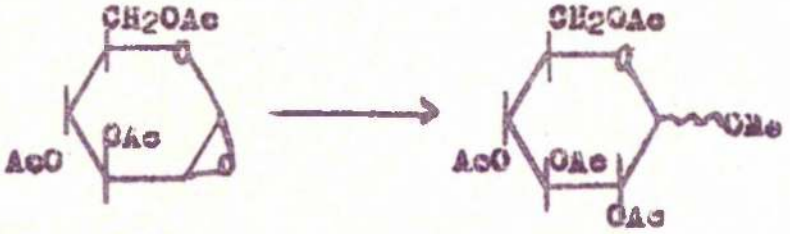
A mechanism has been discussed by Lemieux[387] who suggested that the sugar acetate dissociates to give carbonium (CXXXII) and acetate ions, followed by reaction of the carbonium ion with the phenol giving structure (CXXXIII).



(CXXXII) (CXXXIII)

The Helferich reaction is limited to the phenyl glycosides and there is a scarcity of information concerning its application to glycofuranoside synthesis.

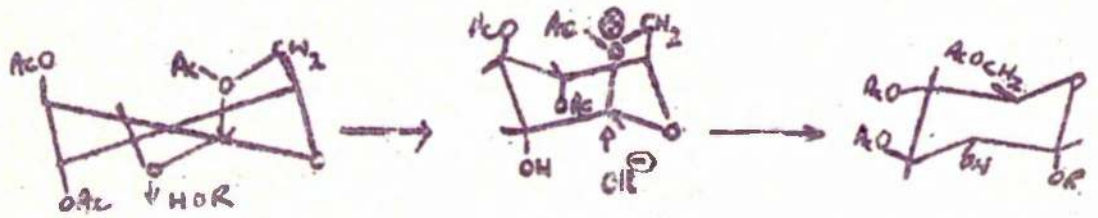
The treatment of 1,2-anhydro-3,4,6-tri-O-acetyl-D-glucose (Brigl's anhydride)[388](CXXXIV) with alcohols gives glucopyranosides,[389](CXXXV)



(CXXXIV) (CXXXV)

Lemieux and his co-workers have used this reagent for the synthesis of the important naturally occurring glycosides sucrose[390], maltose[391], and trehalose[392], by treating Brigl's anhydride with the appropriate O-acetyl sugar -1,3,4,6-tetra-O-acetyl-D-fructose in the case of sucrose. These workers[393] also rationalise the ability of the anhydride to form α - and β -D-glucopyranosides on the basis of its conformation. The most probable conformation of Brigl's anhydride (CXXXVI) has the $-CH_2OAc$ group in the axial orientation of a half chair form.


(CXXXVI) (CXXXVII)



(CXXXVIII) (CXXXIX) (CXL)

This reacts normally with an alcohol to give the β -glycoside (CXXXVII). The mechanism of the abnormal reaction of the anhydride to form α -D-glucopyranosides (CXL) is less clear. A possible route is that involving participation of the C-5 $-CH_2OAc$ group in the first stage of the reaction through (CXXXVIII) to yield the 1,2 diaxial carboxonium ion (CXXXIX). The yields in these syntheses were very poor, and the

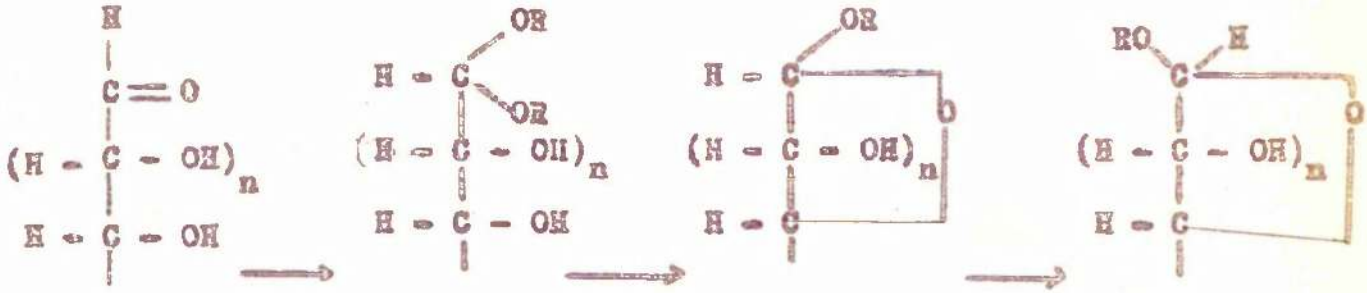
reaction course unpredictable. There is a scarcity of information concerning the 1,2 anhydrides of furanose sugars.

When a sugar is alkylated with one equivalent of dimethyl sulphate and alkali the glycosidic hydroxyl is preferentially alkylated[394]. D-Mannose treated in this manner gave a mixture of α-and β-methyl D-mannopyranosides[395] substances not readily obtained by the Koenigs-Knorr synthesis due to orthoester formation. Tetra-O-acetyl-β-D-fructopyranose treated with silver oxide and methyl iodide gave methyl-β-D-fructopyranoside tetra-acetate[396]. This method is seldom used in alkyl glycoside formation.

One of the oldest and simplest methods of glycoside synthesis introduced by Fischer (1893)[397] involved the treatment of the free sugar with an alcohol containing hydrogen chloride. Most studies of this reaction used methanol when methyl glycosides were formed. Fischer accomplished the formation in a sealed tube at 100° [397], but later workers have modified the method refluxing the methanolic hydrogen chloride[398]. Cation-exchange catalysis with methanol as solvent[399] has also been introduced. Hudson found that, by increasing the concentration of acid the method was improved[400]. Fischer later found that treatment of glucose with methanolic hydrogen chloride at room temperature gave a different product which he termed the γ-glycoside[401]. This was shown by Haworth[402] to be a mixture of α- and β-furanosides, which frequently occur as intractable syrups in this reaction.

The Fischer synthesis is of limited application in glycoside preparation. It is of value only in the formation of glycosides with the lowest aliphatic alcohols and, because most disaccharides are cleaved by alcoholysis, the reaction is not applicable to them. Glucosaminides are however an exception owing to the stability of the glucosaminide link to alcoholysis. A further disadvantage of this method is that there is no way of altering the ratio of $\alpha$ to $\beta$-anomer in the final equilibrium mixture, also the anomers are not easy to separate[386]. Newer methods of separation however, including cellulose column chromatography[403] silicate earth chromatography[404] and gas liquid chromatography[405] (of methylated derivatives) have led to a reappraisal of the Fischer-glycoside synthesis so useful for furanoside formation. The reaction mechanism has also been the subject of recent studies which previously had depended on hydrolytic rather than synthetic work.

Fischer suggested[401] a reaction course for the synthesis of sugar glycosides expanded by Campbell and Link[406] as follows (CXLI) to (CXLIV)



Sugar (CXLI) → Sugar Acetal (CXLII) → $\alpha$-glycoside (CXLIII) → $\beta$-glycoside (CXLIV)

Postulated Intermediates in the Hydrolysis and Formation of

Glycosides (Shafizadeh)

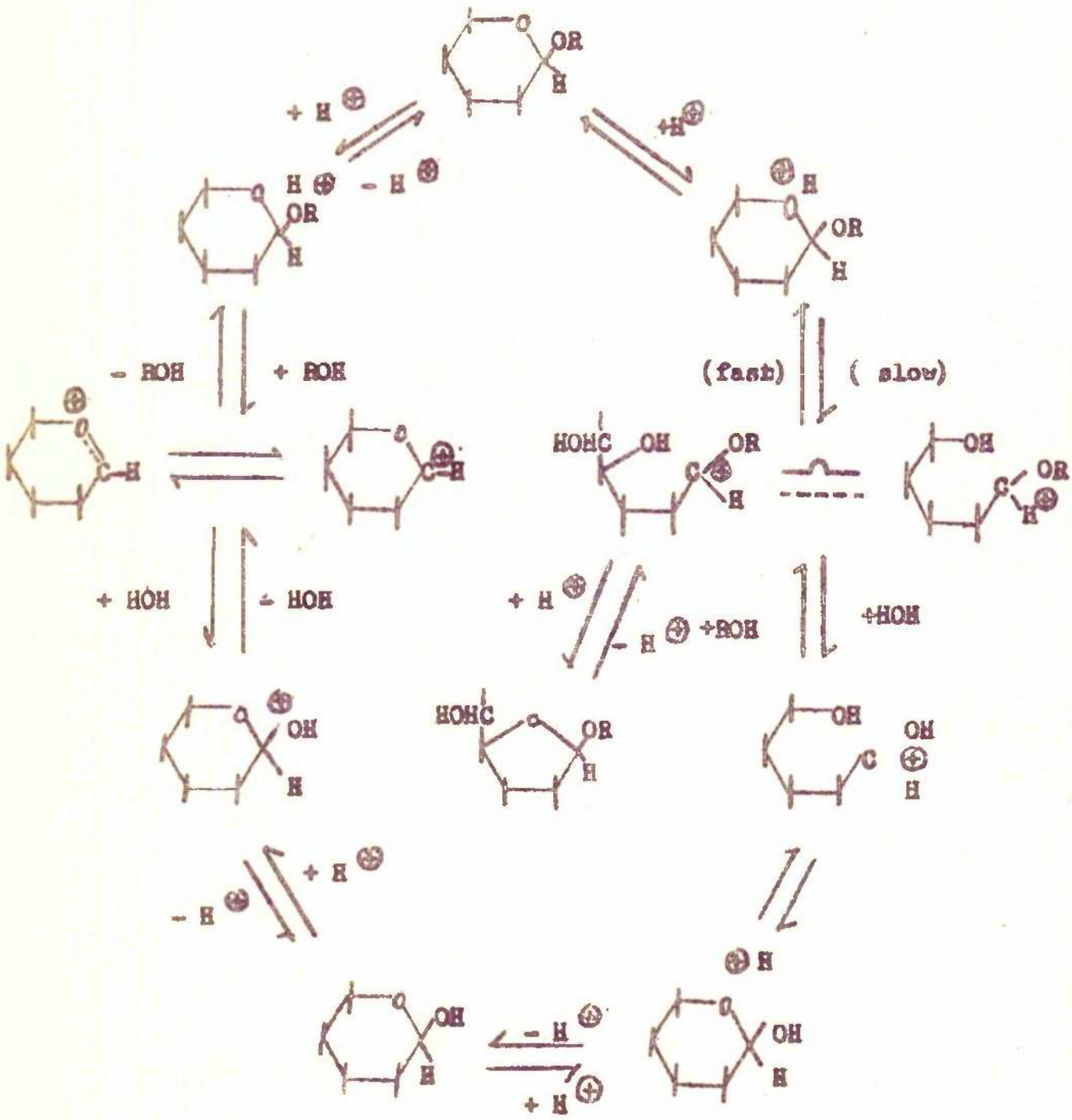



Figure (XIII)

They also suggest the following scheme (Fig.XIII)for glucose in which the equilibrium is shifted in the direction of pyranoside formation at elevated temperatures.

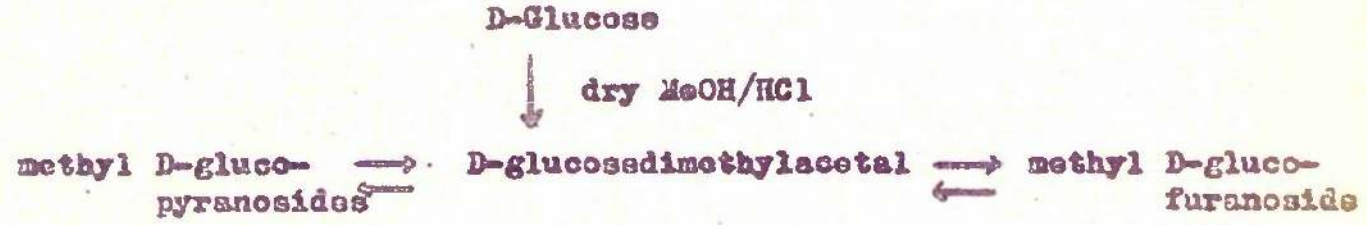



Fig. XIII

The work of Levene, Raymond and Dillion[407] lends support to this hypothesis by investigation of the relative rates of methyl furanoside and methyl pyranoside formation among various monosaccharides, though each sugar had a different reaction time furanosides formed first and thereafter pyranoside.

Levene[407] found that ribose lost its reducing power more rapidly than xylose in methanolic hydrogen chloride. Ribose is known to assume the furanose form with greater ease than the majority of sugars, indeed it occurs largely in nature as ribofuranose[408]. Glycosidation to the furanosides occurs rapidly when ribose is treated with methanolic hydrogen chloride and treatment with benzyl alcohol containing 1% hydrogen chloride results in formation of benzyl glycosides[408]. Campbell and Link[406] obtained D-galactosedimethyl-acetal which on treatment with methanolic hydrogen chloride gave rapid furanoside formation followed more slowly by pyranoside. Shafizadeh[344] gives a general scheme of glycoside formation and hydrolysis supported by the above work. (Fig. XIV).

The early work of Levene, Raymond and Dillion[407] has been largely vindicated by recent studies in which the initial extent of furanoside formation and the furanoside/pyranoside composition at equilibrium was more accurately determined. Mowery and Ferrante[404] re-examined the methanolysis of galactose using modern column chromatographic procedures for product analysis and found that β-anomers formed initially changing later to the α-form. This latter action seemed more "important" than the change of furanoside to pyranoside. Methanolysis of D-mannose was also investigated[409] when it was found that α-anomers predominate at all times and furanosides change quickly to pyranosides.

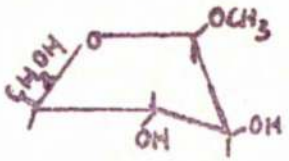
Bishop and Cooper have investigated the kinetics of the Fischer methanolysis of D-xylose[405], D-arabinose, D-lyxose and D-ribose[410] and since dihydrostreptose has been shown to be a branched chain L-lyxose with probably a fixed furanoside ring, their findings are pertinent to the present study. The products of the methanolysis reaction were analysed at different times by gas-liquid chromatography of their fully methylated derivatives. Bishop and his co-workers[411] state that caution must be observed whilst interpreting results of separations by gas-liquid chromatography in the carbohydrate field as, under the conditions used in the technique, a number of changes occur, in sugar molecules including change of sugar ring size and rearrangement of acetal or ketal groups.

With these limitations in mind, the rate of reaction data indicated that methanolysis of a pentose involves the following sequence of reactions:- 1. Pentose → furanosides 2. Anomerisation of furanosides. 3. Furanosides → pyranosides. 4. Anomerisation of pyranosides. These reactions are competitive but it has been possible to establish conditions where a single reaction predominated[410]. Furanosides were formed first, the relative rates of reaction of this form reflecting the relative conformational stabilities of the pentafuranosides.

The furanoid ring was regarded as essentially planar until recently when nuclear magnetic resonance studies allowed a specific conformation to be assigned to D-ribofuranose in nucleotides[412]. Bishop and Cooper propose a conformational system for the furanosides[410] The strain on this ring form can be relieved by slight puckering brought about by movement of one or two atoms out of the plane. Where only one atom is out of the plane the E (envelope) form is said to exist, that with three atoms co-planar by T (twist). The atoms out of plane are indicated by subscripts or superscripts to show respectively displacement below or above the plane of reference; carbon atoms are given numbers and the ring oxygen by O.

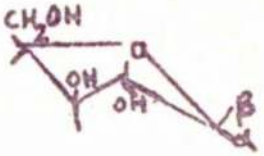
α-D-arabinoside

$T_2^3$ (CXLV)



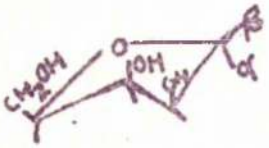
β-D-arabinoside

$E_2$ (CXLVI)



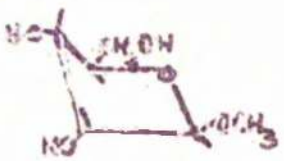
α - or β-D

xyloside $T_3^2$ (CXLVII)



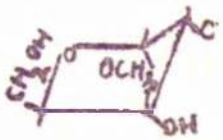
α- or β- D

lyxoside $T_2^3$ (CXLVIII)



β- D - riboside

$E^3$ (CXLIX)



α - D riboside

$E^2$ (CL)

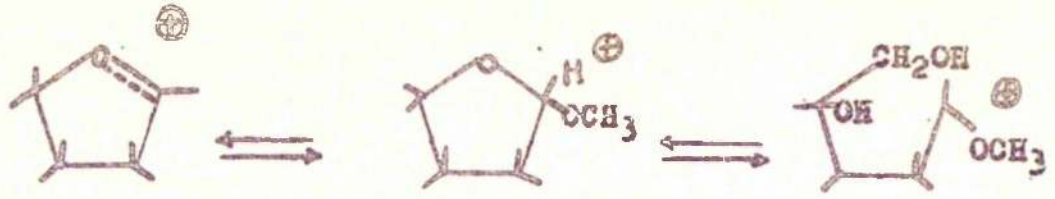
The preferred conformation is as usual predictable where the bulkier groups avoid non-bonded interactions. In the furanoid ring the effective interactions are those between eclipsed groups on adjacent carbon atoms and the most favoured conformations will allow maximum staggering. Conformations with C-1, C-4 or O-displaced have a fully or nearly eclipsed pair of carbon atoms and should be less stable than those in which C-2 or C-3 are displaced from the plane. An example is methyl α-D-arabinofuranoside (CXLV) which has all trans orientation of large substituents and the strain is relieved by maximum staggering afforded by a $T_2^3$ or $T_3^2$ conformation. Methyl D-lyxofuranosides have eclipsed interactions between C-2 and C-3 as well as C-3 and C-4 substituents. The molecule should therefore

adopt a $T_2^3$ conformation, (CXLVIII) which provides maximum distances between O substituents with an ideally staggered form. An alternative for methyl α -D-lyxofuranoside could be $E_3$. The preferred conformation of methyl pentafuranosides is given in Table 8.

TABLE 8.

| Methyl D-pentafuranoside | | Conformation | | Hydroxyl interactions Total |
|---|---|---|---|---|
| Arabinose | α | $T_2^3$ $T_3^2$ | (CXLV) | — (0) |
| | β | $E_2$ | (CXLVI) | C-1 ····· C-2 (1) |
| Riboside | β | $E_2$ | (CXLIX) | C-2·····C-3 (1) |
| | α | $E^3$ | (CL) | C-1 ··· C-2, C-2···C-3 (2) |
| Xyloside | α | $T_3^2$ | (CXLVII) | C-3····C-4 (1) |
| | β | $T_3^2$ ($E_3$) | (CXLVII) | C-3···· C-4 (1) |
| Lyxoside | α | $T_2^3$ ($E_3$) | (CXLVIII) | C-2····C-3, C-3··C-4 (2) |
| | β | $T_2^3$ | (CXLVIII) | C-1··· C-2, C-2··C-3 C-3···· C-4 (3) |

These authors[405] and Capon, Loveday and Overend[413] fail to agree on the form of ring expansion to pyranosides. Bishop and Cooper consider that the change occurs without alteration of configuration, whilst Overend and his colleagues quote evidence from the study of α - and β -D-glucosides indicative of anomeric change. Possible intermediates for the furanoside anomerisation (reaction 2) and furanoside ⟶ pyranoside conversion (reaction 3) are shown according to Bishop and Cooper[410]



(CLI)  (CLII)  (CLIII)

both arising from the protonated furanoside carbonium intermediate (CLII). The non-bonded interactions between the large eclipsed groups in the furanosides will be relieved by ring opening to (CLIII) whilst in (CLI) dissociation at C-1 removes C-1···· C-2 interactions and the displacement of C-3 in $E_3$ or $E^3$ conformation removes C-2···· C-3 and C-3····C-4 interactions. The relative orders of reactivity for the four pentoses should therefore be the same for reactions 2 and 3 and should depend on the strength and number of eclipsed interactions in the furanosides.

The order predicted for the proposed conformations was confirmed by experimental data. The most reactive furanoside is lyxose with two adjacent eclipsed interactions followed by xylose with one eclipsed interactions involving the $CH_2OH$ group, less reactive ribose with one interaction, and the slowest arabinose with no interactions.

The anomeric reversion is also predictable - as in lyxose where $\beta \rightarrow \alpha$ -furanosides inversion is so rapid that no $\beta$ -form is detectable at any stage, a situation accounted for by the extra

C-1-C-2 interaction instability introduced by $\beta$-lyxofuranoside.

The results for the pentoses may be summed up as follows:-[410] Lyxose gives a very unstable $\beta$-furanoside which reverts on formation to the slightly more stable $\alpha$-furan ring $T_2^3$ which expands to the pyranosides of which the $\alpha$-anomer is more stable conformationally and forms rapidly. D-Xylose gives $\alpha$- and $\beta$-furanoside with almost equal stability in $T_3^2$ form. Change from furanoside to pyranoside ring is aided by the C-3-C-4 interaction. The Xylopyranosides show the slowest rate of anomerisation as there are no axial substituents present.

In D-ribose the $\alpha$-glycofuranoside is less stable than the $\beta$- as it has one more eclipsed interaction, the change from furanoid to pyranoid is slower though there is conformational interaction in the ribofuranoside C-2 to C-3. The pyranosides showed anomalous behaviour on conformational predictions thought to be due to the axial C-3 hydroxyl.

In D-arabinose the $\beta$-arabinofuranoside with one eclipsed atom is less stable than the $\alpha$- anomer which has none. This lack of eclipsed interactions stabilised the $\beta$-furanoside and the change from furanoside to pyranoside is slower than with the other sugars. The pyranosides anomerise more rapidly $\beta$- to $\alpha$- than ribosides.

The percentage compositions at equilibrium are quoted in Table 9.

Table 9.

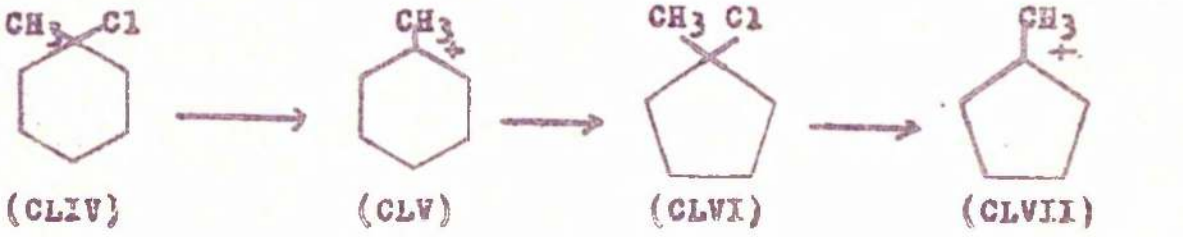
| Sugar | α-Furanoside | β-Furanoside | α-Pyranoside | β-Pyranoside |
|---|---|---|---|---|
| D-Xylose | 1.9 | 3.2 | 65.1 | 29.8 % |
| D-Arabinose | 21.5 | 6.8 | 24.5 | 47.2 |
| D-Lyxose | 1.4 | not detected | 88.3 | 10.3 |
| D-Ribose | 5.2 | 17.4 | 11.6 | 65.8 |

Brown and his colleagues[414] have attempted to rationalise the general differences in behaviour of furanose and pyranose sugars in terms of the I-strain concept[415], a general stereochemical theory proposed by these workers for cyclic carbon systems. I-strain is that change in internal strain which results from change in co-ordination number of a ring atom involved in a chemical reaction. In 5-, 6- and 7- membered ring systems the effect of I-strain is not so obvious as the angles within the ring are close to the tetrahedral angle (109.5°). However it is suspected that comparatively small differences in internal strain can have large effects in rates and equilibria of reactions of these ring compounds. Thus any enlargement of the ring carbon angles by nucleophilic substitution in cyclohexane will cause conformational change, increase non-bonded H-H-repulsions, decrease the symmetry and increase the internal strain (positive I-strain). These reactions of cyclohexane derivatives requiring a change in covalency of any atom from 4 to 5 or from 4 to 3 is opposed by I-strain. The converse is also true.

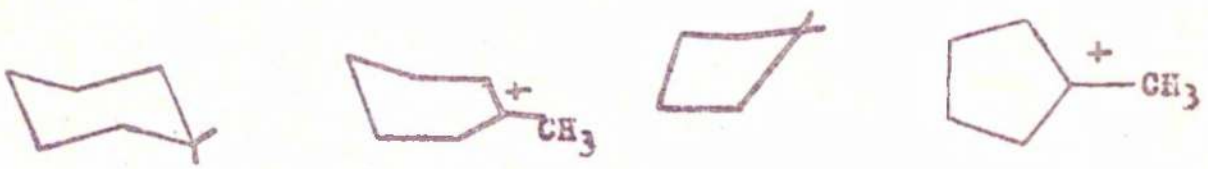
In the case of 5- and 7- membered rings which occur with some strain caused by distortion of the C-C angles, the introduction of an atom with a preferred 120° angle leads to a decrease of this strain and I-strain favours such reactions involving change in covalency from 5 to 4 and 4 to 3 and opposes change in co-ordination number from 3 to 4.

Brown[414] suggests that differences in behaviour of furanose and pyranose forms of sugars may be explained in terms of this postulate. On treatment of a sugar which exists in solution as an equilibrium mixture of ring forms (mostly pyran) with methanolic hydrogen chloride, furanoside is preferentially formed.

The rate of hydrolysis of 1-methyl-1-chlorocyclopentane (CLVI) is 100 times greater than that of the cyclohexane analogue[414] (CLIV). This increased rate is explained by the above concept where formation of carbonium ions (CLVII) and (CLV) respectively with C-bond angle




(CLIV) (CLV) (CLVI) (CLVII)



of 120° gives greater stability (less strain) to the five membered and greater strain to the six membered ring. Presumably such a theory also explains the preferential activity of the five membered furanose sugar form in the initial stages of glycodisation yielding furanosides. Such a cyclic furanoid carbonium ion is indicated by Bishop and Cooper[410](page 119 ).

TRANSGLYCOSYLATION.

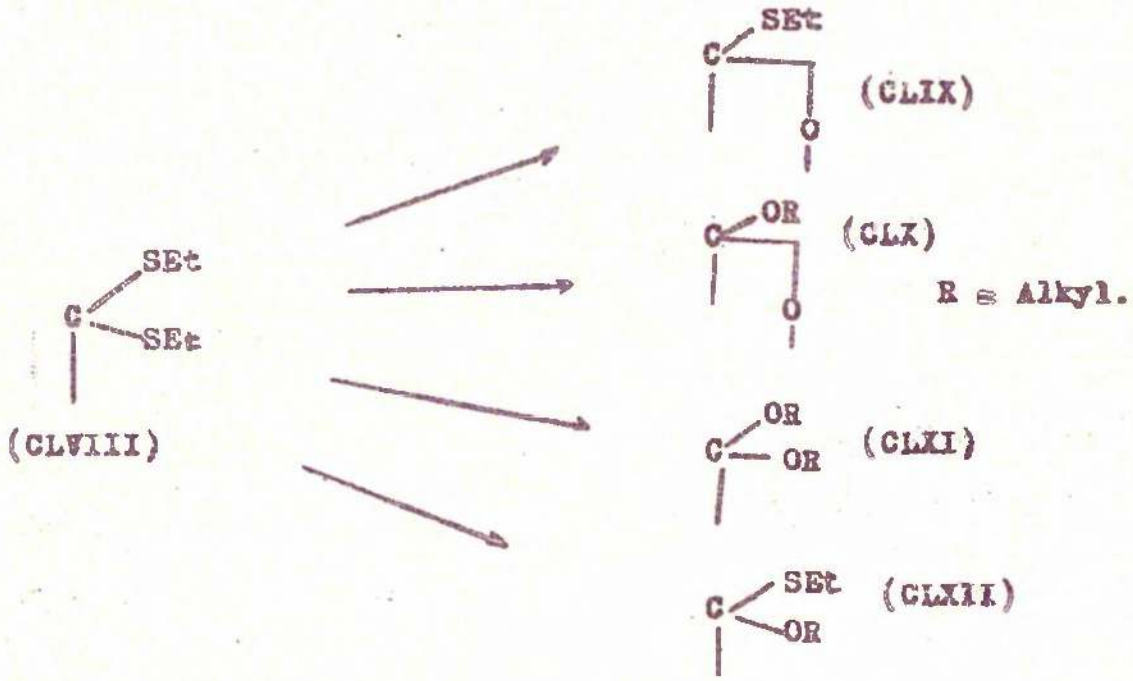
The route of glycoside synthesis followed in this work while almost certainly having the mechanism of solvolysis involved in the Fischer synthesis is a form of transglycosylation and a brief mention of these similar reactions from the literature is made here.

Pigman[417] observes that the alkyl group of the glycoside may be exchanged if the alkyl group of the solvent alcohol differs from that of the initial glycoside. In methanol containing hydrogen chloride ethyl α-D-glucopyranoside gives methyl α-D-glucopyranoside[419]. Purves and Hudson[418] prepared benzyl β-D-fructopyranoside from methyl α-D-fructofuranoside with dry benzyl alcohol containing hydrogen chloride.

Vernon and his colleagues[340b] prepared the methyl glucopyranoside of 2,3,4,6-tetra-O-methyl-D-glucose and D-glucose from the phenyl glucopyranosides a reaction that took place in dry methanolic hydrogen chloride with predominant inversion. They give the formation of a pyranoid carbonium ion as intermediate (Scheme A page 85 ) as already outlined under hydrolysis of glycosides. Furanoside formation

appears to be avoided with this reaction.

Related to the methanolysis action in glycoside formation is mercaptolysis treatment of sugars with ethane thiol (ethyl mercaptan). This rapidly yields from an aldose sugar the diethyl mercaptal (CLVIII) from which thioglycosides (CLIX), glycosides (CLX), acetals (CLXI) and mixed acetals (CLXII) may be prepared by desulphuration with mercuric chloride and appropriate treatment[417b,419].

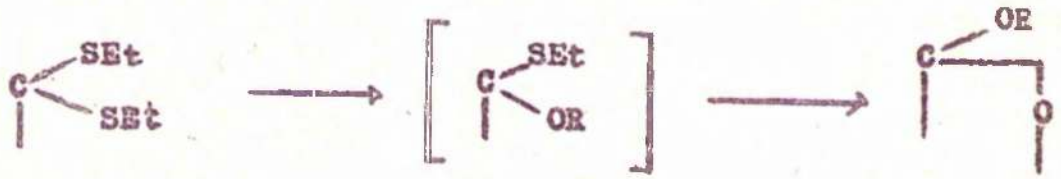



Green and Pacsu[420] noted that glucose and galactose diethylmercaptal treated with an ethanolic solution of mercuric chloride in the presence of excess mercuric oxide (to neutralise any acid formed) gave the β-ethyl glycofuranoside. In the case of glucose the α-thioglycoside could also be obtained. The β-propyl and β-benzyl galactofuranosides

were also obtained from this reaction[421].

In the case of rhamnose diethyl mercaptal, treatment with methyl alcohol in the presence of mercuric salts gave some dimethyl acetal[422] though it is considered to be a by-product.

Paesu and Green[422] considered that during alkylglycoside formation from the acyclic mercaptal a mixed acetal with one alkyloxy and one thioethyl group was an intermediate (CL II).




(CLXII) (CLX)

Wolfrom[423] and his colleagues tested this theory by preparation of the mixed acetals of glucose and galactose and observed the following. D-glucose diethyl mercaptal with methanol and mercuric chloride at room temperature gave ethyl α-thio-D-glucofuranoside. The S-ethyl-O-methylmonothioacetal gave methyl β-glucofuranoside indicating that the mixed acetal is not an intermediate in this reaction.

D-galactose mixed acetal with mercuric chloride in ethanol gave ethyl β-D-galactofuranoside D-galactose diethyl mercaptal gave the same product indicating that the mixed acetal is in this case an intermediate.

Folkers[259] and his co-workers as previously reported applied the mercaptolysis reaction first used by Fischer[424], to streptomycin the streptidine was replaced in the streptobiosamine moiety with a thioethoxyl group - a case of transglycosylation under Fischer conditions. The free aldehyde of streptose formed a thioacetal the product being ethylthiostreptobiosaminide dithioacetal hydrochloride (LXVC) (page 58).

These workers also found that treatment of methyl streptobiosaminide dimethylacetal with ethyl mercaptan containing hydrogen chloride gave the above product by transglycosylation.

Methyl dihydrostreptobiosaminide also undergoes this reaction to yield the $\alpha$- and $\beta$-thioglycoside ethylthiodihydrostreptobiosaminide hydrochlorides (LXVb)[244] separated as the tetra-acetyl derivatives.

Structural studies of mannosidostreptomycin have also utilised this reaction. Treatment of this streptomycin derivative with ethyl mercaptan containing hydrogen chloride was shown to give ethylthiostreptobiosaminide dithioacetal and two ethylthiomannosides[277], products which led Fried and Walz[425] to apply mercaptolysis to the simple mannosides $\alpha$ and $\beta$-methyl mannopyranoside treated with ethyl mercaptan containing hydrogen chloride for eighteen hours gave ethyl 1-thio-$\beta$-D-mannopyranoside characterised as the tetra-acetyl derivative. Better yields were obtained with D-mannose as starting product, the mannose diethyl mercaptal (dithioacetal) being an intermediate.

It was noted that D-galactose gave a similar glycoside but L-arabinose gave its dithioacetal indicating that steric factors are important in this replacement[425].

Synthesis of Dihydrostreptobiosamine Glycosides.

The anomeric benzyl glycosides were first prepared by condensing the methyl dihydrostreptobiosaminide with benzyl alcohol containing 2.5 N hydrogen chloride for 48 hours at 45°. Concentration of the reaction mixture gave crystals m.p. 220°. The mother liquors were reduced to dryness, triturated with ether to remove traces of benzyl alcohol, leaving a lower melting very hygroscopic solid.

A second preparation of the benzyl glycoside which has become the model for the remainder of the series, utilised ether precipitation to give the product and, after trituration with ether, the hygroscopic solid was reprecipitated from dry ethanol and dry ether.

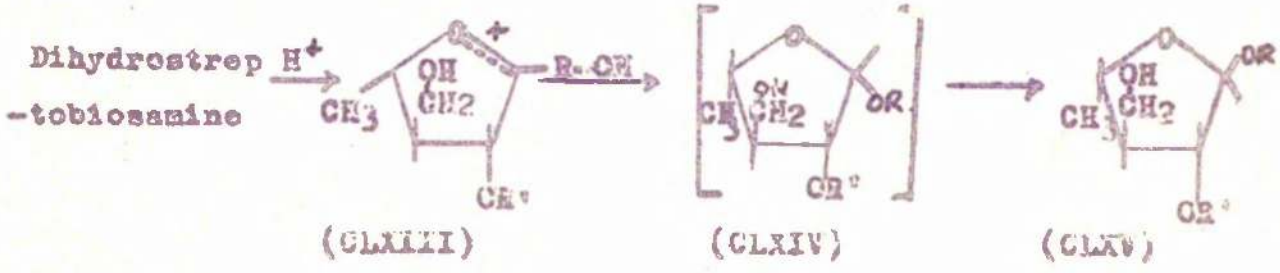
The hygroscopicity of these glycosides is probably attributable to adherent traces of hydrogen chloride. A similar difficulty was reported by Augestad and Berner[403a] in the preparation of galactose, arabinose and xylose methyl furanosides by the Fischer synthesis.

Three glycosides, benzyl, 2-bromoethyl and phenyl obtained in this way in reasonable yield were reasonably stable. Preliminary experiments indicate that cyclohexyl, m-cresyl and anisyl glycosides might be obtained if the hydrogen chloride could be removed.

Polarimetric readings showed slight change from methyl dihydrostreptobiosaminide. The benzyl glycoside gave an absorption maximum at 260 mμ characteristic of the aromatic ring. Infrared analysis for the phenyl and benzyl group showed absorption maxima in the 650 to 850 $cm^{-1}$ region indicative of the presence of aromatic groups.

In preparing glycosides from methyl dihydrostreptobiosaminide hydrochloride it would have been advantageous to prepare the acetyl glycosyl halide as a starting product but so little was known of the properties of such a product that instead of the usual Koenigs-Knorr glycoside synthesis, the modified Fischer method was chosen. The disadvantages of this method have already been cited but, after some neglect, it is regaining popularity for furanoside synthesis.

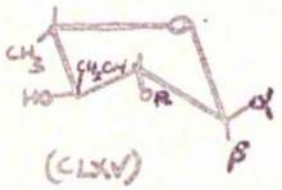
Vernon[340] considers the carbonium ion to have been formed in the methanolysis of phenyl $\alpha$ - and $\beta$ -glucopyranoside. Considering dihydrostreptose as it occurs in dihydrostreptobiosamine (5-deoxy-3-C-hydroxymethyl-L-lyxose) it would seem that on solvolysis it would form a furanoid carbonium ion (CLXIII) intermediate.




R' = N-Methyl-L-glucosamine

This would react with the solvent in large excess with inversion giving the transient $\beta$-glycoside (CLXIV) which would anomerise in the presence of the acid catalyst to the more conformationally stable $\alpha$-anomer (CLXV).

Methyl dihydrostreptobiosaminide hydrochloride occurs as an anomeric mixture which has not been resolved. The penta-acetates have however been separated to show a composition 90% $\alpha$ and 10% $\beta$. These results, confirmed in this work, indicated that the $\alpha$-L-lyxofuranoside predominates. The $T_3^2$ conformation (CLXV) seemed the most likely explanation for this fact. The $\beta$-anomer (CLXV)

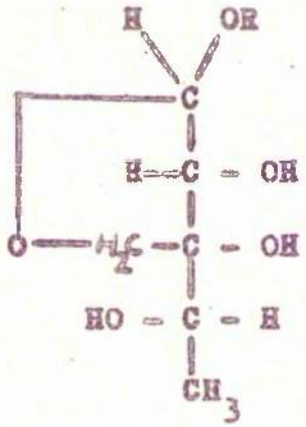


(CLXV)

would entail extra strain on the system which cannot form a pyranoside ring owing to the C-methyl group on C-4.

The mechanism of this transglycosylation which has been outlined is of course only tentative but considered as a solvolysis with the equilibrium favouring aglycone replacement and conformational considerations favouring the $\alpha$-anomer, it would seem feasible. Paper chromatography indicates that probably some $\beta$-anomer occurs but the $[\alpha]_D$ results are all near that of $\alpha$-methyl penta-acetyl-dihydrostreptobiosaminide (-117°) rather than the $\beta$-anomer (-34°)[244] (Table 9).

The ease of formation of these glycosides is consistent with the furanoid ring in dihydrostreptose rather than structure (XXXIX).



(XXXIX)

Table 9.

| Glycoside | $[\alpha]_D$ |
|---|---|
| Methyl dihydrostreptobiosaminide hydrochloride | -125 |
| Benzyl | -110 |
| 2-Bromoethyl | -100 |
| Phenyl | -131 |

Appendix II, p.173 discusses the n.m.r. spectra of some dihydrostreptobiosaminide derivatives which appear to support the anomeric configurations assigned by Wolfrom to streptomycin,[273] on the basis of Molecular Rotation Studies.

2-Bromoethyl and phenyl dihydrostreptobiosaminide have been sent for testing against Mycobacterium tuberculosis but the results are not yet available.

## EXPERIMENTAL

### Materials

Dihydrostreptomycin sulphate and streptomycin sulphate, was supplied by Glaxo-laboratories from a non-sterile, freeze-dried batch.

Pyridine was dried by refluxing over potassium hydroxide and distilling.

Acetic anhydride was redistilled, the early and late runnings being discarded.

Silver carbonate was prepared as required by reaction of silver nitrate and sodium carbonate, the product being filtered and thoroughly washed.

### Paper Chromatography

Paper chromatographic separations were effected by the descending method on Whatman No. 1. paper, unless otherwise reported, the chromatograms being developed for 16 to 25 hours with the organic phase of one of the following solvents systems. (All solvents were redistilled except when fresh AnalaR or other special reagents for chromatography were available).

(a) n-butanol - ethanol - water; 4:1:5,

(b) n-butanol - glacial acetic acid - water; 4:1:5,

(c) n-butanol - pyridine - water; 4:2:3.

After irrigation the chromatograms were dried and treated with one of the following reagents.

1. Aniline hydrogen phthalate (Partridge 1949)[428] for reducing sugars.
2. Ninhydrin (Consden, Gordon and Martin 1944)[429] for compounds containing primary or secondary amino groups.
3. Silver nitrate (Trevelyan, Proctor and Harrison 1950)[430] for carbohydrates.

The papergrams were first moistened with a solution of silver nitrate in acetone and allowed to dry at room temperature. Spraying with ethanolic sodium hydroxide then brought up reducing and non-reducing carbohydrates as black spots. These were fixed by soaking the papers in sodium thiosulphate solution (20%) followed by thorough washing in water.

4. Sodium metaperiodate in alkaline permanganate (Lemieux and Bauer 1952)[431] for glycosides. These show up as yellow-brown spots when the reagent is washed off after 30 to 45 minutes contact.

Papergrams sprayed with aniline hydrogen phthalate or ninhydrin were heated at 100 to 110° for 5 to 15 minutes for colour development.

Thin Layer Chromatography

The thin layer chromatography apparatus used was manufactured by Camag Ltd. Silica gel G. by Merck was spread on the plates in a slurry and activated for 4 hours at 110° [432]. All solvents used were scrupulously dried. Methanol was used except where otherwise noted. Spots were detected by spraying with 5%[433], or concentrated sulphuric acid[434], which gave dark brown spots, or by B.D.H. aerosol with 0.25% ninhydrin in n-butanol for amino sugars which give pink to violet spots. Results were recorded by photocopy where necessary though $R_F$ values against a standard were reproducible for qualitative purposes.

Paper Electrophoresis

Paper ionophoretic analysis was carried out using strips of Whatman No. 3 paper (57 x 11 cm) and a reconstruction of the apparatus described by Foster (1952)[435]. A potential gradient of approximately 16 to 22 volts per cm was usually applied for 1 to 3 hours using 0.2M acetate buffer pH 5 (19g. sodium acetate trihydrate and 3.6g. acetic acid per litre) as electrolyte.

Starting Products

Dihydrostreptomycin trihydrochloride

Dihydrostreptomycin trihydrochloride was prepared from dihydrostreptomycin sulphate (10g.) by treatment with barium chloride (4.35g.) in aqueous solution (100ml). The precipitated barium sulphate was

filtered off and the filtrate evaporated to dryness *in vacuo* at 50°. The residue after evaporation, dried over calcium chloride and in high vacuum at 100° over $P_2O_5$, represented essentially pure dihydrostreptomycin trihydrochloride (6.18g., 61%).

**α- and β-Methyl dihydrostreptobiosaminide hydrochloride**

(a) Preparation after Fried and Wintersteiner[257].

Dihydrostreptomycin sulphate (10g.) which had been dried for 3 hours in a high vacuum pistol over phosphorus pentoxide at 100°, was dissolved in N methanolic hydrogen chloride (192ml) and the solution was kept at room temperature for 48 hours. Dry ether (400ml) was added at the end of this time precipitating streptidine hydrochloride (5.45g.) which was filtered off as a white hygroscopic solid and formed a dipicrate, m.p. 283° (lit.[249] 284-285°).

The methanol - ether filtrate was concentrated *in vacuo* to small volume (25-30ml), shaken and left at 0 - 5° for 2 hours. The pale yellow oily precipitate was separated from the methanol - ether solution by decantation, washed with dry ether (2 x 55 ml) and dried *in vacuo* over calcium chloride and potassium hydroxide (4 - 5 days). The hygroscopic solid obtained was a mixture of the anomeric methyl dihydrostreptobiosaminide hydrochlorides (4.88g., 92.5%) $[\alpha]_D^{19}$ -120° (c. 1% methanol). (Fried and Wintersteiner give $[\alpha]_D^{25}$ -135°)[257]. molecular weight (potentiometric) 380 requires 389.

Found: C, 42.6; H, 7.0; N, 3.5. Calculated for $C_{14}H_{27}NO_9HCl$ C, 43.12; H, 7.2; N, 3.6%.

Methanolysis of dihydrostreptomycin trihydrochloride under the same conditions gave similar yields.

(b) Preparation after Bartz, Countrouliś, Crooks and Rebstock[258].

Dihydrostreptomycin sulphate (2g.) was treated with N methanolic hydrogen chloride (122ml.) for 72 hours at room temperature. Anhydrous ether (250ml.) was added and the precipitated streptidine hydrochloride centrifuged. The supernatant solution was neutralised with 10% methanolic sodium hydroxide, the sodium chloride removed, and the solution evaporated to dryness under reduced pressure. The white residue was dissolved in dry ethanol (10ml.) any insoluble material filtered off and again evaporated to dryness under reduced pressure below 50°. The yellowish-white solid remaining (0.84g., 86%) was an anomeric mixture of methyl dihydrostreptobiosaminides $[\alpha]_D^{20}$ -110°, m.p. 120°.

Methyl N-(2,4-dinitrophenyl)-dihydrostreptobiosaminide

[(1-O-methyl-α(β)-3-C-hydroxymethyl-5-deoxy-L-lyxofuranosyl-(4→1)-O-α-2-deoxy-2-N-(2,4-dinitrophenyl)-N-methylamino-L-glucopyranoside)].

(a) Methyl dihydrostreptobiosaminide hydrochloride (0.84g.) and sodium bicarbonate (0.42g., 2 mol) were dissolved in 50% aqueous ethanol (25ml). 2,4-Dinitrofluorobenzene (0.46g.) was added and the mixture shaken at room temperature for two hours. Concentration of the yellow opalescent solution to dryness at pH 9 and at 50° *in vacuo* gave a sticky orange-yellow residue. Sodium chloride and sodium fluoride were removed by dissolving the residue first in dry methanol (10ml), filtering, removing the solvent under reduced pressure and then treating in a like manner with dry ethanol. The residue was dissolved in distilled water (*ca.* 5ml) and extracted with ether (5ml). The aqueous solution was evaporated to dryness under reduced pressure at 50°, dried *in vacuo* ($CaCl_2$) and redissolved in n-propanol. Insoluble matter was filtered off and after several days at 0 - 5° when crystals failed to form the propanol solution was evaporated to dryness *in vacuo* at 50° to yield a hygroscopic, brown - orange, amorphous brittle solid (0.53g., 42%) gradually melting between 90 - 100° (hot stage).

Found: N, 8.4; $C_{14}H_{29}O_{13}N_3$ requires N, 8.1%.

(b) In a second experiment after condensation of the methyl dihydrostreptobiosaminide hydrochloride (0.51g.) with 2,4-dinitrofluorobenzene (0.28g.) as above, the reaction mixture was concentrated at 50° under reduced pressure. Water (5ml) was added to the sticky orange residue and extracted with benzene (3 x 5ml)

and ether (5ml.), and the aqueous phase evaporated to dryness as before. The residue was dissolved in dry n-propanol, the solution left overnight, filtered to remove inorganic salts, concentrated to dryness and redissolved in ethanol (repeated thrice), to give methyl N-(2,4-dinitrophenyl)-dihydrostreptobiosaminide as a yellow, hygroscopic solid (0.44g., 60.3%) m.p. 150-170°.

Found: C, 43.9; H, 5.7; N, 7.5; $C_{20}H_{29}N_3O_{13}$ requires C, 43.2; H, 6.0; N, 7.6%.

The above experiment was repeated using methyl dihydrostreptobiosaminide sulphate (0.42g.) and yielded methyl N-DNP-dihydrostreptobiosaminide dihydrate (0.2g., 34%) m.p. 160°.

Found: N, 7.8; $C_{20}H_{29}N_3O_{13}.2H_2O$ requires N, 7.5%

Paper chromatograms (ascending) of this material using butanol-acetic acid-water, gave a fast running spot $R_F$ 0.9 with a long tail.

2-Deoxy-2-N-(2,4 dinitrophenyl)-D-glucosamine was prepared by a modification of the method of Kent[352].

D-glucosamine (0.5g.) was treated in alkaline solution with 2,4-dinitrofluorobenzene (0.43g.) for 2 hours at 50°. After concentration to small volume (10ml.) under reduced pressure the reaction mixture was shaken with benzene (12 x 5ml.) and ether (1 x 5ml.) and the aqueous extract evaporated *in vacuo*. The residue

was dried in a vacuum desiccator ($CaCl_2$,KOH) leaving an orange - yellow solid (0.34g., 42%) m.p. 168° (Kent[352] gives 167-169°, Annison James and Morgan[436] give 202-204° and Wang-Yu 206° $[\alpha]_D^{20}$ +55° 350) (c. 1% ethanol).

Found: N, 11.85. Calculated for $C_{12}H_{15}N_3O_9$. N, 12.2%.

1,3,4,6-Tetra-O-acetyl-2-deoxy-N-(2,4-dinitrophenyl) D-glucopyranose.

N-2,4-dinitrophenyl-D-glucosamine (0.2g.) was dissolved in pyridine (10ml) and acetic anhydride (10ml) and left for 2½ days at room temperature and the solvents removed under high vacuum at 50° to leave an orange - brown residue. Recrystallisation from chloroform - ether and n-propanol gave tetra-N-acetyl-2,4-dinitrophenyl-D-glucosamine as an orange-red solid mp. 196° $[\alpha]_D^{20}$ +42° (c. 1% chloroform).

(Kent gave m.pt 161° $[\alpha]_D^{20}$ +72° 352, Wang Yu gave m.p. 214-5° $[\alpha]_D^{20}$ +12° 350.

Found: N, 7.9. Calculated for $C_{20}H_{23}N_3O_{12}$. N, 8.2%.

Methyl N-acetyldihydrostreptobiosaminide

(a) Methyl dihydrostreptobiosaminide hydrochloride (2g.) was dissolved in distilled water (92ml) and methanol (10ml), stirred for 90 minutes at 0 - 5°C with De-acidite FF carbonate form (108ml) and acetic anhydride (2.4ml). The mixture was filtered and the filtrate and washings passed down a column of Amberlite IR 120 ($H^+$ form)

(18.4ml). The colourless effluent and washings were freeze-dried yielding an off-white residue (0.42g., 20%) m.p. 190° which gave white crystals of methyl N-acetyldihydrostreptobiosaminide (0.14g.) from 90% aqueous ethanol $[\alpha]_D^{18}$ 124° (c. 1%, methanol).

Found: N, 3.2; $C_{16}H_{29}NO_{10}$ requires N, 3.54%.

The Amberlite IR 120 was washed with 0.3N hydrochloric acid (1 litre) and distilled water (500ml); the acid solution and washings were neutralised with 10% di-n-octylmethylamine in chloroform when starting product (1.5g.) was recovered by freeze-drying.

(b) [359] Methyl dihydrostreptobiosaminide hydrochloride (4g.) was dissolved in dry methanol (40ml) which contained sodium (0.23g.). The sodium chloride which formed was centrifuged and acetic anhydride (1.3ml., 1.5 equivalents) was added to the supernatant solution at room temperature. After shaking for one hour the solvents were removed under reduced pressure below 45° to yield a creamy white residue which was dissolved in dry ethanol. The Sodium chloride was removed by filtration and the solution again evaporated to dryness under vacuum, to yield a product which differed from the starting material as shown by a slight shift in the infrared absorption spectrum.

The product was dissolved in distilled water (50ml) and passed down a column (1 x 12") of Zeo Karb 225 ($H^+$ form), the eluate was collected in 5ml fractions on a fraction cutter and each fraction

spotted out on a papergram and developed overnight. The resultant chromatograms showed that fractions 4 to 12 had sugar residues present but gave four distinct spots $R_F$ range 0.05 to 0.5. These fractions were mixed and concentrated to yield a light tan residue (1.2g.) which appeared neutral on electrophoresis.

This material was passed down a cellulose powder column (1 x 8") irrigated with n-butanol - acetic acid - water, (4:1:5), and 5ml fractions were collected and examined on papergrams. Fractions 4 and 5 contained a fast moving fraction ($R_F$ 0.7) which after evaporation gave a charred residue (100mg.) of uncharacterised material. Fractions 7 and 8 contained a residue ($R_F$ 0.4, 200mg.) m.p. 150-160° which failed to give a satisfactory analysis for methyl N-acetyl-dihydrostreptobiosaminide.

(c) Methyl dihydrostreptobiosaminide hydrochloride (2g.) was passed down a column of Amberlite IRA 400 (OH form). The base was eluted with distilled water (1 litre) and the eluate concentrated to small volume on a rotary film evaporator at 40°. Ethanol (10ml) was added and the solution evaporated to dryness to give the colourless base (1.3g, 71.7%) m.p. 130°.

This base (1g.) was dissolved in dry pyridine (10ml) and acetic anhydride (0.3g.) and heating continued at 45° for 48 hours or until the ninhydrin spot test was negative. When the ninhydrin test was extinguished the solvents were removed in high vacuum at 25 to 30°.

The residue was treated with chloroform, and the chloroform-soluble matter removed. The major portion remaining was redissolved in dry ethanol (10ml) and evaporated to dryness at 40° in a rotary film evaporator, and finally dried *in vacuo* over concentrated sulphuric acid. Extensive fractionation with dry ether to remove traces of pyridine left methyl N-acetyldihydrostreptobiosaminide as a light tan hygroscopic solid (0.83g., 74%) m.p. 170° $[\alpha]_D^{18}$ -125° (c. 1%, water).

Found: C, 47.9; H, 6.9; N, 3.5; $C_{16}H_{29}NO_{10}$ requires C, 48.6; H, 7.4; N, 3.5%.

The material was neutral but gave two spots on papergrams.

Dodeca-acetyldihydrostreptomycin

Dihydrostreptomycin sulphate (10.18g.) was dried *in vacuo* at 100° ($P_2O_5$) and acetylated first with acetic anhydride, pyridine and fused sodium acetate in methanol for 24 hours, at room temperature, then after removal of solvents, with pyridine and acetic anhydride for 74 hours at room temperature and 5 hours at 45°. The white solid obtained by pouring the acetylation mixture on ice and extracting with chloroform was redissolved in benzene and precipitated with light petroleum (b.p. 80-100°). gave dodeca-acetyldihydrostreptomycin (11.8g., 76%) m.p. 150° $[\alpha]_D^{20}$ -70° (c. 1% chloroform) as a creamy white solid (Mital[360a] gives m.p. 152-155° -67°). $[\alpha]_D^{20}$

Found: C, 48.5; H, 6.8; N, 8.4; Calculated for $C_{45}H_{64}N_7O_{24}$ C, 48.1; H, 6.2; N, 8.7%.

N-Acetyldihydrostreptomycin

Dodeca-acetyldihydrostreptomycin (9.76g.) was de-O-acetylated using dry methanolic ammonia[360b] to yield N-acetyldihydrostreptomycin (5.24g., 92.5%) m.p. 180° (decomp.) with sintering at 80° $[\alpha]_D^{19}$ -80° (c. 1%, water).

Found: C, 41.95; H, 7.7; N, 14.7. Calculated for $C_{23}H_{43}N_7O_{13}{}^{1}\!/_{2}H_2O$

C, 42.4; H, 7.1; N, 15.0%.

(d) Methyl N-acetyldihydrostreptobiosaminide

N-Acetyldihydrostreptomycin (1.2g.) was dried at 100° over phosphorus pentoxide for 2 hours, dissolved in N methanolic hydrogen chloride (61ml) and kept at 17° for 3 days. Anhydrous ether (122ml) was added and the precipitated streptidine dihydrochloride removed by filtration. The filtrate was neutralised with 10% methanolic sodium hydroxide, sodium chloride filtered off and the filtrate concentrated to dryness at 40° under reduced pressure. The residue was dissolved repeatedly in dry ethanol and the sodium chloride filtered off. A yellow product (400mg.) was obtained m.p. 165-170° $[\alpha]_D^{18}$ -120° (c. 1%, water).

α- and β-Methyl penta-acetyldihydrostreptobiosaminide

Methyl dihydrostreptobiosaminide hydrochloride (2.53g.) dried in vacuo over phosphorus pentoxide at 60° was dissolved in dry methanol (25ml) and pyridine (7.5ml). Acetic anhydride (12.5ml)

was added with constant stirring over 50 minutes. Fused sodium acetate (1.35g.) was added and stirring continued over 24 hours. The mixture was filtered and the filtrate concentrated in vacuo below 50°, to a semi-solid mass which was fractionated and washed with ether. This mass was further treated with pyridine (25ml) and acetic anhydride (25ml.) for 44 hours at room temperature and heated at 50° for 4 hours, filtered and the filtrate poured on to crushed ice (190g.). The aqueous solution was extracted with chloroform (5 x 10ml) and the chloroform extract evaporated to dryness at 50° under reduced pressure. The syrupy mass on fractionation with crushed ice (40g.) gave an aqueous solution which was extracted with chloroform, the chloroform dried ($CaCl_2$) and evaporated under reduced pressure to a brown solid mass which remained unchanged on treatment with benzene-petroleum ether (b.p. 80-100°) to yield a light brown powder, which on drying over phosphorus pentoxide in vacuo gave a white powder (1.28g.) m.p. 125°, $[\alpha]_D^{19}$ -105° (c. 1%, chloroform) an amorphous mixture of anomeric methyl penta-acetyldihydrostrepto-biosaminides.

Found: N, 3.1. Calculated for $C_{24}H_{37}NO_{13}$ N, 2.5%.

α-Methyl penta-acetyldihydrostreptobiosaminide[244]

The mixture of α- and β-methyl penta-acetyldihydrostrepto-biosaminide (2.4g.) was boiled with 200ml dry ether for 2 minutes. The ether insoluble material was crystallised from chloroform-ether

yielding $\alpha$-methyl penta-acetyldihydrostreptobiosaminide (1.86g.) m.p. 175° $[\alpha]_D^{19}$ -116.5° (c. 1%, chloroform) which on recrystallisation from dry ethanol gave needles m.p. 190° (Brink, Kuehl, Flynn and Folkers give m.p. 198° $[\alpha]_D$ (117°)[244].

Found: N, 2.7. Calculated for $C_{24}H_{37}NO_3$, N, 2.5%.

$\beta$-Methyl penta-acetyldihydrostreptobiosaminide

This other soluble material from the mixture of $\alpha$ - and $\beta$-methyl penta-acetyldihydrostreptobiosaminide gave crystals (0.25g.) m.p. 150° $[\alpha]_D^{19}$ -36° (c. 1%, chloroform) on addition of light petroleum and recrystallisation from dry methanol (Brink, Kuehl, Flynn and Folkers gave m.p. 155.5-157.5°) $[\alpha]_D^{25}$ -34° (c. 1%, chloroform)[244].

Methyl N-acetyldihydrostreptobiosaminide

(a) $\alpha$-Methyl penta-acetyldihydrostreptobiosaminide (1.38g.) was dissolved in dry methanol (15ml) which had been previously boiled and cooled. Dry ammonia was passed into the solution at 0° when the clear solution became straw coloured. The ammonia saturated solution was left for 24 hours at room temperature, the undissolved material centrifuged and the supernatant evaporated to dryness at room temperature under reduced pressure. The residue was washed with chloroform but as it floated on top as a syrup, the chloroform was extracted with water and the aqueous layer evaporated to dryness at 45° *in vacuo* to yield a white solid which was dried *in vacuo* ($P_2O_5$) overnight. The residue was redissolved

in dry ethanol (2.3ml) and precipitated with dry ether (50ml) as a white hygroscopic solid. This procedure twice repeated before drying in vacuo ($P_2O_5$) afforded methyl N-acetyldihydrostreptobiosaminide as a colourless solid m.p. 145°, $[\alpha]_D^{20}$ -125° (c. 1% methanol) Found: N, 3.95; N, 3.7% $C_{16}H_{29}NO_{10}$ requires N, 3.5%.

1.3.4.6-Tetra-O-acetyl-2-N-methylacetamido-2-deoxy-α-D-glucopyranose (Penta-acetyl-N-methyl-α-D-glucosamine)[260b].

α-D-Glucosamine hydrochloride (10g.) was treated with dimethyl sulphate (5ml) in alkaline solution (N sodium hydroxide 50ml) for half an hour. After removal of the solvents under reduced pressure, the ethanol soluble material was acetylated with pyridine (50ml) and acetic anhydride (50ml) at 0° for 4 days. Recrystallisation of the crude product (4.5g.) alternately from dry methanol (3 times) and dry chloroform/dry ether (3 times) gave white needles (1.8g.) of penta-acetyl-N-methyl-α-D-glucosamine m.p. 156-157° $[\alpha]_D^{20}$ -100° (c. 1%, chloroform). (Knehl, Flynn, Hully, Mozingo and Folkers found m.p. 160.5-161.5° $[\alpha]_D^{25}$ -101° [260b], (Wolfrom, Thompson and Hooper 158.5-159.5° $[\alpha]_D^{25}$ -102° [261b].

2-N-Methylacetamido-2-deoxy-α-D-glucopyranose (N-Acetyl-N-methyl-α-D-glucosamine)[260b].

Penta-acetyl-N-methyl-α-D-glucosamine (1.7g.) was dissolved in dry methanol (40ml) which had been previously boiled and cooled.

The solution was saturated with ammonia at 0° , when the solution turned brown, and left at room temperature for 24 hours. The methanol and ammonia were evaporated *in vacuo* at room temperature and the dark brown syrupy residue washed with dry chloroform. The chloroform insoluble residue was dissolved in methanol (10ml) and added to dry ether (75ml) when a light brown hygroscopic precipitate deposited (0.8g., 80%).

Recrystallisation several times from methanol-ether and once from dry ethanol gave crystals of *N*-methyl-D-glucosamine m.p. 162° $[\alpha]_D^{20}$ +54° (c. 0.5, water).

Found: N, 5.4. Calculated for $C_9H_{17}NO_{10}$, N, 5.95%.

(For L-isomer Kuehl *et al* reports m.p. 165-166° $[\alpha]_D^{25}$ -51° [260b]. (c. 0.4, water).

*2-N-Methylamino-2-deoxy-α-D-glucopyranose.* (*N-Methyl-D-glucosamine hydrochloride*).

Penta-acetyl-*N*-methyl-α-D-glucosamine (0.36g.) was treated[260b] with 2.5N hydrochloric acid yielding *N*-methyl-D-glucosamine hydrochloride (85mgm.) m.p. 160-161°; (Kuehl *et al*[260b] reports m.p. 164-166°, Wolfrom, Thompson and Hooper 160-162° [261b]).

*2-N-Methylamino-2-deoxy-α-L-glucopyranose hydrochloride.* (*N-methyl-L-glucosaminehydrochloride*), was prepared by degradation of streptomycin sulphate (5g.) as white crystals m.p. 160-162°. $[\alpha]_D^{20}$ -100° initial (c. 0.5, in water) (Kuehl *et al* found m.p. 160-163° $[\alpha]_D^{25}$ -103° 88° (c. 0.61, in water).[260b]

2-Deoxy-2-N-(2,4-dinitrophenyl)-2-methylamino-D-glucopyranose (N-(2,4-dinitrophenyl)-N-Methyl-D-glucosamine.

N-Methyl-D-glucosamine (0.35g.) was treated with 2,4-dinitrofluorobenzene as described (page 137) for the preparation of N-(2,4-dinitrophenyl)-D-glucosamine. N-(2,4-Dinitrophenyl)-N-methyl-D glucosamine was obtained as an orange-yellow solid (0.2g., 40%). m.p. 105-110°, $[\alpha]_D$ +7.3 (c. 1, methanol).

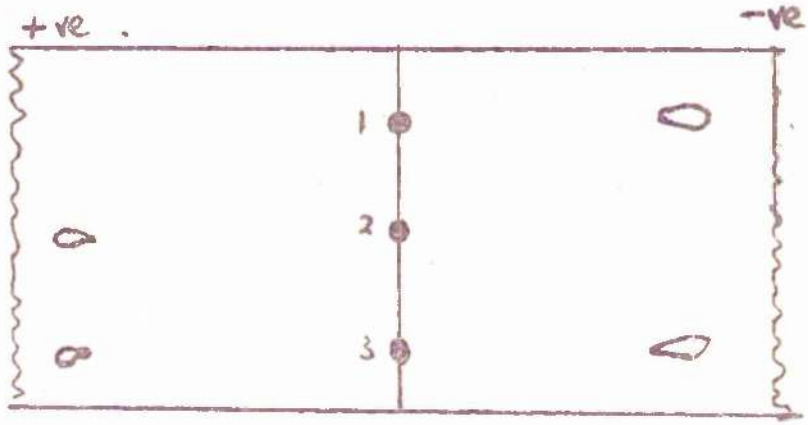
Found: C, 43.1; H, 4.4; N, 11.8; $C_{13}H_{17}N_3O_9$ requires C, 43.5; H, 4.5; N, 11.7%.

Acid Hydrolysis of Methyl N-(2,4-dinitrophenyl)-dihydrostreptobiosaminide

Methyl N-(2,4-dinitrophenyl)-dihydrostreptobiosaminide (0.5g.) was dissolved in N hydrochloric acid containing 50% methanol. An initial polarimeter reading was observed and the solution stirred under reflux. Every 12 hours the solution was examined for evidence of hydrolysis by means of the Fehlings test, polarimeter readings and paper chromatography and the acid strength was increased by 0.5N every 48 hours until changes were observed. The first evidence of hydrolysis was observed when the Fehlings test gave a slight positive after 6 hours with 2N acid. The papergrams then exhibited three spots but the polarimeter value was not obtained due to turbidity. After 48 hours with 2.5N hydrochloric acid, separation of the products of hydrolysis was attempted after preliminary examination by paper chromatography and paper electrophoresis.

Paper Chromatography In the n-butanol-acetic acid-water solvent developed with the silver nitrate reagent, the following spots were identified:- $R_F$ 0.9 methyl N-(2,4-dinitrophenyl)-dihydrostreptobiosaminide, $R_F$ 0.25 N-methyl-L-glucosamine and two unknown spots $R_F$ 0.3 and 0.55 to 0.6.

Paper electrophoresis In the acetate buffer pH 5, the hydrolysis mixture exhibited a basic sugar N-methyl-L-glucosamine migrating towards the cathode and free 2,4-dinitrophenyl migrating towards the anode. Figure ( XV ) shows a typical paper after developing with aniline phthalate.




1 N-methyl-L glucosamine spot

2 2,4 Dinitrophenol

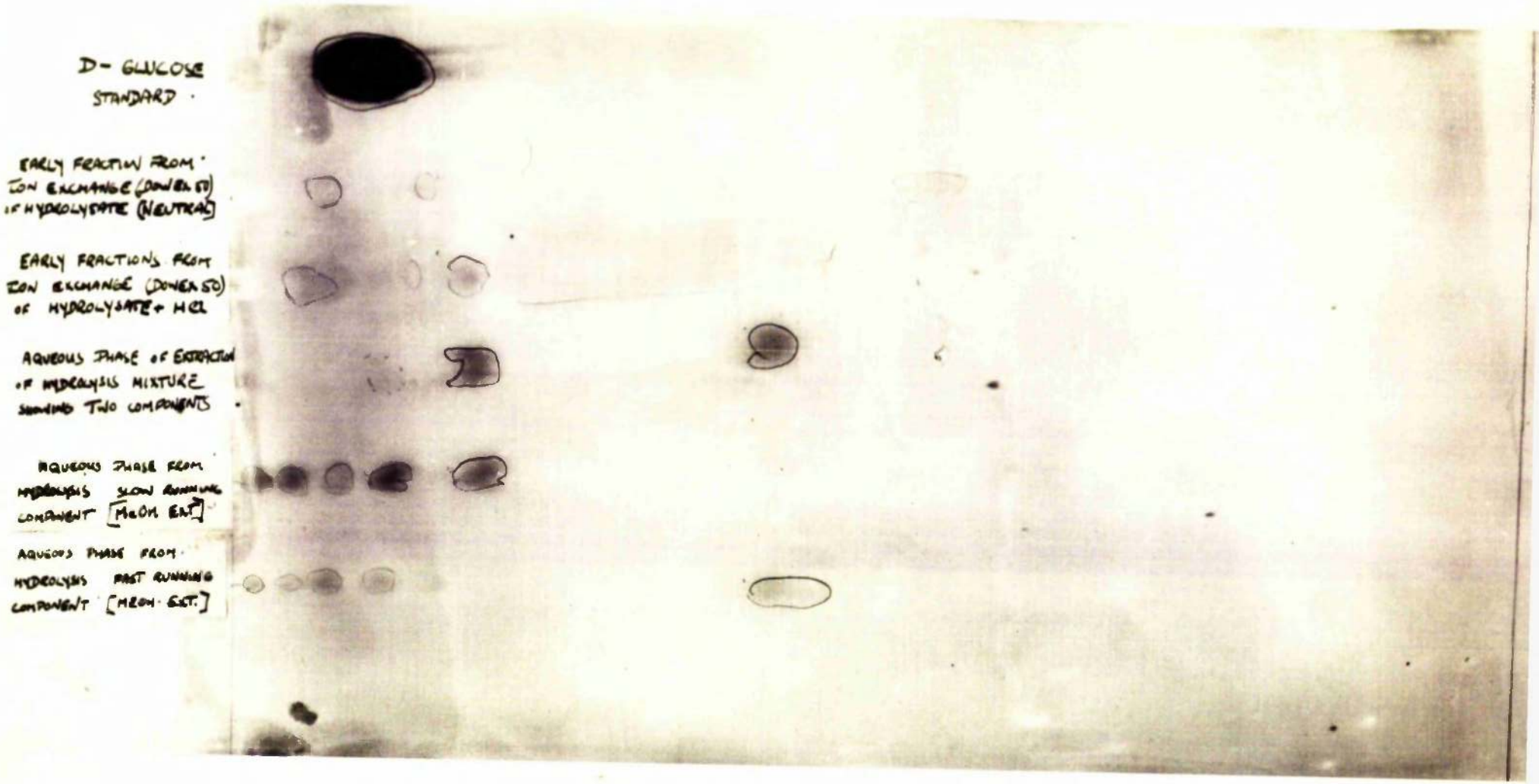
3 DNP-hydrolysate

Fig. (XV).

# PLATE I

## PAPERGRAM - Solvent BUTANOL - ACETIC ACID - WATER

## 16 hours. Developed with SILVER NITRATE REAGENT



Separation Part of the hydrolysate of methyl DNP-dihydrostreptobiosaminide (8ml) was extracted with chloroform (5 x 10ml) and back washed with water (2ml). The cumulated aqueous layers were shaken with a 10% chloroformic solution of di-n-octyl-methylamine (250ml.) which had been purified by repeated alternate extractions with hydrochloric acid and sodium hydroxide. When the clear aqueous layer (10ml) was neutral to B.D.H. universal indicator paper it was washed with chloroform (5ml) and examined by paper chromatography to show the two unknown spots $R_F$ 0.55 and 0.3.

An aliquot (4ml) of the above hydrolysate was streaked out on the base line of Whatman No. 1 sheet (46 x 57cm.) and the chromatogram run for eighteen hours with the butanol-acetic acid-water solvent. The paper was dried at 100°, small longtitudinal strips cut out from each side and the centre and these developed with the silver nitrate reagent to reveal 2 bands corresponding with the above spots $R_F$ 0.55 and 0.3. The areas on the large paper parallel with these bands were cut out and eluted separately with methanol (2 days). The eluates were evaporated to dryness under reduced pressure at 50° and re-examined by papergram. The fast moving spot $R_F$ 0.55 was seen with both solids, faint indeterminate spots $R_F$ 0.1 to 0.3 were also visible (silver nitrate reagent).

The above preparative paper chromatography was repeated, but the paper was first irrigated with distilled water and dried before loading with hydrolysate and the final elution of the strips was done with distilled water rather than methanol before concentration. In this case the paper chromatography showed that two spots were visible corresponding to two substances $R_F$ 0.55 and $R_F$ 0.3 and interference was reduced. After drying in a vacuum pistol ($P_2O_5$) two yellow-white solids (ca. 10mg.) were obtained.

Second Hydrolysis of the methyl DdP-dihydrostreptobiosaminide

Methyl-DdP-dihydrostreptobiosaminide (1g.) was heated for 2 hours in 2.5N hydrochloric acid in 50% aqueous methanol (20ml.) Fehlings test, paper chromatography and electrophoresis showed that hydrolysis had occurred as before, and the hydrolysate was extracted with chloroform, neutralised with a 10% chloroformic solution of di-n-octyl methylamine and the yellow aqueous phase examined by paper chromatography. The chromatograms showed the two spots at $R_F$ 0.3 and 0.6 together with spots corresponding with 2,4-dinitrophenyl (0.9) and/or glucosamine derivatives (0.15).

Aliquots of this hydrolysate (2.5ml.) were streaked out on large chromatography paper as before and the band corresponding with $R_F$ 0.3 and 0.6 eluted. The slower running band occurred too near glucosamine for proper separation. Two solids were obtained

as before - one gave $R_F$ 0.6 on chromatograms (33mg. ) the other $R_F$ 0.3 (11mg. ). The former was subjected to periodate oxidation gave the results described in appendix 1.

Column Chromatography

The solvents were purified as described on page 131 for paper chromatography. n-Butanol-acetic acid-water 4:1:5 organic phase was the solvent of choice.

A column 2 feet long, 1 inch bore with a sinter base was filled to within 1 inch of the top with solvent, a slurry of cellulose powder standard Whatman grade mixed with the above solvent was then poured in continuously while the solvent was removed slowly via the tap at the foot of the column. When the cellulose was at a height of 18 inches the tap was closed and the cellulose bed allowed to settle before placing a circle of filter paper on the top of the cellulose. The efficiency of the column was tested with 2% bromo thymol blue which showed that the column was evenly packed.

An aliquot of the hydrolysate (10ml), which had been neutralised was concentrated (ca. 1ml) under reduced pressure at 55°. This was made up with the chosen solvent to 5ml and added carefully to the top of the column to give an even band. The band was eluted with the butanol solvent and fractions (5ml) collected overnight using an automatic fraction collector. The fractions

were examined by paper chromatography using the accustomed solvent, when it was found that the slow moving substance $R_F$ 0.3 appeared in fractions 24 to 32. The faster moving substance did not separate well from the yellow 2,4-dinitrophenol, but it was present in fractions 13 to 18.

Fractions 24 to 32 were vacuum evaporated at 55° to give a yellow brown powder which darkened on heating. This represented 103.5mg crude material $[\alpha]_D^{20}$ -59°(c. 1%, water). The Lassaigne test gave a positive nitrogen (N-methyl-L-glucosamine $[\alpha]_D^{25}$ -51°).

Hydrolysis of Methyl N-acetyldihydrostreptobiosaminide

Methyl N-acetyldihydrostreptobiosaminide (0.53g.) was dissolved in 2 N hydrochloric acid (10ml) and heated for 24 hours at 50°. The specific rotation changed from -125° to -93°, and the Fehlings test was now positive. Paper chromatography with n-butanol-acetic acid-water, 4:1:5 as solvent, also indicated that hydrolysis had occurred a new spot ($R_F$ 0.4) appeared with periodate - permanganate reagent. Positive ninhydrin spots (violet) indicated that de-N-acetylation had also occurred.

The solution was neutralised with silver carbonate, filtered, and the residue washed with 20ml of distilled water. The filtrate and washings were saturated with hydrogen sulphide, centrifuged and the supernatant passed down an Amberlite IR 45 ($OH^-$) column washed with distilled water (1 litre). The eluate was evaporated to

dryness at 50° in a rotary film evaporator to yield a yellow sticky residue (0.35g.). Free amino sugar was removed by passage of the residue down a Dowex 50 ($H^+$) X x 8, 200-400 mesh cation exchange resin column (1 x 10cm.) irrigated with distilled water from which 20 ml. fractions were collected. The first fraction gave dihydrostreptose as a creamy yellow hygroscopic solid (0.127g., 57%) on evaporation to dryness at 50°C on the rotary film evaporator m.p. 135-140° $[\alpha]_D^{24}$ -70° (c. 1, water 24hrs.).

Found: C, 43.5; H, 6.95; $C_6H_{12}O_5$ requires

C, 43.9; H, 7.3%.

Chromatography (ascending) using n-butanol-ethanol-water 4:1:5 gave $R_F$ 0.4. A thin layer chromatogram run in dry methanol gave $R_F$ 0.7, which is equidistant with arabinose.

Dihydrostreptose acetate. Dihydrostreptose (0.1g.) was dissolved in pyridine (2ml) and acetic anhydride (5ml), the mixture left at room temperature for 4 days, and the solvents removed in vacuo at 50°. The product m.p. 173° (112mgm) was reprecipitated from dry ethanol and dry ether.

Found: C, 46.9; H, 6.5; acetyl, 28.7; $C_8H_{14}O_6$ requires

C, 46.6; H, 6.5; acetyl, 20.87%.

Dihydrostreptose Tosylhydrazone. Dihydrostreptose (20mg.) was condensed with toluene-p-sulphonylhydrazine (purified by crystallisation) in refluxing ethanol. The tosylhydrazone (7mg.) recrystallised from ethanol had m.p. 145° (Wang Yu[355] quotes 137°).

Cation-exchange hydrolysis of Methyldihydrostreptobiosaminide.

Methyl dihydrostreptobiosaminide hydrochloride (1g.) was dissolved in distilled water (3ml.) and Dowex 50 (X8, 200-400 mesh, ca. 20g.) ($H^+$ form) added as a thick slurry (ca. 15 ml. water). The mixture was heated for 4½ days at 50°, papergrams being run after 24 and 48 hours, when new reducing spots appeared. The resin was then placed in a column (1 x 20cm.), washed with distilled water (500ml.) and the eluate evaporated to dryness under reduced pressure at 50° to yield a residue (0.55g.) which gave a positive ninhydrin colour on papergrams.

Passage down a fresh Dowex 50 ($H^+$) column (1 x 8") gave a ninhydrin negative, electrophoretically neutral material as a dark brown syrup (0.33g.), most of which was soluble in methanol (20ml.). The solution was filtered and the filtrate evaporated to dryness under reduced pressure yielding a residue (0.27g.) which smelt of concentrated hydrochloric acid. This acidic residue was passed down a column (1 x 3") of Zeo Karb 225 ($OH^-$ form) anion exchange resin yielding, after concontration of the eluate (500ml.), a methanol soluble brown glossy syrup (0.05g.). Preparation of the tosylhydrazone from this residue yielded N-methyl-L-glucosamine tosylhydrazone (30mg.) of off-white crystals.

Found: N, 12.43; $C_{14}H_{23}O_6N_3$; requires

N, 12.7 %.

Paper chromatography. The above hydrolysis followed by paper chromatography using n-butanol-acetic acid-water indicated that some change occurred. Detection with periodate-permanganate reagent showed a faint new spot $R_F$ 0.4 and ninhydrin showed the presence of N-methyl-L-glucosamine ($R_F$ 0.15).

Methyl streptobiosaminide dimethyl acetal

Streptomycin sulphate (12g.) which had been dried for 2 hours over phosphorus pentoxide at 100° was dissolved in N methanolic hydrogen chloride (200ml.) and left for 48 hours at room temperature. Anhydrous ether (400ml.) was then added, the precipitated streptidine hydrochloride filtred, and the filtrate concentrated *in vacuo* to *ca.* 30ml. Further anhydrous ether (250ml.) was added to yield a yellow brown "oily" hygroscopic precipitate which on drying *in vacuo* (KOH, $CaCl_2$) gave crude methyl streptobiosaminide dimethyl acetal hydrochloride (5.3g., 74%) as a light brown hygroscopic solid. $[\alpha]_D^{20}$ -124° (Folkers[244] gives $[\alpha]_D$ -143°)

Found: N, 3.2 ; calculated for $C_{16}H_{38}ClNO_{10}$

N, 3.2%

Methyl streptobiosaminide dimethyl acetal hydrochloride (5g.) was passed through a column (1 x 10") of De-acidite FF($OH^-$) form eluted with 200mls. of distilled water after which the washings became acidic. The eluate was evaporated to dryness under reduced pressure at 50° to yield crude methyl streptobiosaminide dimethyl acetal (4.1g., 89%).

Methyl N-acetylstreptobiosaminide dimethyl acetal.

The crude base (4g.) was acetylated in dry pyridine (25ml.) with acetic anhydride (1.5ml.) for 7 days, the course of the N-acetylation being followed by ninhydrin spot tests (positive to negative). The solvents were removed *in vacuo* at 45° , the residue dissolved in methanol (20ml.) and the methanol insoluble material removed by filtration Anhydrous ether (200ml.) was added and the resultant precipitate collected redissolved in methanol (20ml.) the insoluble material filtered and the

filtrate added to anhydrous ether (200ml.). The precipitated methyl N-acetylstreptobiosaminide dimethyl acetal was triturated extensively with dry ether, and dried in vacuo over concentrated sulphuric acid to yield a light brown solid (3g., 75%).

Acid hydrolysis of methyl N-acetylstreptobiosaminide dimethyl acetal.

Methyl N-acetylstreptobiosaminide dimethyl acetal was dissolved in N hydrochloric acid (20ml.) and heated at 45° for 3 days. The acid was neutralised with silver carbonate, hydrogen sulphide passed into the solution for 30 minutes and the silver sulphide removed by filtration. The filtrate was evaporated to dryness yielding dark brown crystals (2.25g.) which were redissolved in distilled water (20mls.) and passed through a (1 x 7") column of Dowex 50 ($H^+$) form X8 200-400 mesh eluted with distilled water. The combined eluate was collected in fractions (10 x 50ml.) each one of which was concentrated and examined by paper and thin layer chromatography for evidence of separation. Fractions 3 to 7 exhibited ninhydrin positive spots $R_F$ 0.15 with n butanol-acetic acid-water. Fractions 1 and 2 exhibited long brown streaks in the same solvent. These latter fractions were again passed through a fresh Dowex 50 ($H^+$) column 1 x 7 collected in fractions (30ml.) which were concentrated and examined on papergrams. Fraction 1 from this new column on evaporation to dryness under reduced pressure gave a brown charred solid (0.1[illegible]g.) which exhibited a long indeterminate streak on papergrams giving a blue-violet colour with ninhydrin.

Attempt to prepare N-methyl-L-glucosamine penta-acetate.

The above residue (0.13g.) was acetylated by treatment in dry

pyridine (8ml.) with acetic anhydride (15ml.) for 4 days at room temperature. The solvents were removed in vacuo at 50° the residue dissolved in water and extracted with chloroform (5 x 10ml.). The chloroform solution was evaporated to dryness under reduced pressure, the residue triturated with ether which was decanted to leave a dark brown hygroscopic solid. Repeated treatment with dry ethanol followed by evaporation gave a similar product which could not be crystallised. Papergrams of this crude substance and authentic penta-acetyl-N-methyl-D-glucosamine showed the same $R_F$ value.

Attempted ninhydrin degradation of methyl dihydrostreptobiosaminide.

Methyl dihydrostreptobiosaminide (0.2g.) was dissolved in distilled water (100ml.) with ninhydrin (180mg.). Hydrochloric acid (0.5N)was added dropwise until the pH was 5 and the reactants boiled under reflux for half an hour. The dark brown solution was filtered, evaporated to dryness in vacuo and examined by paper chromatography. Extensive charring had occurred but no evidence of deamination was apparent, a spot for methyl dihydrostreptobiosaminide being detected on paper chromatography $R_F$ 0.2. (Periodate - permanganate reagent, n-butanol-acetic acid-water).

In a second experiment methyl dihydrostreptobiosaminide hydrochlorid (0.2g.) was dissolved with ninhydrin (0.15g.) in distilled water (5ml.) and heated in a boiling water bath for 3 hours. The dark brown solution was cooled, filtered and passed through a small Dowex 50 ($H^+$) form column (200-400 mesh X x 8, 1 x 12cm.) eluted with distilled water (50ml.). The eluate was extracted with chloroform (3 x 20ml.) and butanol(3 x 20ml evaporated to dryness under reduced pressure at 50° and the residue(0.14g

examined by paper chromatography.

The residue was acetylated with pyridine (5ml.) and acetic anhydride (5ml.) for a week at room temperature. The solvents were removed under reduced pressure at 50° , the residue dissolved in water neutralised with sodium carbonate and extracted with chloroform (20ml.). The chloroform extract was dried overnight ($CaCl_2$) and evaporated to dryness leaving a red brown residue which was redissolved in benzene (15ml.) and poured into 200ml. of petroleum ether to yield a red-brown precipitate (22mg. ) which possessed the same $R_F$ as methyl dihydrostreptobiosaminide in chromatograms.

Attempted enzymatic hydrolysis with N-acetyl-β-D-glucosaminidase.

Materials Samples of p-nitrophenyl-N-acetyl-β-D-glucosaminide, freeze-dried ram - testes - extract source and crystalline bovine-plasma albumin were used.

Buffers Citrate buffer (0.5M) pH 4.3 was prepared from stock solutions of sodium citrate and citric acid. In order to obtain the desired final pH in reaction mixtures, 0.05M with respect to citrate, allowance was made for the rise of approximately 0.2 pH unit which occurs on dilution.

Borate buffer (0.2M) pH 9.5 was made from boric acid and sodium hydroxide.

Estimation of p-nitrophenol released. This was measured spectrophotometrically on a Unicam SP,300 spectrophotometer $\lambda$ max. 400$\mu$. A standard absorption curve of p-nitrophenol was prepared for concentrations of 10, 20, 30 and 40 $\mu$g per ml. respectively.

p-nitrophenol Standard Curve

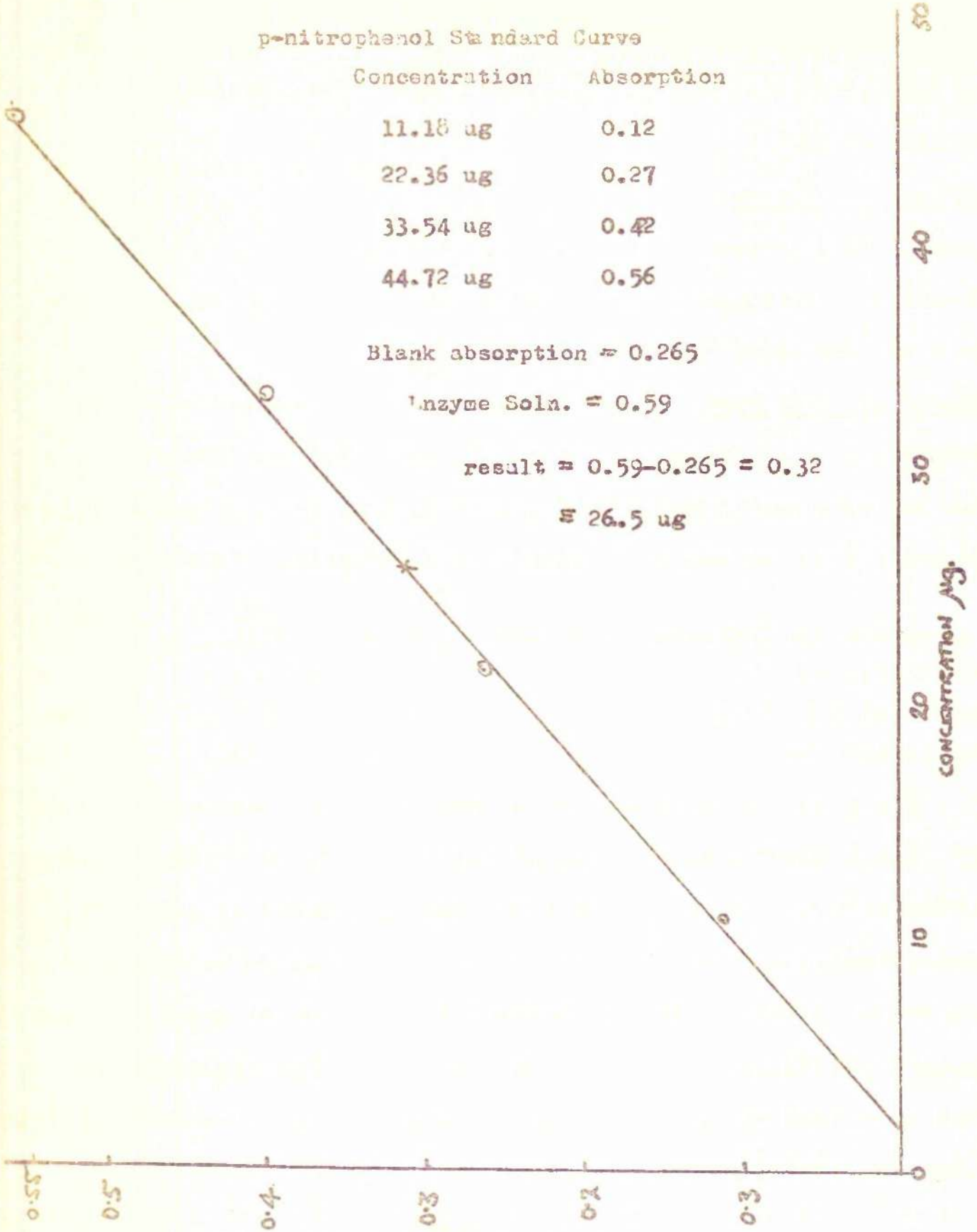
| Concentration | Absorption |
|---|---|
| 11.18 ug | 0.12 |
| 22.36 ug | 0.27 |
| 33.54 ug | 0.42 |
| 44.72 ug | 0.56 |

Blank absorption = 0.265

Enzyme Soln. = 0.59

result = 0.59–0.265 = 0.32

= 26.5 ug




Enzymatic Hydrolysis of p-nitrophenyl-N-acetyl

β -glucosaminidase Graph I

Enzyme solution. Ram-testes-extract (60mg.) was suspended/dissolved in distilled water (100ml.) containing crystalline bovine-plasma albumin (0.1g.).

Substrate Solutions. p-Nitrophenyl-β-N-acetylglucosaminide (12mg., 36mM.) was dissolved in distilled water (2ml.).

Methyl N-acetyldihydrostreptobiosaminide (80mg., 40mM.) was dissolved in distilled water (5ml.).

Enzyme Activity Test. Three tubes were set up, one test and two controls (one enzyme and one substrate) such that the total volume was 1ml. and concentrations 0.06% enzyme, 7.2mM substrate (p-nitrophenyl-N-acetyl-β-glucosaminide), and pH 4.5, 0.05M citrate buffer.

| | I | II | III |
|---|---|---|---|
| Enzyme 0.6% in 0.1% albumin solution | 0.1ml. | 0.1ml. | 0.0ml. |
| Substrate 36mM. | 0.2ml. | 0.0ml. | 0.2ml. |
| Buffer pH 4.3, 0.5M | 0.1ml. | 0.1ml. | 0.1ml. |
| Distilled water | 0.6ml. | 0.8ml. | 0.7ml. |

The tubes were incubated in a constant temperature water bath at 37° for 30 minutes and hydrolysis arrested by the addition of borate buffer pH 9.7. The amount of p-nitrophenol released is estimated absorptiometrically at 400 mμ corrected with reference to substrate and enzyme blanks. The results are summarised in the graph (Fig. XVI ).

Enzymatic Hydrolysis of Methyl N-acetyl dihydrostreptobiosaminide. This experiment was set up as with the p-nitrophenyl-N-acetylglucosaminid substrate, but larger volumes (5ml.) were used as follows:-

| | I | II | III |
|---|---|---|---|
| Enzyme 0.6% suspended in 0.1% albumin solution | 0.5ml. | | 0.5ml. |
| substrate 40mg. | 0.5ml. | 0.5ml. | |
| buffer citrate 0.5M.pH 4.3 | 0.5ml. | 0.5ml. | 0.5ml. |
| distilled water | 3.5ml. | 4.0ml. | 4.0ml. |

The solutions were incubated at 37° for 2 hours, centrifuged to remove insoluble (enzyme) matter and papergrams run. The polarimetric readings did not differ between commencement and conclusion of hydrolysis. For this concentration of enzyme and substrate the chromatograms exhibited no evidence of hydrolysis (periodate-permanganate).

A repeat experiment doubling the concentration of substrate and enzyme used also showed no signs of hydrolysis.

## GLYCOSIDES OF DIHYDROSTREPTOBIOSAMINE.

Benzyl dihydrostreptobiosaminide hydrochloride. Methyl dihydrostreptobiosaminide (0.5g.) was scrupulously dried (60° *in vacuo* over $P_2O_5$), dissolved in 2.8N benzyl alcoholic hydrogen chloride (25ml.) and left for 48 hours at room temperature. The solvents were concentrated to about 10ml. under high vacuum at 55° and left over 48 hours when creamy crystals were deposited. The crystals (80mg.) were washed with dry petroleum ether (b.p. 80/100°), (3 x 10ml.) and acetone and petroleum ether were added to give white micro crystals (22mg.) m.p. 220°. The ultraviolet spectrum showed absorption at 255mμ characteristic of the aromatic ring. Confirmatory weak peaks at 1490, 770, 740 and 680cm$^{-1}$ were obtained in the infrared.

The mother liquors were concentrated to dryness in high *vacuo* to leave a brown syrupy residue, which was dried in a high vacuum desiccator for 3 days ($CaCl_2$, KOH), triturated with ether to yield an off-white solid (224mg.). Reprecipitation from ethanol, with acetone and dry ether gave benzyl dihydrostreptobiosaminide hydrochloride as a white hygroscopic solid (119mg.) m.p. 210°, $[\alpha]_D^{20}$ −110°

Found: N, 2.6; $C_{20}H_{32}ClNO_9$ requires

N, 3.0%.

The above experiment was repeated with methyl dihydrostreptobiosaminide hydrochloride (1g.) except that after 48 hours the reaction mixture was flooded with dry ether (*ca.* 250ml.) when a hygroscopic precipitate was obtained. Recrystallisation from ethanol with acetone and dry ether followed by desiccation ($CaCl_2$KOH) gave white crystals (0.67g.) of benzyl dihydrostreptobiosaminide hydrochloride m.p. 210°

Found: C, 49.2; H, 6.5; N, 3.0; $C_{20}H_{32}ClNO_9$ requires

C, 51.5; H, 6.7; N, 3.0.

2-Bromoethyl dihydrostreptobiosaminide hydrochloride.

2-Bromoethanol was redistilled and gaseous HCl passed in to give a 2.8N solution. Carefully dried methyl dihydrostreptobiosaminide hydrochloride (0.5g.) was dissolved in 2N, 2-bromoethanolic hydrogen chloride (25ml.) and left for 48 hours at room temperature. The solvent was removed in a high vacuum at 60° to leave a violet syrup, which was redissolved in dry ethanol (15ml.) and separated from any insoluble material. The ethanolic solution was evaporated to dryness at 50° and the syrupy violet residue redissolved in the minimum of dry methanol. Dry ether (25ml.) was added, yielding a pale violet precipitate which on filtering and drying in vacuo ($CaCl_2KOH$) gave 2-bromoethyl dihydrostreptobiosaminide hydrochloride as a violet powder (0.42g.) m.p. 125-130° $[\alpha]_D^{20}$ -100°(±2°).

Found: C, 35.3; H, 6.0; N, 3.1; $C_{15}H_{29}BrClNO_9$ requires

C, 37.3; H, 5.8; N, 2.9%.

Phenyl dihydrostreptobiosaminide Pure phenol (50g.) was melted in a hot water bath at 60° and kept molten whilst dry gaseous hydrogen chloride was bubbled in for an hour. The weight gain was noted and phenol added to give a normal solution with respect to hydrogen chloride.

Carefully dried methyl dihydrostreptobiosaminide (1g.) was added to the phenolic hydrogen chloride (30ml.) and left for 48 hours at 55°. Dry acetone (200ml.) was added to give a white hygroscopic precipitate, which was filtered, fractionated with dry ether extensively to remove traces of phenol, and dried in vacuo (KOH, $CaCl_2$) 24 hours. The crude

product was dissolved in the minimum of dry ethanol (ca. 5ml.), and precipitated with dry ether (50ml.), affording phenyl dihydrostrepto biosaminide hydrochloride as a hygroscopic yellowish white pwwder (1.013 85%) m.p. 115° $[\alpha]_D^{20}$ -131°(c, 1% water). The infrared spectrum showed aromatic peaks at 690, 760, 830, and 1500 $cm^{-1}$. The ultraviolet spectrum also showed benzenoid absorption at 260 mμ.

Found: C, 51.1; H, 6.7; N, 2.9; $C_{19}H_{30}ClNO_9$ requires

C, 50.5; H, 6.5; N, 3.1%.

Cyclohexyl glycoside. Redistilled cyclohexanol (50ml.) was treated with hydrogen chloride as before and diluted to give a normal solution of hydrogen chloride.

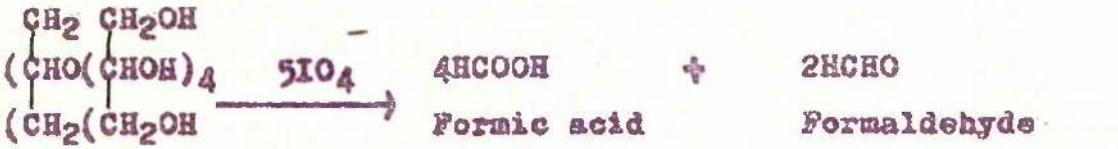
Methyl dihydrostreptobiosaminide hydrochloride (0.5g.) was treated with the N hydrogen chloride in cyclohexanol (25ml.) for 48 hours at 50°. Dry ether (100ml.) was added to yield a white hygroscopic precipitate. Reprecipitation from ethanol (5ml.) by dry ether followed by washing with ether gave crude cyclohexyl dihydrostreptobiosaminide hydrochloride as a hygroscopic solid (0.21g., 42%), m.p. 110-120°.

# APPENDIX I.

## Periodate oxidation of carbohydrates

For this most important degradation a number of methods[437] are available and after some weeks of preparative analysis, a micro determination was worked out combining two of these. Points to watch when interpreting results were - non-specific or over oxidation reactions which occur if conditions have not been carefully controlled. Oxidation may also be hindered by steric effects and formate esters may be formed.

Mannitol which is very readily oxidised was used as standard for degradation.




Method 1:[355] Oxidations were carried out in the dark in glass stoppered flasks using an approximately 10 fold molar excess of sodium metaperiodate (0.01 to 0.04 M). Reactants (10 to 30 mgm.) were dissolved in water and the appropriate volume of 0.04 M periodate solution added. A blank solution was prepared at the same time.

Consumption of oxidant: At suitable time intervals, e.g. 3, 6, 12, 24 and 48 hours, aliquots were withdrawn from each solution and sodium bicarbonate (0.29g.) added. Excess sodium arsenite (25 ml. 0.02 M) was then immediately added, followed by potassium iodide (ca. 0.29g.) Under these conditions, unreduced sodium metaperiodate was reduced by arsenite and the excess arsenite was then titrated after standing for 10 minutes with standard iodine solution (ca. 0.02M) using "Thyodene" indicator.

$$H_2AsO_3^- + IO_4^- \longrightarrow H_2AsO_4^- + IO_3^-$$

$$H_2AsO_3^- + I_2 + H_2O \longrightarrow H_2AsO_4^- + 2I^- + 2H^+$$

$$H^+ + HCO_3^- \longrightarrow H_2O + CO_2$$

The difference between blank and the oxidising solution titre is proportional to the oxidant consumed.

$$I \equiv \frac{As_2O_3}{4} \equiv \frac{IO_4^-}{2}$$

1ml. 0.01 M iodine $\equiv \frac{0.01}{1000}$ moles iodine

$$\equiv \frac{0.01}{1000} \times \frac{1}{2} \text{ moles } IO_4^-$$

$$\equiv 0.005\text{m. moles } IO_4^-$$

Therefore if sample weight is m mg., molecular weight of reductant M, and titre difference X ml. of 0.01 iodine solution, the uptake of oxidant in mole/mole is given by $\frac{0.05 \times X \times M}{m}$

As solutions used were 0.02 M, this gives the expression

$$\frac{2 \times 0.05 \times X \times m}{M}$$

Experimental: In the initial experiments mannitol and glucose were oxidised. Mannitol was more rapidly oxidised than glucose unless a bicarbonate buffered periodate solution was used (q.v. chromotropic acid HCHO estimation). A 10 mg. scale was used initially.

Periodate solution: A 0.04 M solution of sodium metaperiodate was prepared (2.139 in 250 ml. distilled water.)

Arsenite solution: An 0.02 M solution was used; 1 g. of arsenic

trioxide was dissolved in N sodium hydroxide solution (warmed, if necessary) and N hydrochloric acid added to pH 7, and diluted to 1 litre with distilled water.

Iodine solution: 2.6 g. of iodine with 8 g. of potassium iodide in 1 litre of distilled water gave an 0.02 N solution.

Method: For a ten-fold excess, mannitol (10 mg.) was dissolved in 0.04 M periodate (12.84 ml.) with distilled water (2.16 ml.). This solution and a blank solution containing no mannitol were placed in a 25 ml. graduated flask (covered in black paper with ground glass stoppers). All solutions were kept away from direct light. Aliquots (1.5 ml.) (i.e. $\frac{1}{10}$) were taken and sodium bicarbonate (ca. 0.2 g.) added. Excess 0.02 N sodium arsenate (25 ml.) was immediately added followed by potassium iodide (ca. 0.2 g.) and after 10 min., titrated with iodine. From the difference between the blank and test titres the number of moles of oxidant per mole of sugar used was found, e.g. for mannitol after 1 hour

Blank $I_2$ = Arsenate 25 ml. ≡ 21.9 ml. 0.02N iodine

Test ≡ 23.1 ml. iodine

i.e. Amount of periodate used in oxidation of mannitol (standard)

= 23.1 - 21.9 = 1.2 ml. of 0.02N arsenite solution

No. of moles oxidant per mole sugar (mol. wt. - 182.17)

$$= \frac{0.1 \times 1.2 \times 182.17}{10}$$

= 5.308 mole/mole (high)

This method was rather wasteful if an unknown sugar was being used (10-30 mg.) so a smaller scale (2-5 mg.) was tried in combination

with the chromatropic acid estimation of the formaldehyde produced, a description of which follows.

Micro determination of formaldehyde liberated during oxidation of carbohydrate with periodate using chromatropic acid

Eegrwe (1937)[438] first noted that formaldehyde reacted with chromatropic acid (1,8-dihydroxynaphthalene-3,6-disulphonic acid) on heating in strong $H_2SO$ (60% w/v) to give a strong, apparently specific colour.

Originally the formaldehyde was distilled off but this was inaccurate in small concentrations and an in situ method was developed.[438a] As chromatropic acid is itself rapidly oxidised with periodate, complete removal of periodate along with any inorganic reaction products is essential.

This is best done by preparing insoluble salts and, as the optimum pH for formaldehyde formation is 7.5–8 this limits the precipitation choice. Best results were with lead salts, but even here the choice was limited as some of these interfere with the colour test. Lead dithionate was especially suitable, principally because of its high solubility and also since the dithionate ion decomposes in acid into sulphate and sulphur dioxide. The lead sulphate is conveniently removed by centrifuging and sulphur dioxide does not interfere with the reaction and, in fact, stabilises the chromatropic acid against oxidation by air and light

$$PbS_2O_6 \xrightarrow{H^+} SO_2 + PbSO_4$$

Reagents

(a) Chromotropic acid reagent: Chromotropic acid (1 g.) was dissolved in hot water (100 ml.) and the solution filtered through glass wool to remove any insoluble sulphones; stannous chloride (0.1 g.) was then added (stabilising agent) and the turbid solution obtained diluted to 500 ml. with sulphuric acid 66% w/v. All operations were carried out in the dark and the solution stored in a stoppered brown glass bottle can be used up to about 10 days.

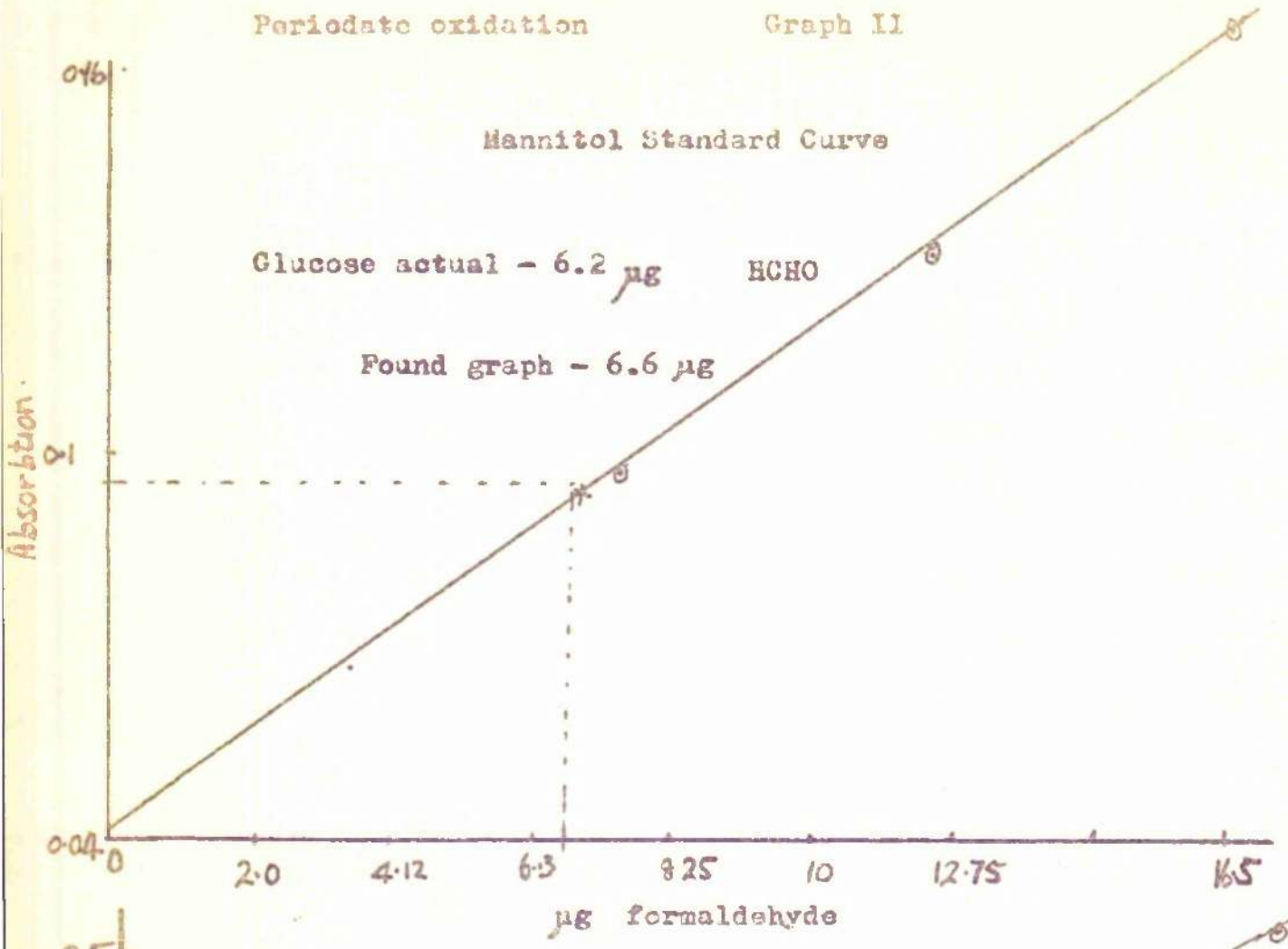
Periodate solution: For mannitol a buffered solution is unnecessary as the oxidation is rapid at most pH values, but for glucose a slow reaction requiring optimum pH, a buffer is necessary.

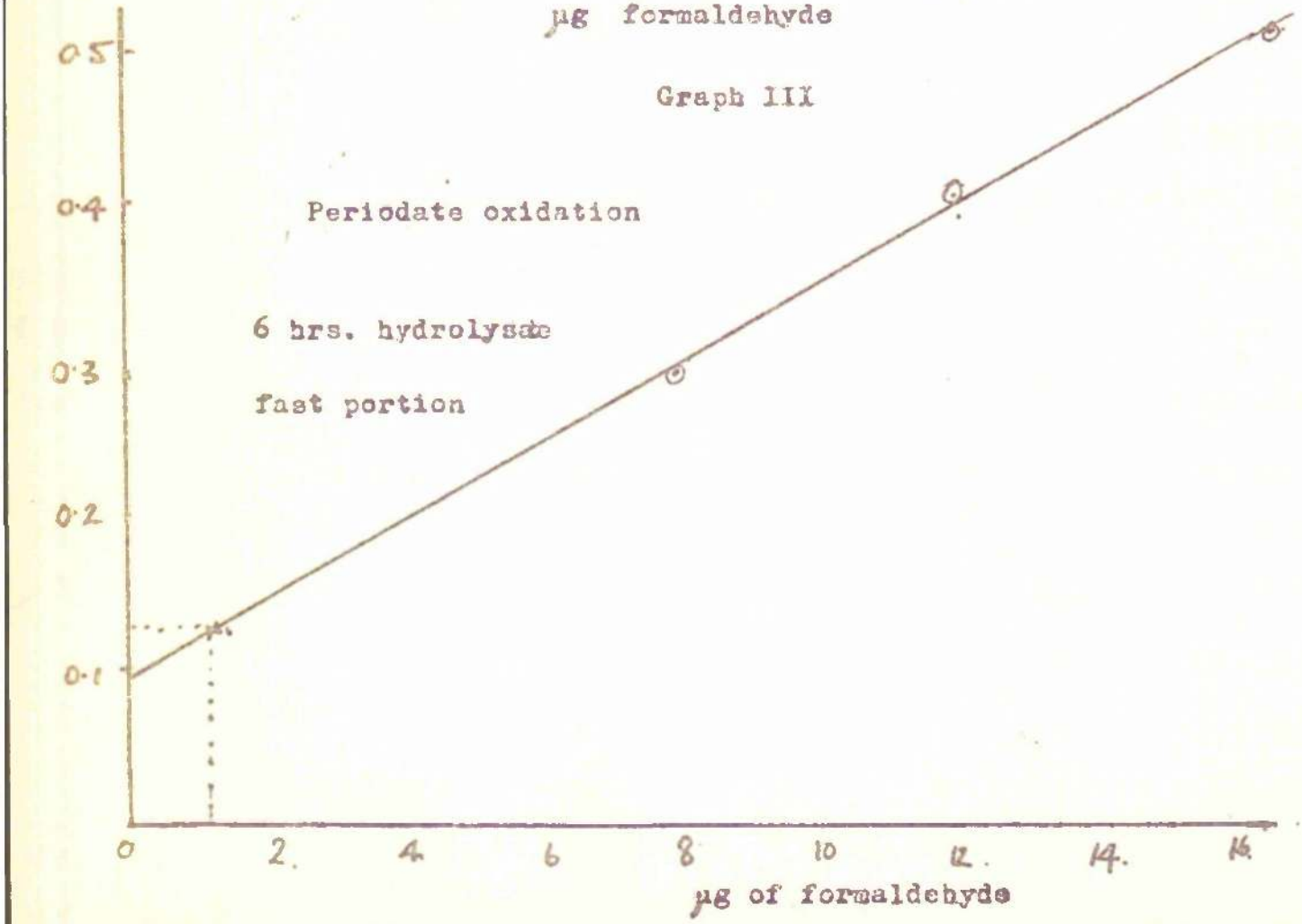
Sodium metaperiodate (0.015 M) in 0.045 N sulphuric acid was diluted before use with an equal volume of N sodium bicarbonate. This reagent is suitable for simple sugars but for sugars of higher molecular weight, a stronger solution should be used.

Lead dithionate: An equal molar quantity of sulphuric acid was added to a solution of barium dithionate ($BaS_2O_6$ 20% w/v), the barium sulphate was removed by centrifugation and a slight excess of lead carbonate was added to the acid solution. After the reaction was complete the excess lead carbonate was removed by centrifugation, and the supernatant solution added to the ethanol (2-3 vols.). After standing at $0^\circ$ overnight, the crystals ($PbS_2O_6$ 4 $H_2O$) obtained were washed with ethanol, and dried over calcium chloride. The reagent was used as a 20% w/v solution in water. Solutions frozen at $-10^\circ$ are stable over long periods.

Method: The example for mannitol standard is given. One volume (5 ml.) of a freshly prepared periodate-bicarbonate solution (sodium metaperiodate 0.015M in 0.045N sulphuric acid diluted with an equal volume of sodium

Periodate oxidation Graph II



bicarbonate solution) was added to an equal volume (5 ml.) of an 0.01% aqueous solution of mannitol. The oxidation was allowed to proceed in the dark at room temperature in 10 ml. ground glass stoppered graduated flasks, and at suitable time intervals, e.g. 0.5, 1 and 2 hours (with mannitol oxidation is complete in 30 minutes) aliquots were withdrawn and pipetted into 1 ml. of lead dithionate reagent 10% w/v contained in a 2 ml. centrifuge tube. After mixing and centrifuging, a 1 ml. aliquot of supernatant was removed, care being taken to avoid inclusion of any precipitate, placed in a 10 ml. centrifuge tube, and the chromotropic acid reagent (9 ml.) added. (This and subsequent operations were performed away from direct light.) After standing 30 minutes, the precipitated lead sulphate was centrifuged off and the supernatant solution transferred to a test tube and heated in a boiling water bath for at least 30 minutes when the colour developed. The absorption was read at 570 mμ using 1 cm. cells in a Unicam SP. 500 spectrophotometer.

The procedure was simultaneously used for 0.005% and 0.0025% mannitolsolutions using 0.005% glucose as a test unknown. Thus a standard curve of absorption against formaldehyde produced was shown using the fact that mannitol is oxidised to give 2 moles formaldehyde per mole of sugar. See graph I.

This was repeated several times. The optimum time of heating for colour development was 1 hour.

<u>Combination of the two methods for Periodate.</u>

This entailed the scaling down of the oxidant consumption titration to the 2.5-5 mg. scale. The weight of mannitol oxidised to give a

standard curve in the chromotropic acid formaldehyde determination were 5 mg., 3.75 mg. and 2.5 mg.

Procedure: A 0.5% mannitol solution was used

For 5 mg. take 7 ml. 0.04M periodate + 1 ml. mannitol 0.5% + 2 ml. water;

3.75 mg. take 7 ml. periodate + 0.75 ml. mannitol 0.5% + 2.25 ml. water

2.5 mg. take 7 ml. periodate + 0.5 ml. mannitol 0.5% + 2.5 ml. water.

Blank take 7 ml. periodate (0.04M) with 3 ml. water.

These were mixed in 10 ml. graduated flasks, fitted with ground glass stoppers. After ½, 1, 6 and 12 hours, 1 ml. aliquots were taken, N sodium bicarbonate (0.5 g.) added and 0.02N sodium arsenate (5 ml.) followed by 0.5 g. potassium iodide. After 10 minutes the excess arsenite was titrated with iodine using "thyodene" indicator.

For the formaldehyde estimation, 1 ml. aliquots of the oxidising solution were added to 10 ml. of distilled water and 1 ml. of the diluted solution was pipetted into lead dithionate and the procedure adopted as described before in the chromotropic acid estimation.

For the fast running unknown from the hydrolysate of methyl N=2,4-dinitrophenyldihydrostreptobiosaminide ($R_F$ 0.55) a 0.01% solution was prepared and the 3.75 mg. scale used, i.e. Aqueous solution (3.75 ml.) with 0.04M solution of sodium metaperiodate (17 ml.)

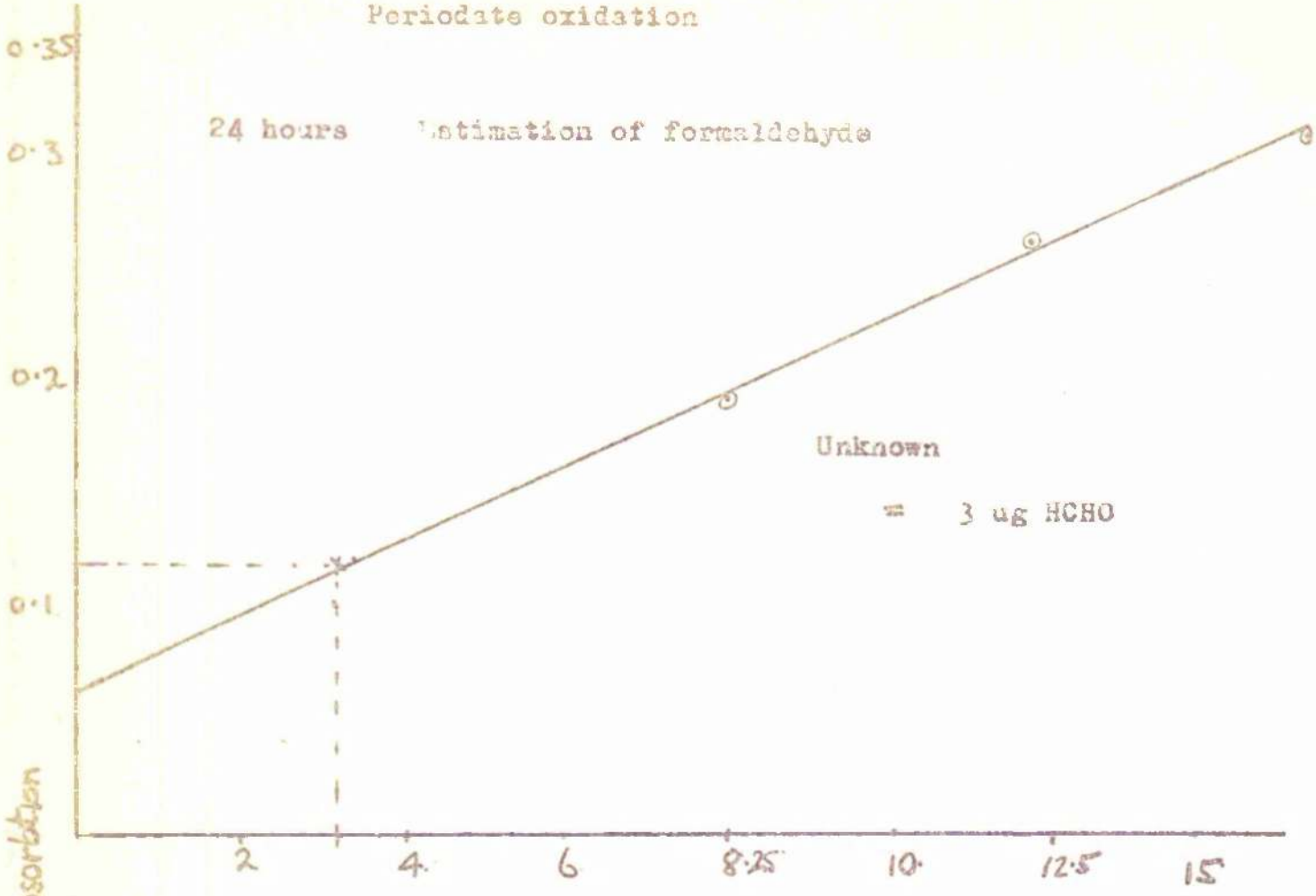
Results - after 6 hours oxidation

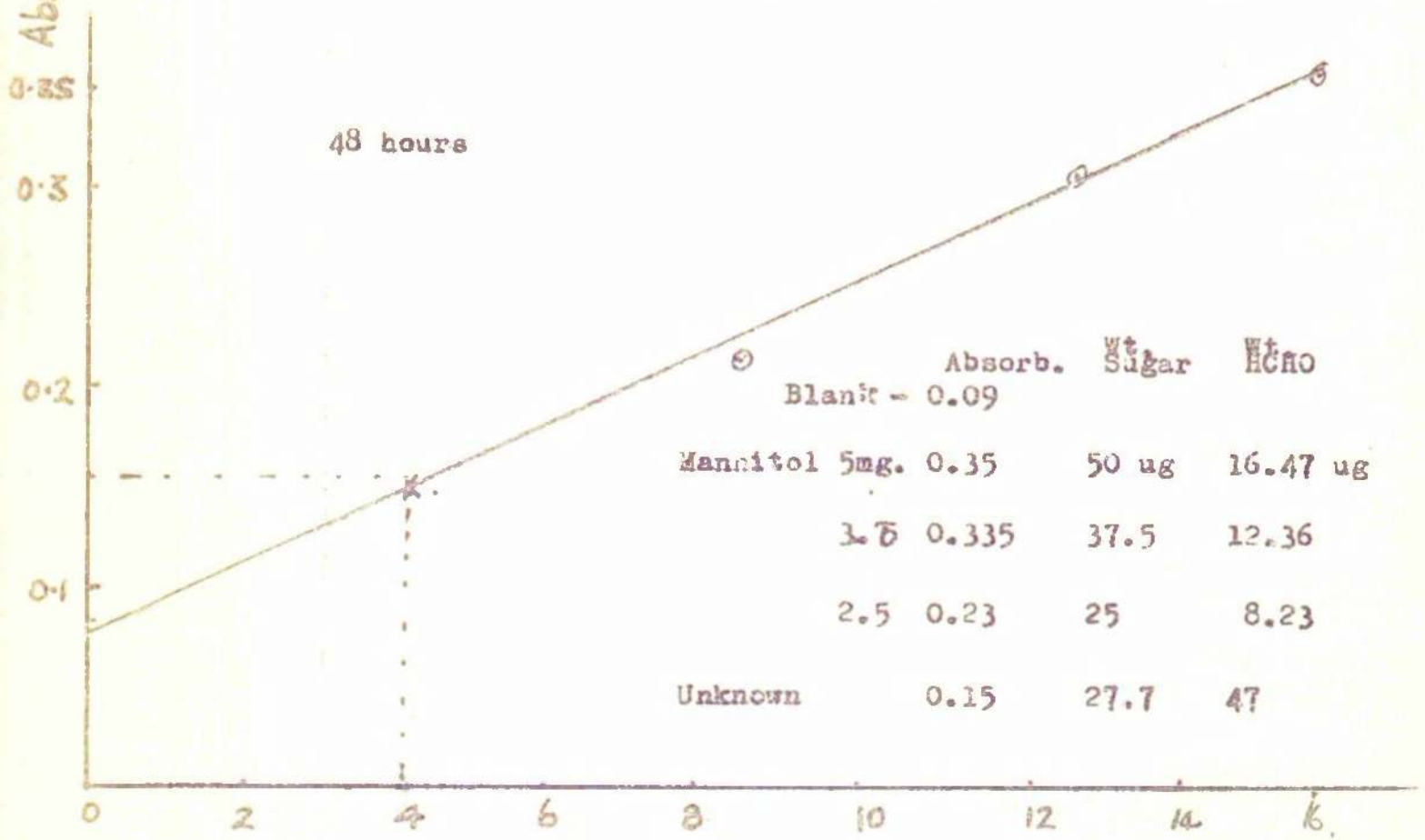
Titration for periodate

I ≡ As Mannitol standard Blank = 2.53 ml.

Periodate oxidation

24 hours — Estimation of formaldehyde




| | Absorb. | Wt Sugar | Wt HCHO |
|---|---|---|---|
| Blank | 0.09 | | |
| Mannitol 5mg. | 0.35 | 50 ug | 16.47 ug |
| 3.75 | 0.335 | 37.5 | 12.36 |
| 2.5 | 0.23 | 25 | 8.23 |
| Unknown | 0.15 | 27.7 | 47 |

Titre

5mg. = 3.86 ml. i.e. Difference = 3.86 - 2.53 = 1.33 ml.

3.75 mg. = 3.56 ml. " " = 3.56 - 2.53 = 1.03 ml.

2.5 mg. = 3.24 ml. " " = 3.24 - 2.53 = 0.71 ml.

Since strength of arsenite = 0.02%; molecular weight of mannitol = 182.17

5 mg. No. of moles $IO_4$ used/mole sugar = $\frac{0.1 \times 1.33 \times 182.17}{5}$ = 4.95

3.75 mg. = $\frac{0.1 \times 1.03 \times 182.17}{3.75}$ = 5.01

2.5 mg. = $\frac{0.1 \times 0.71 \times 182.17}{2.5}$ = 5.174

Unknown

2.77 mg. Titre $I_2$ ≡ As = 2.64

Difference in titre = 2.66 - 2.53 = 0.13 ml.

molecular weight of unknown ~ 160

No. of moles $IO_4$ per mole sugar = $\frac{0.1 \times 0.13 \times 160}{2.77}$ = 0.7544

= 0.8 mole/mole.

Formaldehyde from graph

0.0277 mg. unknown 1.3 mg. formaldehyde

No. of moles/mole sugar = $\frac{1.3}{32} \times \frac{160}{27.7}$ = 0.469 mole/mole

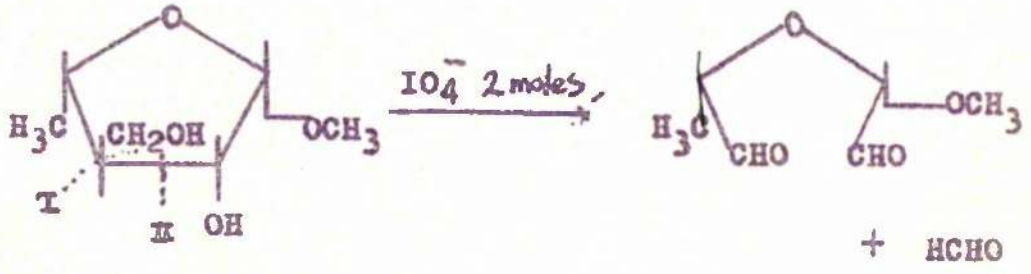
Tabulated results for unknown

| Time | moles oxidant/mole sugar | formaldehyde moles/mole |
|---|---|---|
| 6 hours | 0.75 | 0.46 |
| 24 hours | 1.328 | 0.54 |
| 48 hours | 1.560 | 0.66 |

Conclusions

These figures were only approximate as the unknown material was not crystalline but was contaminated with hemicelluloses from filter paper.

Apparently for every 2 moles of periodate used one mole of formaldehyde is produced which is consistent with a methyl glycoside rather than the free sugar which would be 3 moles of oxidant, as seen in figure XVII.




methyl dihydro

streptose

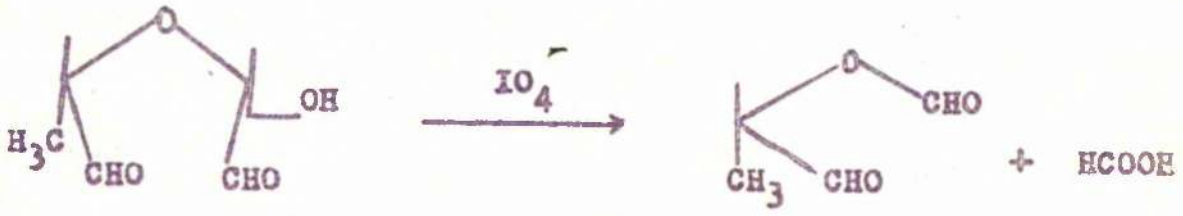



Figure XVII.

## APPENDIX II.

The application of proton magnetic resonance spectra (hereafter termed n.m.r.) to the study of configuration and conformation in carbohydrates was first reported by Lemieux and his associates.[439] Pyranose sugars were first studied when confirmatory chemical evidence allowed certain areas of the spectrum to be assigned to specific protons in similar environments around the pyran ring. Slight changes in the chemical shift signal ($\tau$ values) indicated change from equatorial (lower) to axial (higher) orientation. The fine structure of the hydrogen interactions, or spin-spin coupling constants (J values) could be used to confirm theoretical predictions of the proton orientation.

These workers[440] and Jardetsky[412] later extended this work to pentafuranoses, thus enabling conformations to be assigned to these structures. In certain cases, anomeric configurations which had been assigned on the basis of molecular rotation were revised.

These observations may be summarised as follows:-[439]

a) A shift of 5-10 c.p.s. between the signal for the methyl hydrogens of equatorial and axial acetoxy groups was observed.

b) The anomeric C-1 hydrogens of acetylated aldopyranoses produce signals which are separated by 10 to 26 c.p.s. when these are axial in one of the anomeric forms and equatorial in the other. The anomeric hydrogen is a unique type of hydrogen in the molecule and is responsible for the signal at the lowest field (3-4$\tau$) - attributed to the fact that it is the only hydrogen attached to a carbon which is bonded to two oxygen atoms and is consequently less shielded. This postulate is supported by the doublet signal often seen for

this hydrogen which is only strongly coupled with one adjacent hydrogen thus affording an A,B system. In some cases the doublet is not resolved.

c) The signals from the 5-6 τ region arise from the methylene hydrogens of the acetylated addopyranose ring. The sharpness and intensity of the signals at ca τ-8 definitely relate to the 12 protons of the four acetoxy groups in the pentose tetra-acetates (15 protons in the hexoses).

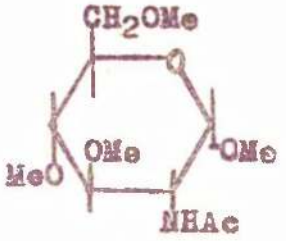
d) The magnitude of the coupling constants derived from measuring the spacing is consistent with the generalisation that spin-spin coupling is 2-3 times greater when neighbouring hydrogens are in axial-axial positions.

The data presented indicates that a change in the configuration at the anomeric centre produces a chemical shift towards a lower field when the hydrogen passes from an axial to an equatorial orientation. This shift varies greatly from one pair of anomers to another.

Rinehart[202] and his colleagues, as reported in p.37 assigned the glycosidic linkages to neomycins by means of n.m.r. spectroscopic study of various derivatives. The spectra of hexa-N-acetyl neomycin B and C show the anomeric proton as a singlet (J = 1 c.p.s.) at -0.5 p.p.m. relative to solvent H.O.D. This negligible coupling is only possible between trans (β) protons at C-1 and C-2 as cis (α) in five membered rings should have J = 6c.p.s. and have been observed with some other ribosides as 4 c.p.s.[440]

Barker and his colleagues[441] working with the aminoglycosides from the antibiotic novobiocin has collated data concerning methyl

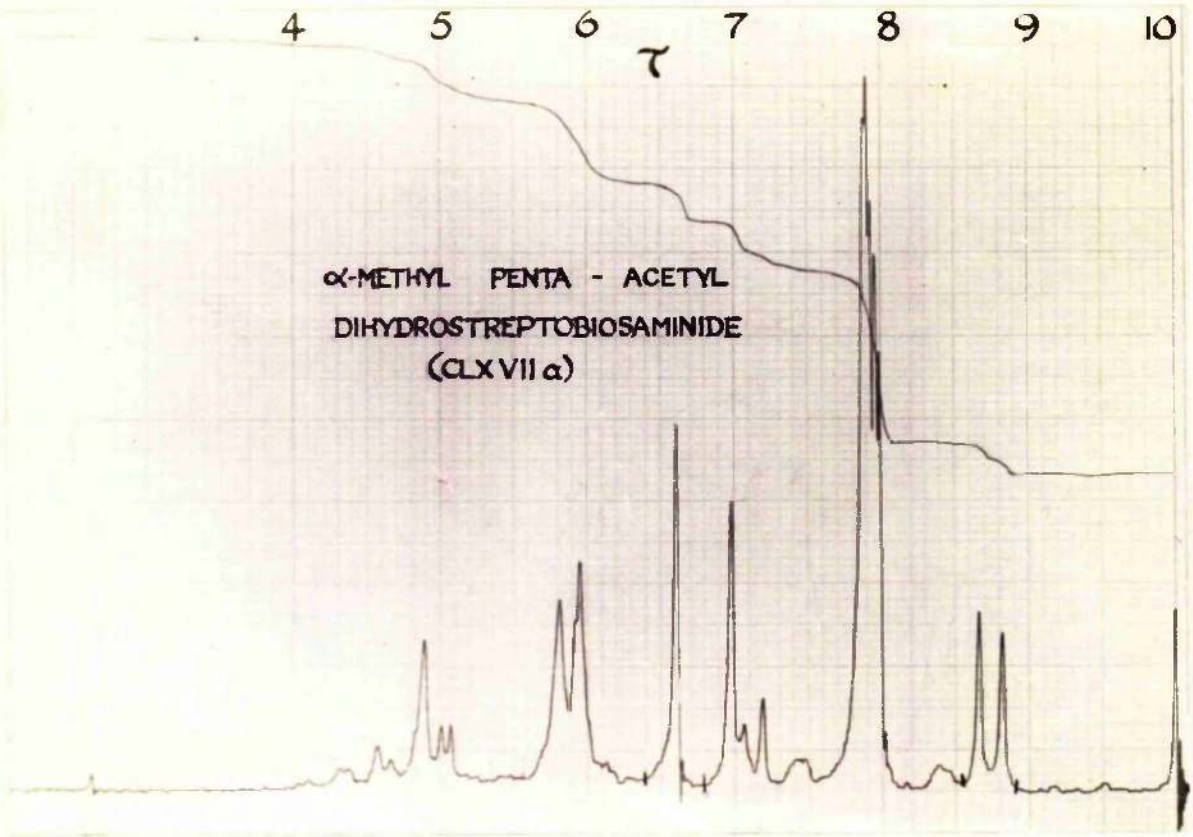
glycopyranosides 1-methoxy and acetoxy protons. The protons of the 1-methoxy group of some methyl pyranosides fall into two groups around $\tau$ = 6.69 and $\tau$ = 6.85. If the probable C 1 conformation of the hexose derivatives is assumed, these values correspond respectively to the equatorial and the axial orientations of the group. Acetylation of the methyl pyranosides gives a uniform signal of 6.7 which is attributed to the spatial arrangements of the acetoxy groups. A comparison of the spectra of the free glycopyranoside and its acetyl derivative therefore provides a means of determining the orientation of the methyl glycoside group, by observing if the methoxy signal is unchanged - (equatorial ) or altered by about 0.16 to a lower field (axial). For the methyl 2-acetamido -2-deoxy-3,4,6-tri-O-methyl- $\alpha$ - D-glucoside (CLXVI) the 1-O-methyl sigal gives $\tau$ = 6.84 which is consistant with an axial position.

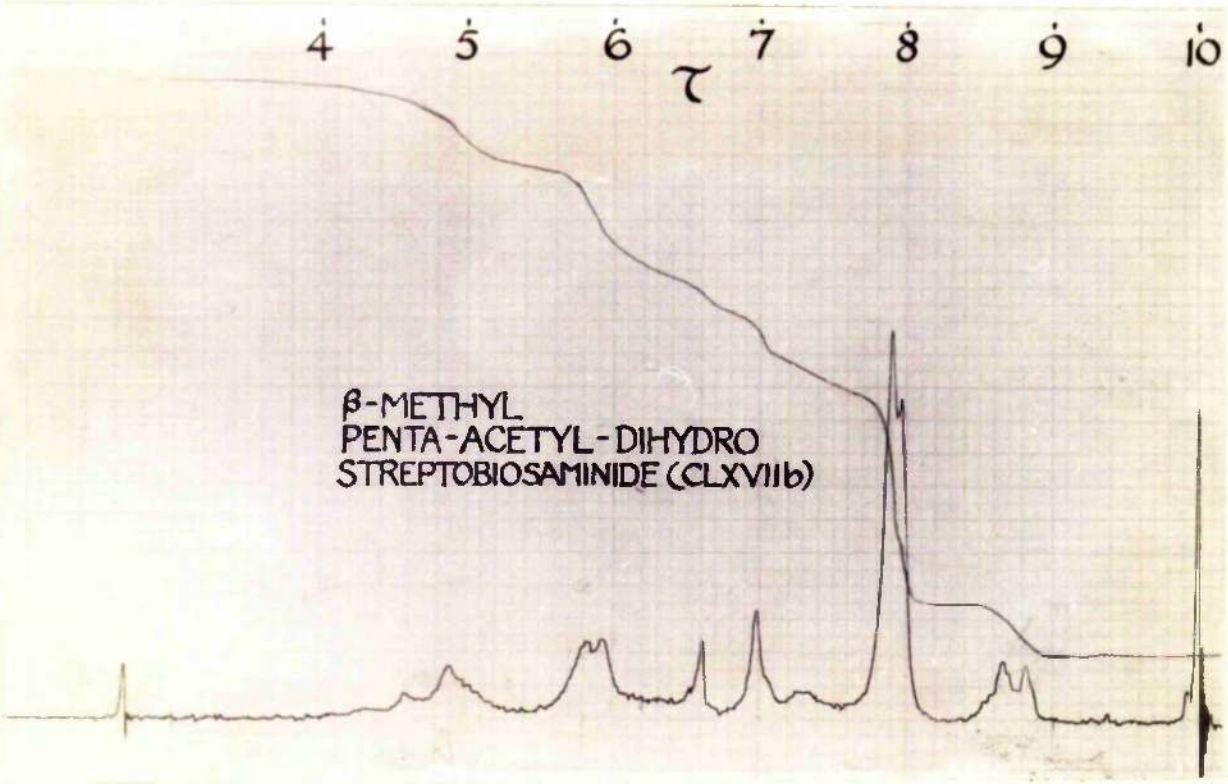


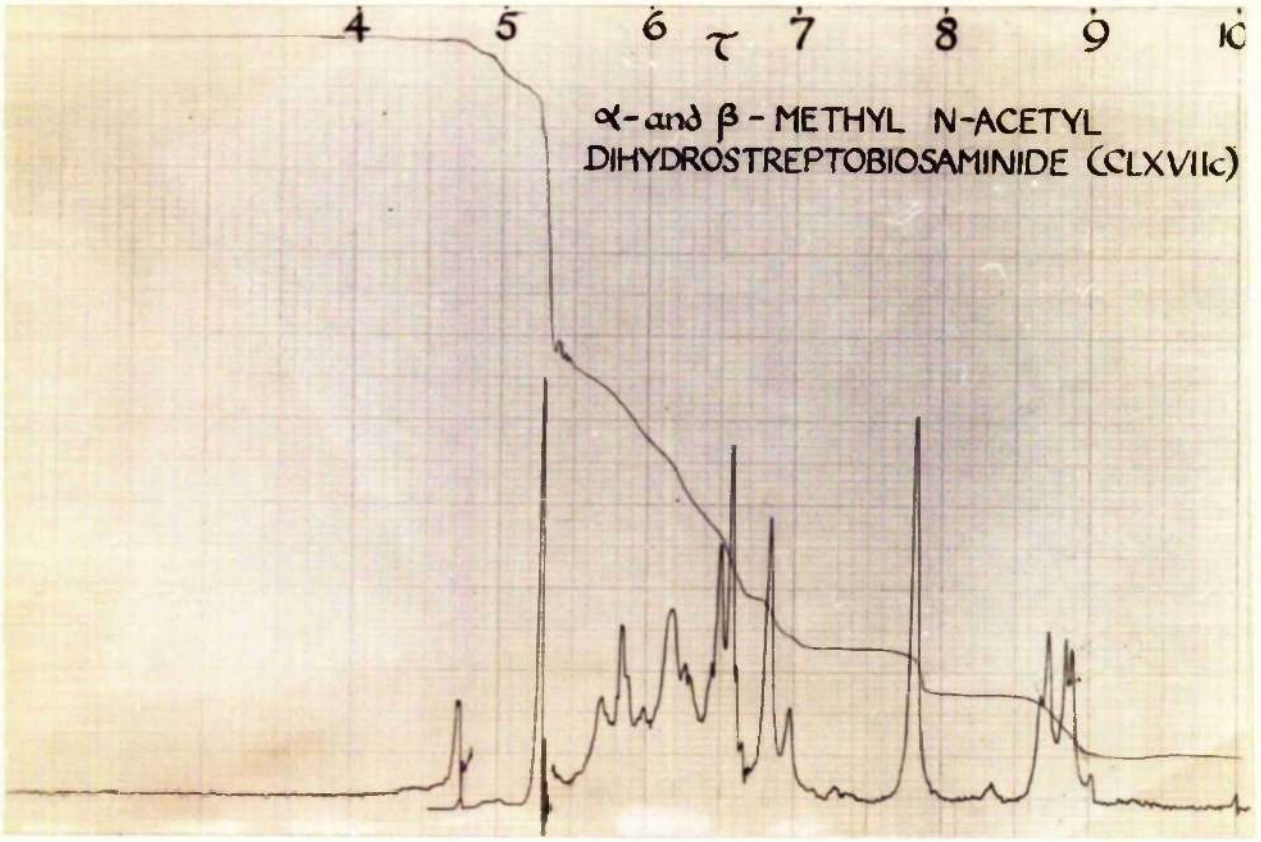

(CLXVI)

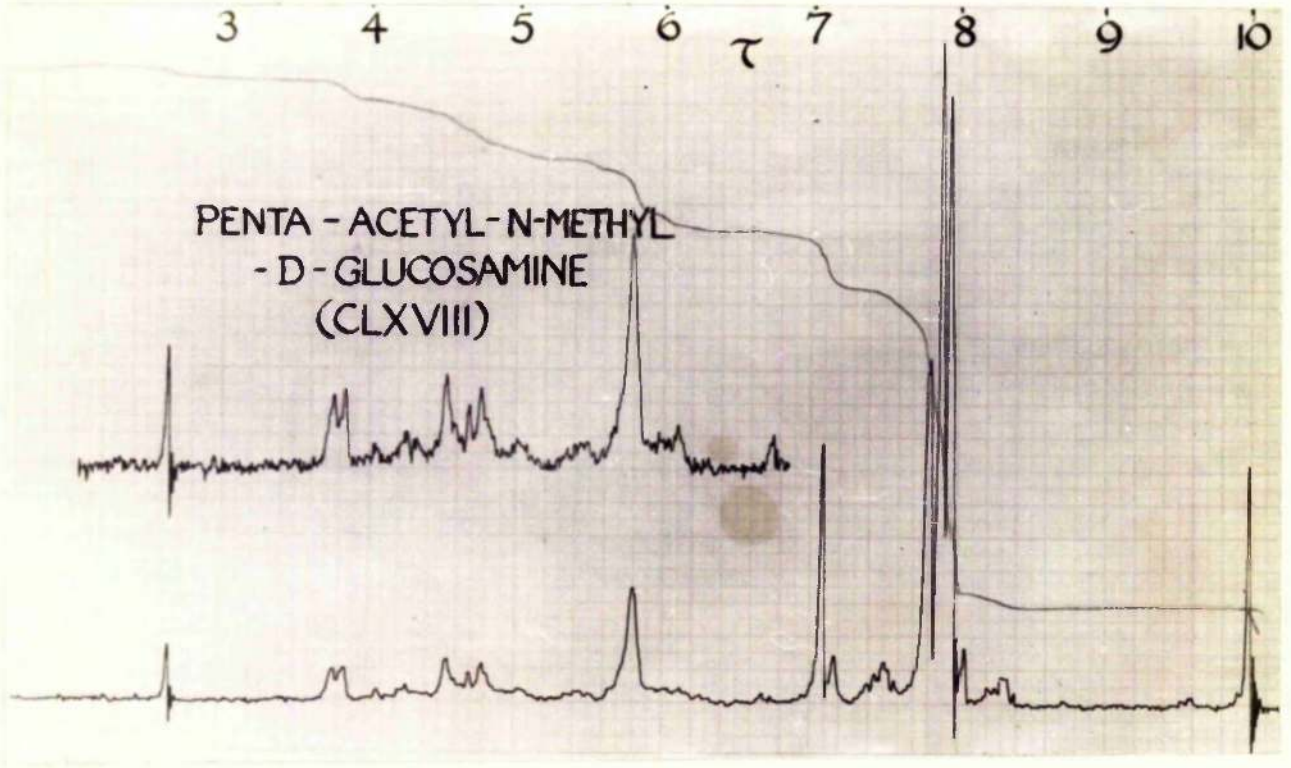
Barker and his associates[441] also examined and compared the acetoxy group signals of the fully acetylated pyranoses and whilst this study was complicated by the uncertainty of the spatial arrangements of the elongated acetoxy groups with consequent proton shielding variations reasonable consistency was observed.

The object of the present n.m.r. study was to confirm the glycoside linkages in methyl $\alpha$ - and $\beta$ - dihydrostreptobiosaminide, already assigned from molecular rotation studies [273] and to report any conformational


4
5
6
τ
7
8
9
10
α-METHYL PENTA - ACETYL
DIHYDROSTREPTOBIOSAMINIDE
(CLX VII α)

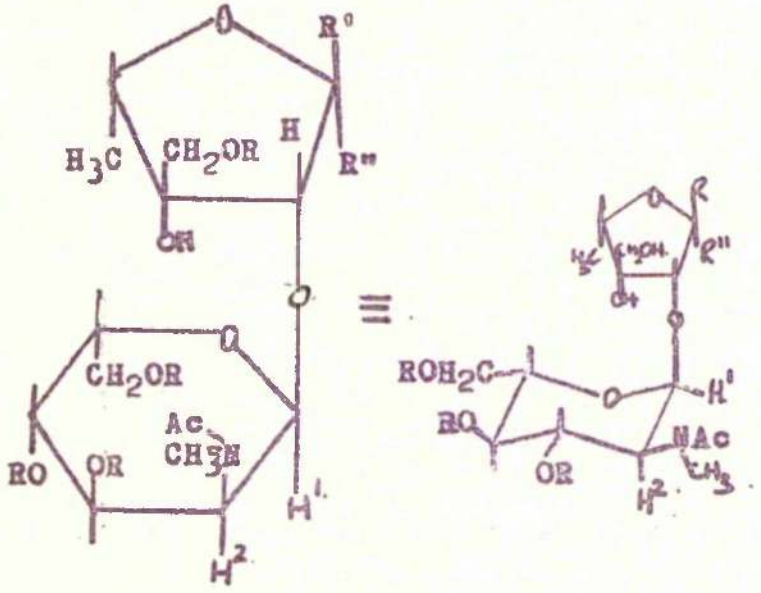

4
5
6
τ
7
8
9
10
β-METHYL
PENTA-ACETYL-DIHYDRO
STREPTOBIOSAMINIDE (CLXVIIb)


4
5
6
τ
7
8
9
10
α- and β - METHYL N-ACETYL
DIHYDROSTREPTOBIOSAMINIDE (CLXVIIc)


3
4
5
6
τ
7
8
9
10
PENTA - ACETYL - N-METHYL
- D - GLUCOSAMINE
(CLXVIII)

evidence available for the furanoside ring of dihydrostreptose. Difficulties in interpretation, however, have been encountered due to lack of model compounds, some of which were available but gave spectra of poor resolution. (Penta-acetyl-N-methyl-L-glucosamine and dihydrostreptose mono-acetate),

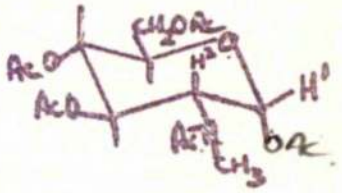
The spectra of $\alpha$ - and $\beta$.- methyl-penta-acetyldihydrostreptobiosaminide (CLXVIIa and b) were however obtained in deuterated chloroform ($CDCl_3$) and compared with the spectrum of the unresolved anomers of methyl-N-acetyldihydrostreptobiosaminide (CLXVIIc) run in deuterated water ($D_2O$ or HOD). The spectrum of penta-acetyl-N-methyl-$\alpha$-D-glucosamine (CLXVIII) also gave some useful information.



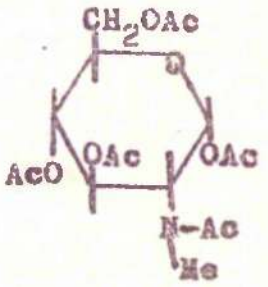
(CLXVII)a R = Ac, R' = OMe, R" = H

b R = Ac, R' = H, R" = OMe

c R = H, R' = H or Me, R" = OMe or H



III.



(CLXVIII)

Barker and his colleagues[441] gave $\tau$ = 6.7 as the 1-methoxy signal for the acetylated pyranosides - the signal occurs at $\tau$ = 6.62 in the spectra

shielding differences caused by the furanoside ring. For methyl-N-acetyldihydrostreptobiosaminide the unresolved $\alpha$- and $\beta$- anomers are clearly present as two signals are seen in this area, $\tau = 6.5$, and $\tau = 6.6$.

The methylene hydrogens of both the furanoside $\tau = 5.95$ and pyranoside $\tau = 5.82$ rings CLXVIIa and b are well resolved and identified by comparison with the spectra from the penta-acetyl-$\alpha$-D-glucosamine derivative.

The N-methyl and acetoxy signals are seen in both spectra at $\tau = 6.98$ and $\tau = 7.8$ to 8 respectively. The acetoxy signals differ slightly, the $\alpha$-anomer showing a quintet and the $\beta$-isomer a doublet.

The spectra of the D-glucosamine derivative shows a triplet signal for the acetoxy signals which may be assigned $\tau = 6.85$ for the N-acetyl group, 7.92 for the axial and 7.98 for the equatorial groups, respectively.

The most interesting portion of the spectra for the streptomycin derivatives lies in the signals caused by the terminal C-methyl group of dihydrostreptose. In the $\alpha$-penta-acetyl derivative this occurs as a methyl doublet ($\tau = 8.66$ and 8.82, $J = 6$ c.p.s.) whereas in the poorly resolved signal of the $\beta$-anomer it occurs as a quartet ($\tau = 8.65$, 8.71, 8.82 and 8.86; $J = 7$ and 8 c.p.s. - (1,3; 2,4)). This poor resolution is much improved in the spectra of the anomeric mixture of the N-acetyl derivatives, which gives much the same values.

The reason for this difference in signals of the $\alpha$ and $\beta$-isomers is not clear, but must depend upon the configuration at C-1 in the furanoside ring introducing long range coupling in the $\beta$-isomer, which may exist as discussed in page 117 in a number of T(twist) or E(envelope) forms.

Further examination of the spectrum of α-methyl penta-acetyl dihydrostreptobiosaminide leads to the speculation that if the doublet signal at $\tau$ = 4.57 and 4.67, J = 6 c.p.s. may be assigned to the C-1 hydrogen of the N-methyl-L-glucosamine ring moiety, the spin-spin coupling constant is consistent with interactions between an axial and an equatorial hydrogen. This assignment supports the conformational diagram (CLXVIII) in which the most stable orientation has the glycosidic bond in the axial position, the C-1 hydrogen equatorial the bulky N-methylacetamido group exists in the equatorial position and the C-2 hydrogen in the axial position.

Support for the above speculation comes from the spectrum of penta-acetyl-N-methyl-α-D-glucosamine where the anomeric hydrogen signals occur as a doublet $\tau$ = 3.78, 3.88 J - 6 c.p.s., the spin-spin coupling constant of an axial equatorial system as represented in CLXVIIIb, between the C-1 hydrogen and the C-2 hydrogen.

The poor resolution at the lower $\tau$ values in the spectra of the dihydrostreptobiosamine derivatives precluded further assignments.

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# SUMMARY

## The Chemistry of some Streptomycin Derivatives

The history of the chemotherapy of tuberculosis is reviewed, with emphasis on the newer experimental drugs such as ethambutol, kanamycin and 4,4'-di-isoamyl oxythiocarbanilide. The recognised regimens in tuberculo-therapy are discussed briefly in order that the current status of streptomycin may be appreciated.

The chemistry of the aminoglycoside antibiotics is reviewed in detail, evidence of the recently assigned structures of the neomycin-paromycin group and the kanamycins being cited as well as the more classical degradation and structural determination of streptomycin. Suggested biogenetic pathways and recent mode of action studies are noted, the introduction being completed by an inventory of synthetic derivatives of this group of antibiotics.

The remaining points of the chemistry of dihydrostreptomycin to be elucidated are discussed. This includes confirmation of the configuration of the glycosidic linkages, and direct proof of the nature and ring form of dihydrostreptose, the central moiety of dihydrostreptomycin.

Early workers degraded the trisaccharide dihydrostreptomycin with methanolic hydrogen chloride into the diguanidinoinositol, streptidine, and the methyl glycoside of the glucosaminide, dihydrostreptobiosaminide, the latter is composed of the methyl lyxofuranoside, dihydrostreptose, linked glycosidically to the hexosamine, N-methyl-L-glucosamine. Further acid degradation, however, led to destruction of the dihydrostreptose and this was not isolated.

D-glucosaminides are known to be very stable to acid hydrolytic conditions, a stability attributed to the positive charge on the amino nitrogen group causing repulsion of the proton and supressing formation

of the intermediate carbonium ion. N-Substitution of the methyl D-glucosaminides with an electron withdrawing group has been shown to facilitate glycosidic cleavage; the formation of an electronically neutral substituted amino group, such as the acetamido group, apparently allowing protonation of the glycosidic bond. Hydrolysis of the N-substituted glucosaminides, however, is accompanied by removal of the substituting group, and the rate at which this occurs at the expense of glycosidic hydrolysis is apparently dependent on the electrophilic properties of the substituting group.

In this present work dihydrostreptose was obtained from the L-glucosaminide methyl dihydrostreptobiosaminide by application of the above theories. The N-(2,4-dinitrophenyl)-(DNP),N-acetyl- and, N-tosyl-derivatives of methyl dihydrostreptobiosaminide have been prepared, the N-acetyl derivative has been obtained by a variety of routes. Samples of N-methyl-D- and L-glucosamine and some novel N-acyl derivatives have also been prepared for comparison by paper chromatography with various hydrolysis products.

The acid hydrolysis of methyl N-(2,4-dinitrophenyl)-dihydrostreptobiosaminide has been investigated in detail; paper chromatographic results showed that hydrolysis had occurred with 2N hydrochloric acid. The hydrolysis of methyl N-acetyldihydrostreptobiosaminide yielded a small sample of dihydrostreptose, which was characterised as the free sugar and the monoacetate. Methyl N-acetylstreptobiosaminide dimethylacetal the analogous derivative of streptomycin under the same acid conditions gave only N-methyl-L-glucosamine, the streptose moiety being degraded.

Deamination of methyl dihydrostreptobiosaminide with 1,2,3-indane trione hydrate was attempted unsuccessfully, as was cation-exchange hydrolysis of this compound.

Enzymatic cleavage of methyl N-acetyldihydrostreptobiosaminide was also unsuccessful under the conditions tried.

The second section of the thesis is devoted to glycosides of dihydrostreptobiosaminide. Methods of glycosidation, the Koenigs-Knorr, the Helferich and the Fischer syntheses are reviewed. Transglycosidatio the acid-catalysed replacement of the methyl by another aglycone was attempted with methyl dihydrostreptobiosaminide and yielded the benzyl phenyl, 2-bromoethyl, cyclohexyl, m-cresyl and anisyl glycosides. No mechanism has heretofore been proposed for this substitution. An examination of its relation to the Fischer glycoside synthesis suggests that the furanose ring of dihydrostreptose would facilitate the reaction and that the ease of glycosidation provides indirect evidence of the nature of this ring in dihydrostreptobiosaminide.

The phenyl and 2-bromoethyl dihydrostreptobiosaminides have been tested *in vitro* against *Mycobacterium tuberculosis*.

Nuclear magnetic Resonance studies of various dihydrostreptomycin derivatives are also interpreted.