STUDIES ON THE POLYSACCHARIDE ELABORATED BY STREPTOCOCCUS SALIVARIUS AND ASSOCIATED ENZYMES

By

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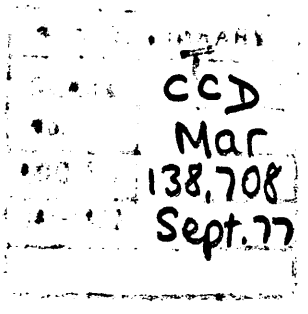
Doctor of Philosophy



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January 1977.

To My Wife Els

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ABSTRACT

Strains of Streptococcus salivarius (ATCC 13419, NCTC 8606 and 51) yielded, when cultured in a sucrose broth, extracellular polysaccharides that proved to be D-fructans possessing β-linkages. Sedimentation and gel-filtration results suggested average molecular weights to lie in the range 10^6 to 25×10^6. Methylation studies indicated these polymers to be branched molecules, degrees of branching being 9.2 - 12.3%, and in addition to possess either 2⟶6-linked fructofuranose or 2⟶5-linked fructopyranose residues with branching in either case occurring at C-1. This ambiguity was resolved by showing the position of the linkage to reducing D-fructose groups in a disaccharide preparation obtained from a partial acid hydrolysate of the fructan of strain 51 to be C-6, thereby demonstrating that the fructan possesses 2⟶6-linked fructofuranose residues and complying therefore with the definition given for a levan.

The extracellular levanase induced by culturing S. salivarius strain 51 in a levan containing medium was isolated and partially purified. It was shown to completely degrade levan in an exo-manner, D-fructose being liberated. A comparative study with the invertase of Candida utilis demonstrated that the latter enzyme possesses a far wider affinity for β-fructofuranosido-substrates such as levan, inulin, sucrose and methyl β-fructofuranoside. The ability of the levanase to degrade oligosaccharides obtained from inulin was investigated.

A study of the lengths of branches in strain 51 levan was made by reducing-end linkage analysis in oligosaccharide preparations obtained from a partial acid hydrolysate of the levan. Chemical ionisation mass spectrometry was shown to facilitate the assignment of structure to the hexitol derivatives obtained.

The exo levanase was used as a probe in an attempted investigation of the type-structure of the levan elaborated by S. salivarius strain 51, the evidence obtained suggesting the structure to be of the tree-like type.

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I.A. Definition of the Term Levan

Because some variation exists in the literature as to the use and meaning of the term levan, it is necessary to give the modern, accepted definition as used in this thesis.

A levan is a homo-polymer of D-fructose in which the majority of the interfructofuranosidic linkages are of the β-configuration and between positions C-2 and C-6, any branch linkages occurring at position C-1.

The practice of calling all homo-polymers of D-fructose (fructans), of bacterial origin, "levans" is not employed. Recently[1,2] "inulin-like" fructans of bacterial origin have been characterised in which the majority of interfructosidic linkages are between positions C-2 and C-1, with branching at position C-6.

I.B. Historical Survey of Bacterial Fructans

Of the substances produced by living organisms, polysaccharides form an important group, a great diversity of biological function being displayed by them. Polysaccharides are widely found as reserve substances, structural materials in higher plants and as protective substances e.g. as components of bacterial cell walls and gums, exuded by certain plants and trees to seal wounds. Polysaccharides are also found as constituents of the exo-skeletons of anthropods, as components of cartilage in vertebrates, in animal joint fluids and many other places.

Polysaccharides (glycans) may be divided into two groups, homoglycans and heteroglycans. The former comprises those polymers containing one kind of polymerised monosaccharide

or monosaccharide derivative, members of the latter group containing more than one kind. In nature, heteroglycans occur in greater diversity, homoglycans in greater abundance.

Of the homoglycans, the glucans (homoglycans of D-glucose) are the most abundant, and are found as both linear and branched polymers. The linear glucans are exemplified by cellulose, which occurs in far greater abundance than any other polysaccharide, by amylose, a component of starch, and by laminarin, which occurs in the brown algae. To the branched glucans belong amylopectin, the other starch component, and most of the dextrans, polymers of D-glucose synthesised from sucrose by many strains of bacteria.

Fructans, homoglycans of D-fructose, are also widespread in nature, mainly occurring as reserve substances in higher plants, notably in species of Compositaea and Graminaea. Fructans in this group are mainly of the inulin type, being unbranched molecules, of molecular weight about 5,000[3,4,5] possessing β-2 ⟶ 1 interfructosidic linkages and terminated by a D-glucose residue at the reducing end.[6,7]. Another group of fructans, found principally in grasses, was termed the phlein group and characterised by the principal interfructosidic linkages being β-2 ⟶ 6.[8,9] There are also fructans, of plant origin, that fall intermediate between the above two groups, e.g. triticin,[10] a grass polysaccharide and avinarin,[8] a very highly branched polymer possessing both 2 ⟶ 6 and 2 ⟶ 1 interfructosidic linkages.

Fructans and glucans, of bacterial origin, first aroused attention in the sugar industry, towards the end of the nineteenth century, when it was realised that unwanted slimy growths on sugar cane and in the refinery liquors were due to microbial infection. Beijerinck[11] made a study of the bacteria and enzymes responsible for these slime formations and recognised that certain bacteria possess the ability to synthesise glucans and fructans from sucrose. He coined the terms "viscosaccharase"[12] to describe an enzyme which produces slime from cane sugar, and "emulsion laevulan"[13]

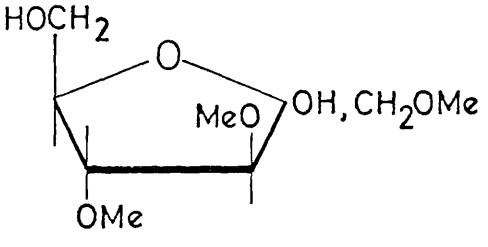
to describe the laevorotatory polysaccharide, identified as a fructan, a major component of some of the growths.

Prior to 1930 levans were defined as bacterial polysaccharides composed of D-fructose as the sole constituent. The term levan was introduced by Grieg-Smith [14,15] to describe the laevorotatory fructan formed by the action of Bacillus levaniformans on sucrose. He postulated that the synthesis involved initial inversion of sucrose, the "levan" being formed from the "nascent" fructose liberated. He discovered, however, that the organism could not produce a fructan from the hydrolysis products of sucrose (D-fructose and D-glucose) in their normal condition. Owen [16-18] on the other hand, showed that gum formation, from sucrose, by so-called "gum forming bacteria" was greatly inhibited by the addition of yeast, indicating that the products of yeast inversion do not possess substrate activity for gum formation. Also, Owen [19] claimed to have isolated the enzyme from Bacillus vulgatus and named it "levanase" as it produced so-called "levan" from sucrose. Kopeloff et al. [20,21], in 1920, found that certain mould spores contained a fructan forming enzyme, which exhibited maximum activity at pH 7. They considered this fructan to be formed from "nascent" fructose and glucose according to the hypothesis of Grieg-Smith. [14,15]

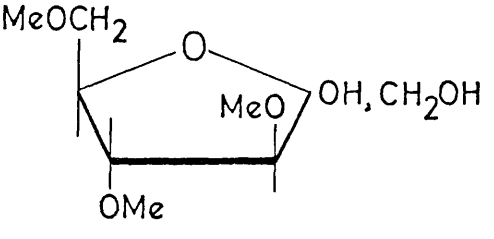
Harrison et al., [22] in 1930, obtained a polysaccharide from cultures of Bacillus mesentericus when grown in a sucrose medium. In addition, they obtained an enzyme preparation from this species capable of synthesising the same polysaccharide from sucrose. An almost quantitative yield of D-fructose was obtained upon hydrolysis of the polysaccharide, showing it to be a fructan, thought, at the time, to be related to inulin. In the following year, however,

Hibbert et al.,[23] partially characterised this fructan by chemical means. Initially they showed it to differ from inulin in the physical properties of the respective tri-acetates and tri-methyl ethers, and on the evidence of rotational data ($[\alpha]_D^{20} = -40^\circ$) considered the anomeric configuration to be of the β -type.

Hydrolysis of the methylated fructan yielded a crystalline tri-O-methyl-D-fructose (claimed to have structure I) which was found to differ from the liquid 3,4,6-tri-O-methyl-D-fructose (II), obtained from methylated inulin, in that an osazone could not be formed and oxidation of I, with nitric acid, yielded a dibasic lactol acid (III), whereas oxidation of compound II yielded monobasic 3,4,6-tri-O-methyl-D-arabino-2-Hexulofuranosonic acid (IV).[4]



I



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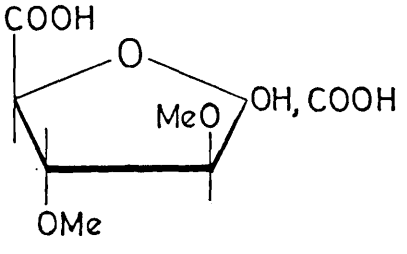

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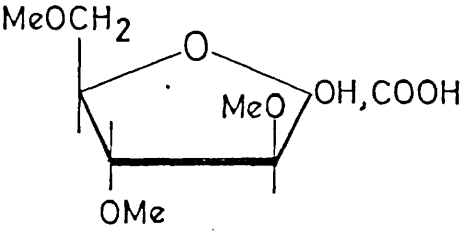
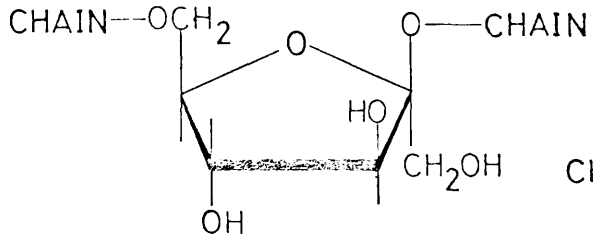


IV

These findings lead to the conclusion that crystalline I is O-methylated at positions 1, 3 and 4. This is compatible with the fructan elaborated by B. mesentericus possessing 2 ⟶ 6 linked

fructofuranosyl residues (V) or 2⟶5 linked fructopyranosyl residues (VI).



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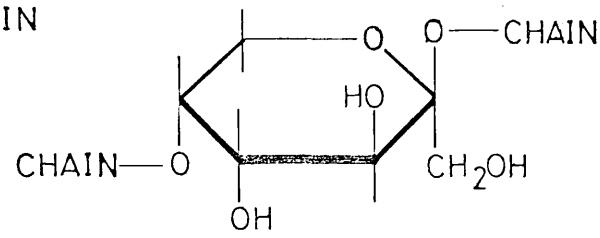
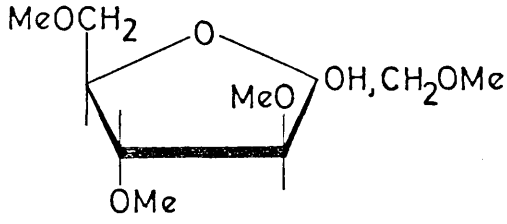
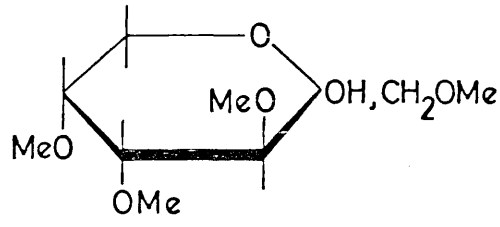


VI

Hibbert et al.[23] further subjected the 1,3,4-tri-O-methyl-D-fructose to methanolysis at room temperature, followed by methylation and hydrolysis to give a tetra-O-methyl-D-fructose. This was identical with that obtained from inulin, sucrose and methyl β-D-fructofuranoside, shown by Avery et al.[24] to be O-methylated at positions 1, 3, 4 and 6 (VII).



VII



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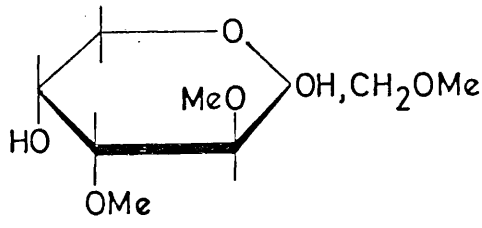
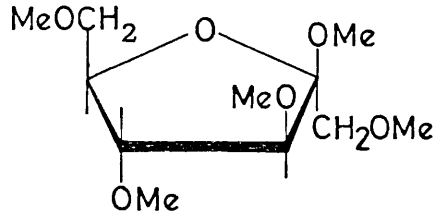


IX



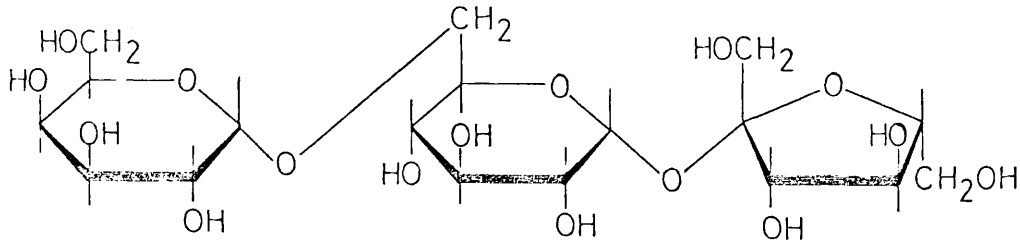
X

It was claimed that this finding indicated the presence of furanosyl residues, and hence 2⟶6 linkages, in the fructan. This

sequence of reactions, i.e. methanolysis, methylation, hydrolysis, can yield 1,3,4,5-(VIII, from the pyranose form, IX) and/or 1,3,4,6-tetra-O-methyl-D-fructose (VII, from the furanose form, I). Such a sequence of reactions does not, therefore, allow a distinction to be made between furanose and pyranose forms of D-fructose in fructans. The fact that Hibbert et al., by methanolysis at room temperature of the 1,3,4-tri-O-methyl-D-fructose, did indeed obtain the methyl 1,3,4,6-tetra-O-methyl-D-furanoside (X) is in agreement with our present knowledge of the methanolysis of monosaccharides. Bishop and Cooper[25,26] have shown that furanosides are the kinetically controlled products.

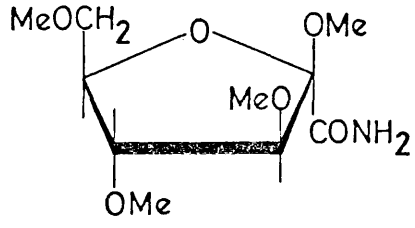
Unambiguous chemical evidence for the ring size of the fructose residues in the so called levans has, to the knowledge of the author, not been obtained. Statements such as "All known fructans contain β-D-fructofuranose residues"[27] are, therefore, not supported by chemical evidence. Circumstantial evidence such as the action of invertases on these polysaccharides, the ease of acid hydrolysis, and, above all, the fact that they are enzymically synthesised from substrates possessing fructofuranosyl end groups (see section I.C.) is however compatible with levans being composed of fructofuranosyl residues. This thesis will provide chemical proof that the fructans of Streptococcus salivarius are in fact levans (for definition see section I.A.).

Hibbert and Brauns[28] found the fructan of Bacillus subtilis to be very similar to that of B. mesentericus and may likewise be termed a levan. Mitchell and Hibbert[29], in 1932, produced the first evidence for the fact that only saccharides terminated by a β-D-fructofuranosyl residue can serve as substrates for the enzymic synthesis of levan. They used raffinose (O-α-D-galactopyranosyl-(1$\longrightarrow$6)-α-D-glucopyranosyl β-D-fructofuranoside, XI) as a substrate with B. mesentericus and obtained a polymer identical with that when sucrose is the substrate.



XI

Challinor et al.[30] estimated chains of B. mesentericus levan to be 10 - 12 residues in length. This they concluded from the fact that the methylated levan, upon hydrolysis and glycosidation, yielded 10 - 11% of methyl 1,3,4,6-tetra-O-methyl-D-fructofuranoside (X) which was identified by its conversion to methyl 3,4,6-tri-O-methyl-D-arabino-2-hexulofuranosidonamide (XII), shown to be identical to the authentic compound prepared from sucrose.[24]



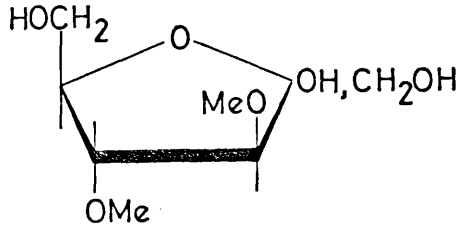
XII

The nature of the reducing terminus was not established and the methods employed did not reveal the presence of any di-O-methyl-fructoside in the hydrolysis mixture, which would have provided the clue to the true nature of these high molecular weight branched molecules. Using essentially the same methods, Lyne, Peat and Stacey[31] investigated the fructans of Bacillus

megaterium, Bacterium (Phytomonas) pruni and Bacterium (Phytomonas) prunicola, the latter two organisms being responsible for serious stone fruit diseases. These polysaccharides were similarly established as being levans and similar results with regard to chain lengths were reported.

Apparently, these early investigators believed the levan molecules to be actually composed of only 10 - 12 fructose residues, which would correspond to a molecular weight range of 1638 - 1962. In 1944, Ingelman and Siegbahn[32], showed by ultracentrifugation sedimentation studies together with electron microscopical observations, the particle weight of Bacillus vulgatus (subtilis ?) levan to be as high as $5 - 10 \times 10^7$. In a later publication[33] Gilbert and Stacey assumed that the levan of Pseudomonas mors-prunorum (Wormald) was an aggregation of repeating units, each unit being a chain 10 - 12 fructose residues in length. However, no explanation was forwarded at that time, for how the repeating units were held together.

There followed, then, a period of development in the understanding of bacterial levan structure, initiated by Bell and Palmer[34], in 1949, who employed partition column chromatography to separate the partially methylated fructoses obtained upon hydrolysis of an unspecified methylated fructan. They obtained 1,3,4,6-tetra-O-methyl-D-fructose (VII), 1,3,4-tri-O-methyl-D-fructose (I) and 3,4-di-O-methyl-D-fructose (XIII).



XIII

Because of the volatility of compound VII it was determined colorimetrically, compounds I and XIII being determined gravimetrically. Compound VII was characterised by comparison of physical properties with literature values of specific rotation[35-38] and methoxyl content.[39-41] Similarly compound I was characterised by comparison of specific rotation[30,31] and refractive index.[23] Compound XIII was characterised by comparison with the synthetically prepared material of McDonald and Jackson.[42]

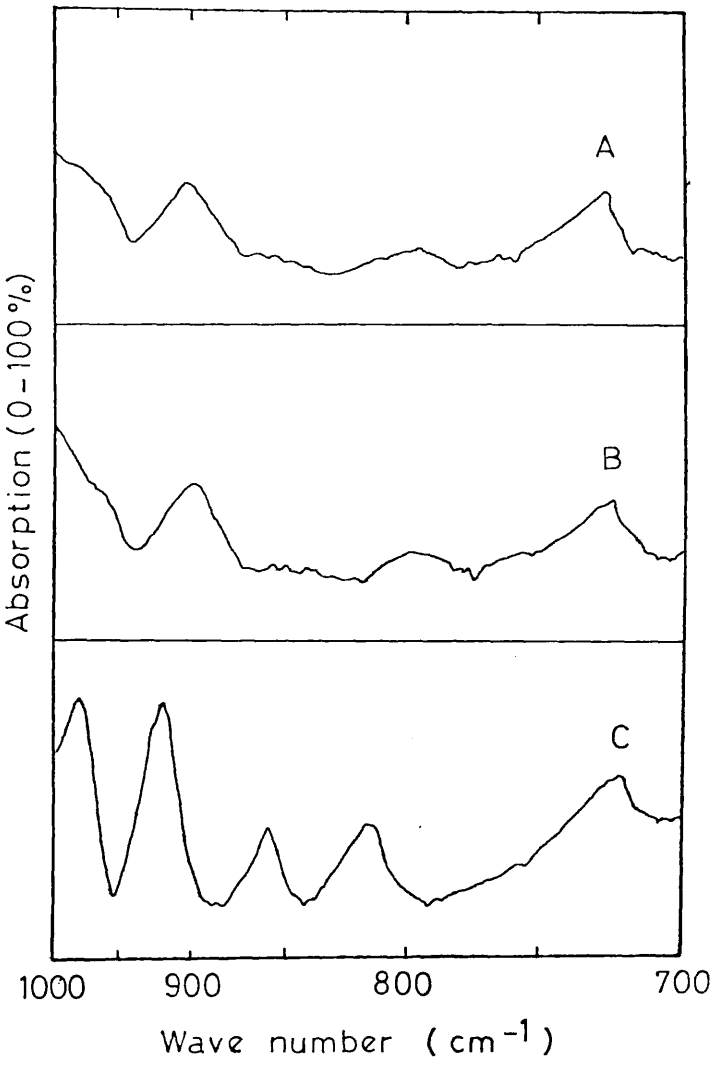
Palmer,[43] in 1951, detected the presence of D-glucose in acid hydrolysates of certain fructans, including the levan of B. subtilis, by the glucose oxidase method. On the assumption that glucose originated from the "reducing" terminus, a molecular weight of 37,700 was calculated for this polymer. Such a method, however, is probably not reliable because the enzymic synthesis of levans from sucrose involves the liberation of glucose, and should any of this material be occluded in the polymer structure it would be co-determined with any glucose from the polymer itself and lead to meaningless results. In the following year, Murphy[44] obtained a fructan from Bacillus polymyxa when grown in a sucrose containing medium. On the basis of identifying 3,4-di-O-methyl-fructose (XIII) as a component of the hydrolysate of the methylated polymer, it was suggested, for the first time, that this levan is a branched polymer, the branch linkages occurring at position C-1. Murphy separated the products of the hydrolysis of the methylated polymer and quantitatively determined the molar ratio of 1,3,4,6-tetra-, 1,3,4-tri- and 3,4-di-O-methyl-fructose by the methods of Bell and Palmer.[34] The oily compound XIII was characterised by comparison of physical properties with those obtained by Schlubach and Sinh[8] and Arni and Percival[10], who isolated this compound from the hydrolysis products of methylated avenarin and tricitin respectively, the latter workers performing its original chemical characterisation.

At about the same time, Hestrin[45] discovered that the organism Aerobacter levanicum conveniently produces large quantities of a

fructan when grown in a sucrose containing medium. A cell-free enzyme preparation, which catalysed the synthesis of the polysaccharide, was easily obtained by sonic disruption of the washed bacterial cells. Bell and Dedonder[46], in 1954, carried out a structural re-examination of the fructans elaborated by strains of Pseudomonas prunicola and Bacillus subtilis. They established that both polymers are branched levans by methylation and hydrolysis, and separation, on a silica column, of the resulting partially methylated fructoses.[34] The average repeating unit for both polymers was found to contain 9 - 10 residues, corresponding to a degree of branching of 10 - 11%. They also searched for the presence of glucose in hydrolysates of the polymers, by the method of Palmer.[43] Failure to detect any glucose prompted them to state that the molecular weights must be very high. This was supported by the fact that the B. subtilis levan could be centrifuged down at 25,000 g. Thus the possibility that a D-glucose residue forms the (pseudo) reducing terminus and the speculation that such polymers are formed by chain lengthening transfer of β-fructofuranosyl residues, ab initio sucrose, could not be ruled out. To support such a hypothesis, Dedonder and Noblesse[47] demonstrated the formation of an ascending series of D-glucose containing oligosaccharides during the enzymic synthesis of levan by the levansucrase of the B. subtilis strain used by Bell and Dedonder.

About the same time, Barker and Stevens[48] showed that fructans exhibited characteristic infra-red (i.r.) spectra dependant upon the positions of linkages. The β-2$\longrightarrow$1 linked inulins displayed markedly different spectra to fructans possessing β-2$\longrightarrow$6 linkages. Indeed, Barker et al.[49], in 1955, proposed the fructan synthesised by a streptococcus isolated from a "ropy-fermentation" to be a levan, on the basis of its i.r. spectrum. Hestrin et al.[50] identified the fructans elaborated by Aerobacter levanicum and cell free enzymic extracts thereof to be levans with branching at C-1 by the then established method of methylation analysis, the partially methylated fructoses, obtained on hydrolysis of the methylated polymers, being identified on the basis of R_f values. The two levans were further shown to be similar with respect to their i.r. spectra and considerably different from that of inulin (Fig. I.1.).

Figure I.1. Infra-red spectra of levans and inulin[50]



Key :

A. A. levanicum culture levan
B. enzyme synthesised levan
C. inulin (Difco)

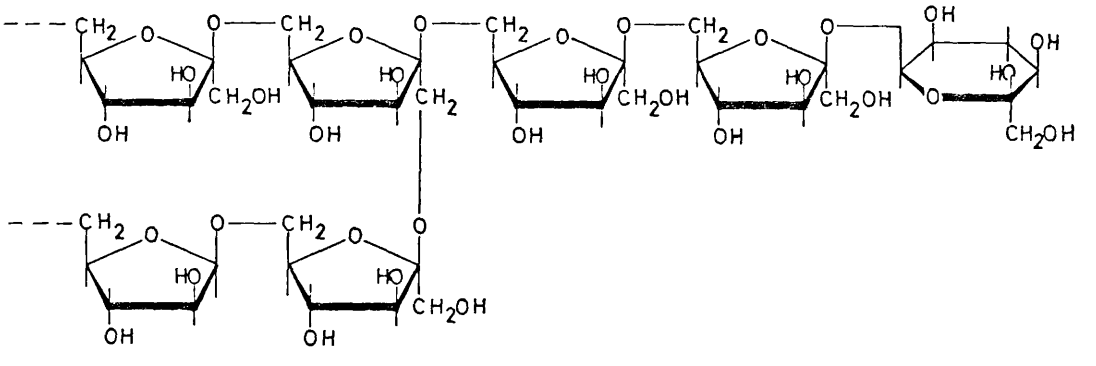
Feingold[51], in 1955, reported a more exact figure (6×10^7) for the molecular weight of A. levanicum levan, and Feingold and Gehatia[52], using the methods of Murphy[44], established that the average repeating unit comprised 9 residues, corresponding to a degree of branching of 11%, for both levans of culture and cell-free enzyme origin. In these experiments the methylated fructoses, obtained upon hydrolysis of the methylated polymers, were separated on a hydrocellulose column.[53] Partial acid hydrolysis of this levan followed by charcoal - Celite chromatography[54] yielded oligosaccharides. Paper chromatography revealed two series of oligosaccharides at degree of polymerisation (DP) values of 4 and above, presumably of the linear and branched type. These workers also established the anomeric configurations to be mainly, if not entirely, of the β - type, by showing that oligosaccharides in the range DP2 - DP10 were completely hydrolysed by yeast invertase, a hydrolase specific for β - D - fructofuranosidic linkages. It was believed that the polymer was formed by sequential addition of single fructosyl groups to the growing molecule[55,56], but no explanation could be found for polymer growth termination, which occurs even in the presence of excess substrate and active enzyme. Investigation of molecular weights revealed that of the "culture" levan to be 17×10^6 and that of the "enzymic" levan to be about 67×10^6.

One bacterial fructan, chemically identified as a levan by Avigad and Feingold[57], proved to be somewhat different from levans previously characterised. This was the levan of a Corynebacterium species which proved to have an average repeating unit of some 15 residues.

Dedonder[58], in 1958, at the start of what proved to be a very extensive series of studies, re-examined the chemical structure of Bacillus subtilis levans and proposed a model of the structure with glucose linked through its anomeric position, as in sucrose, forming the pseudo - reducing terminus of the molecule (Fig. 1.2.). This has since become the accepted structure of all bacterial levans,

although no attempts have been made to determine in greater detail the structure of these polysaccharides. Dedonder and Slizewicz[59], using the methods of ultracentrifugation and light scattering, determined the levan of Bacillus subtilis to be truly macromolecular, its molecular weight lying in the range 6.6×10^3 to 10^8.

Figure I.2. The levan molecule[58]



Very many other organisms have been shown to produce levans from sucrose containing media. Fuchs[60], who was mainly concerned with bacterial strains connected with stone-fruit diseases, on the basis of i.r. spectroscopy, showed that levans are elaborated by several species of Pseudomonas, Serratia kiliensis, Arthrobacter species and again Aerobacter levanicum and Bacillus subtilis. Having studied the i.r. spectra of traditionally characterised levans, he concluded that true levans (principally 2 ⟶ 6 linked) have absorption maxima at 929, 722 and 811 cm^{-1}, whereas inulin, (2 ⟶ 1) linked, typically displays maxima at 984, 932, 873, 817 and 722 cm^{-1} (cf. Fig. I.1.).

Levans have also been shown to be produced by strains of Achromobacter[61], Azotobacter chroococcum[62], Bacillus mesentericus[63], Acetobacter suboxydans[64,65], Zymomonas mobilis[66] and Pseudomonas aurantiaca.[67]

In recent years investigators have shown some degree of renewed interest in specific bacterial fructans because of physiological or immunological properties displayed by them.

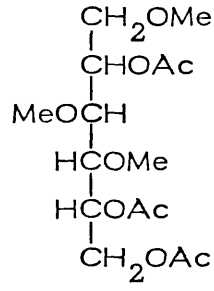
Goldstein and So[68], in 1965, made the observation that certain fructans give a pricipitin reaction with concanavalin A, the globulin from Jack bean (Canavalis ensiformis). Lewis et al.[69] were prompted, by the fact that the fructan of Leuconostoc mesenteroides strain C gives this reaction, to investigate this polysaccharide. By the now traditional method of methylation and hydrolysis followed by separation, characterisation and quantisation of the resulting methyl ethers of fructose, they established the polymer to be a levan with an average repeating unit of 5 to 6 residues which corresponds to a degree of branching of 17 - 20%, the highest figure thus far reported for bacterial fructans.

The filamentous organism, Odontomyces viscosus, responsible for the development of sub-gingival plaque and associated periodontal disease in Syrian hamsters[70], was found to produce a fructan when cultivated in a sucrose or raffinose containing medium. This polysaccharide was extracted by ethanol precipitation and on the evidence of specific rotation ($[\alpha]_D^{20} = -77.3^\circ$) and its i.r. spectrum (absorption maxima at 803, 874 and 919 cm^{-1}) was concluded to be a levan.[71]

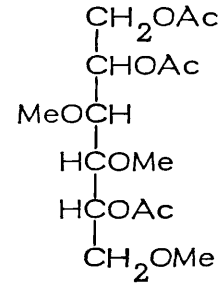
Levans have been shown to be antigenic in man and are precipitated by some myeloma proteins.[72] Differences in the immunochemical behaviour of different preparations have been attributed to differences in the levan structures.[72,73] This prompted Lindberg et al.[74] to investigate the structures of different levans and inulin

by the modern technique of methylation analysis and gas chromatography - mass spectrometry (gc - ms) of the derived alditol acetates.[75,76]

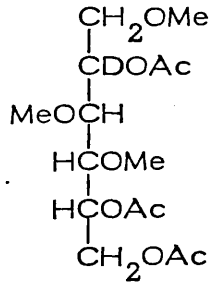
It was recognised that some difficulties might be experienced with fructoses, since on reduction the epimeric glucitol and mannitol derivatives would be expected. However, the two forms did not separate on the columns employed.[77] It was also recognised that the partially methylated hexitol acetate (XIV) derived from 1,3,4-tri-O-methyl-D-fructose (I, from hydrolysed, methylated levans) and that (XV) derived from 3,4,6-tri-O-methyl-D-fructose (II, from hydrolysed, methylated inulin) might not be distinguishable by mass spectrometry. This problem was overcome by the introduction of a deuterium label at C-2, in the reduction step of the procedure, with sodium borodeuteride.



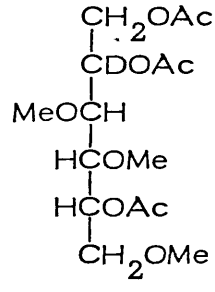
XIV



XV



XVI



XVII

The presence of a 2-d-2,5,6-tri-O-acetyl-1,3,4-tri-O-methyl-hexitol (1,3,4-tri-O-methyl-hexitol acetate, XVI) was taken as evidence of the parent polysaccharide being a levan.

Inulin gave exclusively the 2-d-1,2,5-tri-O-acetyl-3,4,6 tri-O-methyl-hexitol (3,4,6-tri-O-methyl hexitol acetate, XVII). The retention times relative to 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol (R_{tmg} values) and ratios of di-, tri- and tetra-O-methyl hexitol acetates were measured by g.c.-peak integration. The degree of branching, being proportional to the percentage of the di-O-methyl hexitol acetate, was found to be very variable among the substances studied. The differences in immunochemical behaviour were attributed to these structural variations.

Only a very limited number of uses for levans have been found or tried. Feingold and Gehatia[52] prepared fractions of slightly acid hydrolysed levan from Aerobacter levanicum and called them levulans. Preliminary investigations of a levulan of molecular weight 30,000, as a possible blood plasma expander, proved encouraging. Studies in this direction were continued by Schechter and Hestrin[78], who found suitable levan fractions to have a number average molecular weight of 28,000 ± 4,000 and a mass average figure of 115,000 ± 15,000. No toxic effects were observed. They studied the relation of polymer size to behaviour in the body[79] and concluded that the maximum molecular weight of a levan molecule that can filter through the kidneys is less than 75,000. A patent was taken out for the production of levans of controlled molecular weights[80], for use as blood plasma substitute, but the use of levans in this context did not become established, presumably because of the greater availability of dextrans which have found extensive application in this field.

Levans have also found application in the photographic industry as agents for improving the covering power of photographic emulsions.[81]

Apart from the limited applications levans have enjoyed, and their immunochemical properties, these polymers have been found to

have infection promoting activity. For example, levan administered to mice intravenously increases the virulence of intraperitoneally injected bacteria such as Salmonella typhi.[82] This finding has, at least in part, been explained by the discovery that intravenous levan causes sealing of the vascular lining, thus preventing escape of blood constituents into the peritoneal cavity.[83] The potential practical importance of a substance with endothelial sealing activity is apparent, but this line of application has, it seems, not been pursued.

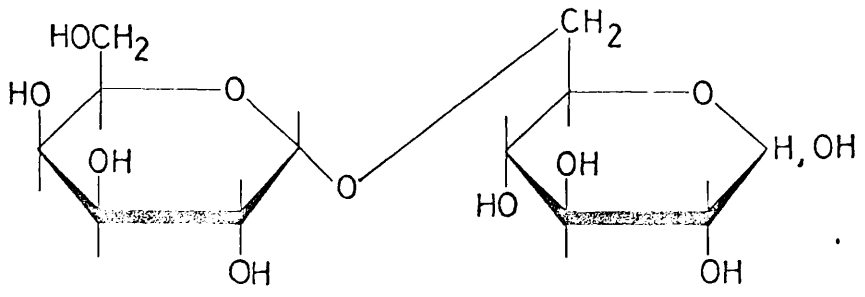
I.C. The Enzymic Synthesis of Levan

Until the mid - 1950's, knowledge on the mode of enzymic synthesis of levans was relatively meagre. A summary of the early work appeared in a review by Barker and Bourne in 1953.[84]

Prominent among the early investigators were Hestrin and his collaborators who, in 1943, obtained cell-free preparations of the enzymes responsible for levan synthesis (levansucrase, (2⟶6)-β-D-fructan:D-glucose 6-fructosyltransferase, EC 2.4.1.10) from cultures of Bacillus subtilis and Aerobacter levanicum.[85,86] It was with preparations from the latter organism, that this group performed an important series of investigations, beginning in 1944, when it was shown that the following reactions are catalysed :[87]

$$(n+m)\ \text{sucrose} + (n)\ H_2O \longrightarrow (n+m)\ \text{glucose} + (n)\ \text{fructose} + [\text{levan}]_m$$

$$(n+m)\ \text{raffinose (XI)} + (n)\ H_2O \longrightarrow (n+m)\ \text{melibiose (XVIII)} + (n)\ \text{fructose} + [\text{levan}]_m$$

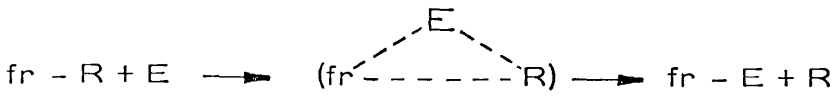


XVIII

The levansucrase preparation was found to have a greater specificity than the invertases with respect to hydrolase activity, methyl β-D-fructofuranoside and inulin, for example, not serving as substrates. In addition, it was shown that D-glucose can serve as a fructosyl acceptor and competitively inhibit levan synthesis. The optimum conditions of pH and substrate and enzyme concentrations for levan synthesis were then established.[88]

Hehre[89] succeeded in obtaining similar levansucrase preparations from Streptococcus salivarius and a spore forming bacillus, the laevorotatory fructans obtained by the action of these preparations on sucrose being classified as levans on the basis of serological tests.[89.90] Similar tests showed that certain organisms, including some strains of group H streptococci[91] and Leuconostoc mesenteroides NRRL B-512[92], were able to synthesise both dextrans and levans simultaneously.

The suggestion that the levansucrase substrate, sucrose, is initially hydrolysed (inverted) to D-glucose and D-fructose, prior to polymerisation of the free fructose units,[14,15] was rejected in 1944.[93] It was replaced by the view that levan and dextran synthesis occurs by repeated transfer of hexosyl residues from donor molecules to the growing acceptor molecules via an enzyme - fructosyl (or enzyme-glucosyl in the case of dextran synthesis) complex,[94,95] the fructosyl - enzyme complex being logically supposed to be formed via a transitional enzyme-substrate species.[94]



where fr - R denotes a substrate molecule capable of donating the fructosyl group, fr, to the enzyme, E, to form the complex fr - E.

This concept was extended in 1955 when Hestrin et al.[96] proposed that the transfructosylation reactions catalysed by the levansucrase of A. levanicum constitute a two-step process :

Initial reversible formation of an enzyme - fructosyl complex

$$fr - R + E \rightleftharpoons fr - E + R$$

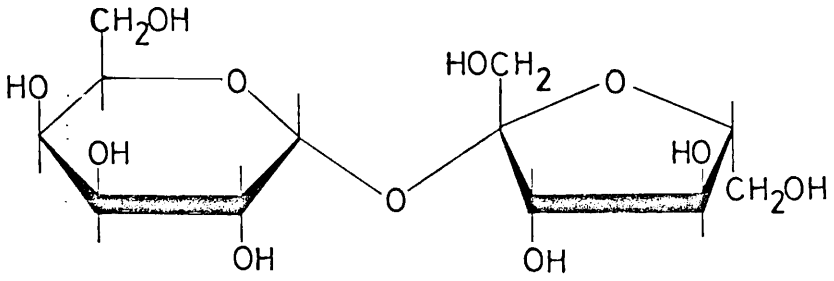
Irreversible transfer to an acceptor molecule

$$fr - E + A \longrightarrow fr - A + E$$

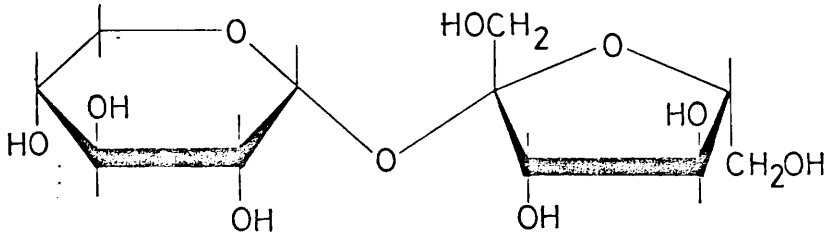
where A denotes an acceptor molecule.

The overall irreversible process was termed a "stepwise" propagation, commonly now called a "multimolecular" process.

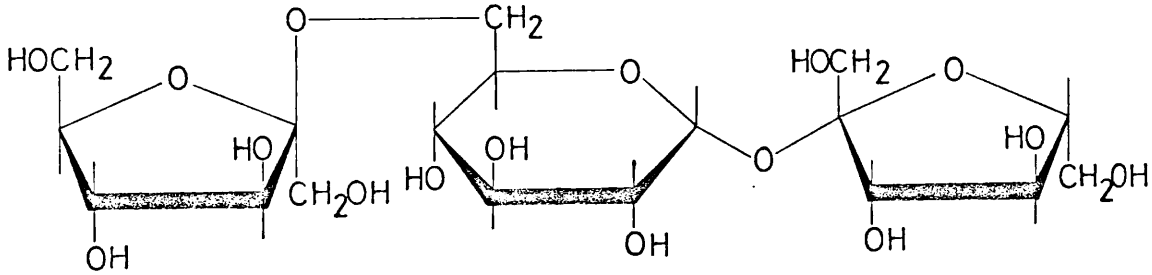
Subsequent studies on the donor and acceptor specificities of this enzyme[50] revealed the important fact that only donor compounds (fr - R) such as sucrose, raffinose (XI), β-D-fructofuranosyl α-D galactopyranoside (XIX), β-D-fructofuranosyl α-D-xylopyranoside (XX), O-β-D-fructofuranosyl-(2 $\longrightarrow$ 6)-α-D-glucopyranosyl β-D-fructofuranoside (neo-kestose, XXI) and O-α-D-galactopyranosyl-(1 $\longrightarrow$ 6)-O-α-D-galactopyranosyl-(1 $\longrightarrow$ 6)-α-D-glucopyranosyl β-D-fructofuranoside (Stachyose, XXII) can serve as donors. All of these sugars are non-reducing, terminated by a β-D-fructofuranosyl residue, the R - residue being liberated in every case.



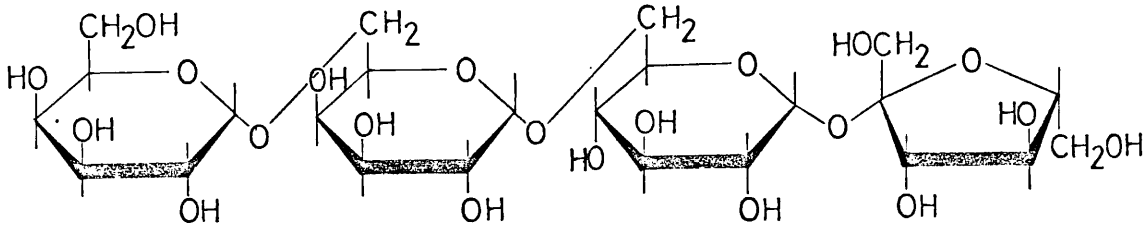
XIX



XX

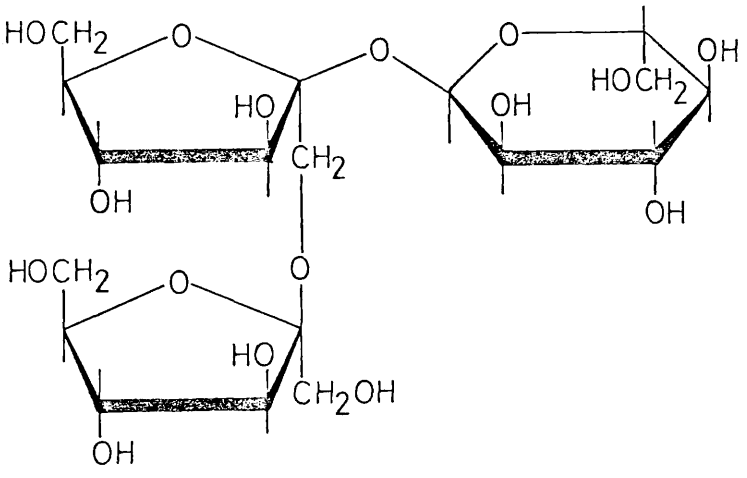


XXI



XXII

Further, it was found that water can act as a fructosyl acceptor and concomitant formation of oligosaccharides can occur. This latter reaction, first noted by Kohanyi and Dedonder[97] with the levansucrase of B. subtilis, involves initial transfer of a fructosyl residue to the carbinol group in the acceptor molecule, the major product being O-β-D-fructofuranosyl-(2⟶1)-β-D-fructofuranosyl α-D-glucopyranoside (1-kestose, XXIII). This lead to the suggestion that branching at C-1 in the growing levan polymer may occur by a similar process.



XXIII

The net overall reaction has been summarised thus:

Part reactions

(m) fr - R + (m) H_2O ⟶ (m) fructose + (m) R
(inversion)

v (q - 1) fr - R + (v) fr - R ⟶ (v) fr_q - R + v (q - 1) R
(oligosaccharide formation)

(n) f - R + P ⟶ fr_n - P + (n) R
(polymerisation)

Adding,

$$(s)\ fr - R + (m)\ H_2O + P \longrightarrow (m)\ \text{fructose} + (v)\ fr_q - R + fr_n - P + (s - v)\ R$$

where P denotes an acceptor leading to polymer formation.

The reversibility of the initial complex formation was demonstrated, by the same workers, by showing that the fructosyl galactoside (XIX) is formed from raffinose (VII) in the presence of levansucrase and D-galactose. Similarly the fructosyl xyloside (XX) is formed in the presence of D-xylose.

e.g. raffinose + E $\rightleftharpoons$ fr - E + melibiose (XVIII)

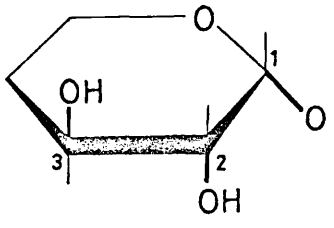
fr - E + D-galactose $\longrightarrow$ fructosyl galactoside (XIX) + E

The levansucrase of B. subtilis was found by Péaud - Lenöel and Dedonder[98,99] to differ from that of A. levanicum in respect to the reversibility of the transfer reaction. Low molecular weight preparations of levan were found to serve as substrates for the transfer of fructosyl residues to aldose acceptors to give compounds of the type fr - R.

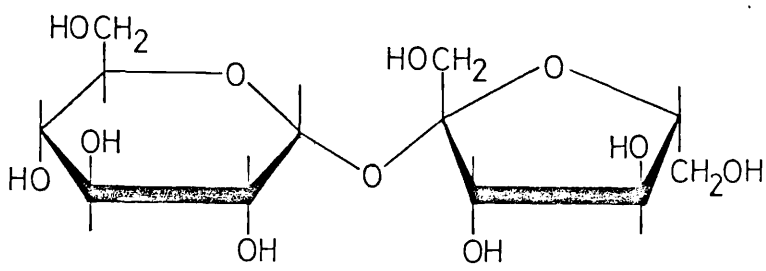
During this period, levansucrases were considered as being similar to yeast and mould invertases since these latter enzymes also possess the ability to transfer fructosyl residues to molecules other than water. Bacon[100] classified the various types of transferase activity exhibited by these enzymes. Whereas the initial formation of the yeast invertase - fructosyl complex is irreversible, the corresponding complex formation with mould invertase and levansucrase is reversible, followed by subsequent irreversible fructosyl transfer to a carbinol position in the acceptor molecule. In this respect the latter two enzymes are similar, although mould invertase possesses the ability to hydrolyse all β-D-fructofuranosyl linkages. Of these three enzymes, the characterising ability of levansucrase to synthesise

macromolecules was considered, by Bealing and Bacon[101], to depend upon the enzyme-fructosyl complex to have a high affinity for large acceptor molecules. This property of A. levanicum levansucrase was demonstrated by Hestrin et al.[50] Levan oligosaccharides, obtained from partial acid hydrolysates of levan, in the range DP4 to DP8, displayed sharply increasing acceptor activity[102], possibly an explanation for the observation that levan molecules, with DP values less than 20, could not be detected in levansucrase-sucrose systems.[103] Addition of low molecular weight levans to systems containing B. subtilis levansucrase systems was found to enhance both the rate of levan synthesis and yield of levan but diminish the average molecular weight of the product.[104.105]

By the end of the 1950's the hypothesis of an enzyme - fructosyl complex was furthered. It was then generally agreed[106] that the transfructosylation reactions involved an initial enzyme - substrate complex formed by chemisorption of the substrate molecule at the enzyme surface. This was thought to involve bonding of the glycosidic oxygen atom of the substrate to the enzyme bond forming site. This hypothesis is supported by the fact that only when the aldopyranosyl residue possesses certain configurational arrangements can the substrate serve as a fructosyl donor. The requirements are that position C-2 be unsubstituted with its hydroxyl group cis disposed to the glycosidic oxygen atom and position C-3 be unsubstituted and carry a hydroxyl group trans disposed to that at C-2 (XXIV). These requirements are met in compounds such as sucrose (XXV), the fructosyl galactoside (XIX), and the fructosyl xyloside (XX).



XXIV



XXV

Péaud-Lenöel[105] advanced the hypothesis that the enzyme can complex reversibly with short chain levans in addition to the substrate molecule. This theory allows an explanation for the reversibility of the transfructosylation reaction found with B. subtilis levansucrase.

$$(\text{levan})_n + E \rightleftharpoons (\text{levan})_n\text{-}E$$
$$(\text{levan})_n\text{-}E + \text{fr-R} \rightleftharpoons (\text{levan})_n\text{-}E\text{-fr-R}$$

The fructosyl residue was then assumed to be transferred to the levan chain liberating the free aldose, R.

$$(\text{levan})_n\text{-}E\text{-fr-R} \rightleftharpoons (\text{levan})_{n+1}\text{-}E + R$$

This is an example of an "insertion" process. It was suggested, on the basis of kinetic evidence, that in the absence of so called "levan primers", oligosaccharides are initially formed from sucrose which subsequently become bound to the specific levan building site for the polyrepetitive "insertion" mechanism to occur. These ideas are summarised in the following scheme :

$$L_n\text{-}E \overset{+S}{\rightleftharpoons} L_n\text{-}E\text{-}S \rightleftharpoons L_{n+1}\text{-}E + G \quad \text{(polymerisation)}$$

$$\uparrow\downarrow + L_n \qquad \uparrow\downarrow + L_n$$

$$E \overset{+S}{\rightleftharpoons} E\text{-}S \xrightarrow{+A} E + A\text{-}F + G \quad \text{(oligosaccharide synthesis)}$$

$$E\text{-}S \xrightarrow{+H_2O} E + F + G \quad \text{(inversion reaction)}$$

where L_n and L_{n+1} denote levan molecules with DP values of n and n+1 respectively, A denotes a fructosyl acceptor molecule leading to oligosaccharide formation and S, F and G denote sucrose, fructose and glucose, respectively.

Ebert and Stricker[107] obtained a highly purified preparation of A. levanicum levansucrase, essentially free of hydrolase activity, and, by means of kinetic studies, established that the polymerisation and inversion reactions follow a Michaelis mechanism, oligosaccharide formation having a different dependence, the rate increasing with sucrose concentration over the entire range.[108,109] Further, it was found that primers are not necessary for the polymerisation reaction which is first order with respect to enzyme concentration.[108] Because of the formation of relatively few large molecules, Ebert and his collaborators proposed that the so-called "polyreaction" follows an insertion type mechanism.

Tsuchiya et al.[110] had used similar reasoning to explain the synthesis of relatively few large dextran molecules, after the initial suggestion of Stacey in 1943.[111]

More recently, further advances have been made by the Dedonder group, focussing its attention on the levansucrase of B. subtilis, an enzyme found to differ fundamentally from that of A. levanicum in that it is inducible.[112] Again, addition of low molecular weight levan initiators was found to increase the rate and yield of levan synthesis. The reversibility of the transfer reaction was demonstrated by the appearance of sucrose and the fructosyl galactoside (XIX), when low molecular weight levans were treated with the enzyme in the presence of D-glucose and D-galactose, respectively. In addition, such levans could be hydrolysed in the absence of fructosyl acceptors. Although the hydrolysing action was found to be much weaker than the synthesising action, it was, nevertheless, clear cut and tended towards a limit of about 50% hydrolysis. As fructose was the only hydrolysis product, detectable

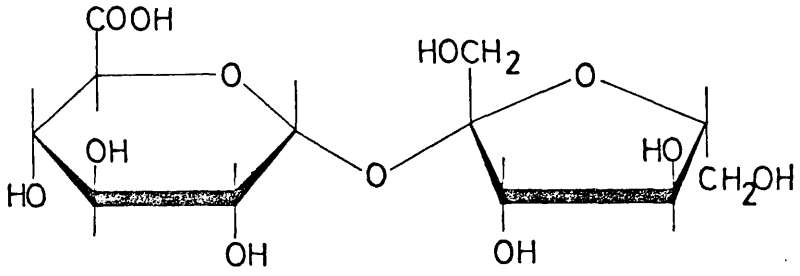
by chromatography, the enzyme was concluded to act as an exo hydrolase, it being suggested that the hydrolysis limit corresponds to the percentage residues on external branches. High molecular weight levans, however, were found not to be substrates for this reaction. The hydrolysing action was concluded to be a property of the levansucrase itself on the evidence of heat deactivation experiments and electrophoretic behaviour.

In a subsequent paper [113], Rapoport and Dedonder studied, in considerable detail, the donor and acceptor specificities of their enzyme preparation. As the results of their experiments led them to propose a mechanism for the enzymic action, these will be briefly discussed. As far as donor specificity is concerned, only fr - R compounds with an aldopyranosyl residue possessing trans disposed hydroxyl groups at C - 2 and C - 3 can serve as donors (see structure XXIV). Modifications at C - 4, e.g. inversion of configuration or substitution by a chain of aldopyranosyl residues (especially 1⟶4 linked D - glucosyl) do not deactivate the donor molecule. Modifications at C - 6 are also permitted. Rapoport and Didenot [114] found that galactosides of sucrose, higher homologues of raffinose (XI) possess donor activity, passing through a maximum at DP4 (stachyose, XXII) and decreasing thereafter to zero at DP7. Further, the presence of substituents such as a carboxyl group at C - 6 of the aldosyl residue does not destroy donor activity, as demonstrated by the incorporation of glucuronic acid in the exchange reaction.

D - glucuronic acid + sucrose

⇅ levansucrase

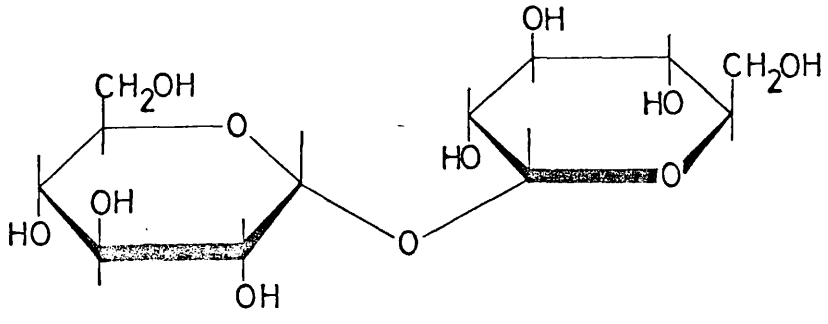
β - D - fructofuranosyl α - D - glucopyranosiduronic acid (XXVI) + D - glucose



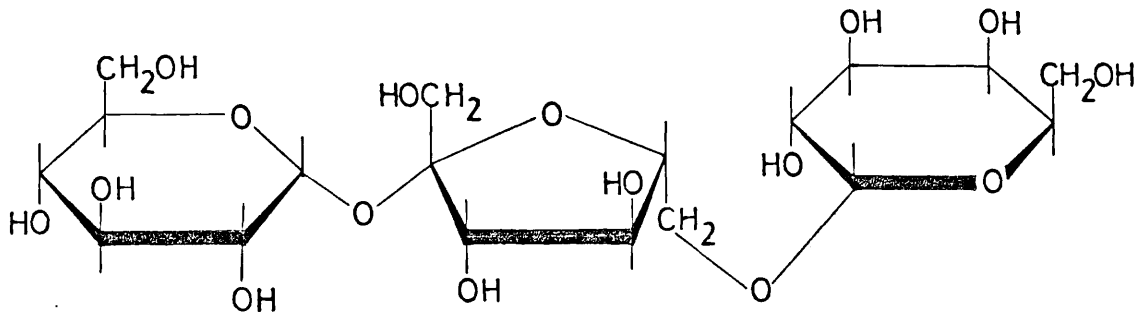
XXVI

The D-glucose liberated in the reaction with sucrose was found, on the evidence of its slowness to react with glucose oxidase (which is specific for the β - form of D-glucose) and polarimetric evidence, to be in the α - form, i.e. it is liberated with retention of configuration.

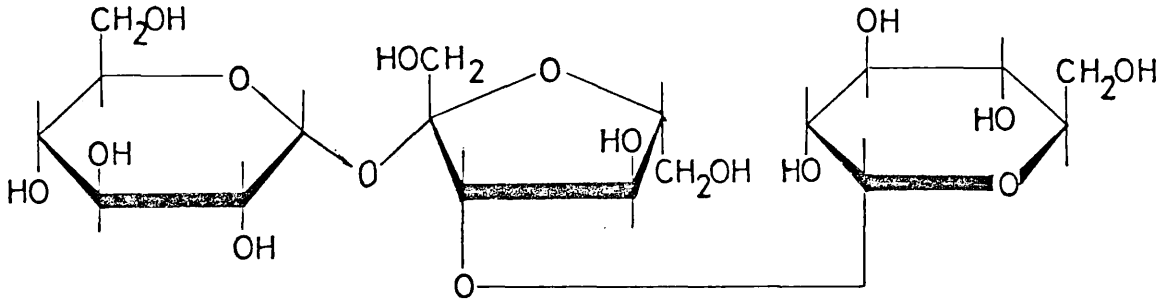
The specificity of the fructosyl acceptor was found to be far lower than thought previously, transfer being possible to a great number of compounds. Among the sugars acting as acceptors, D-fructose was found to have no influence on the rate of D-glucose liberation, whereas trehalose (α-D-glucopyranosyl α-D-glucopyranoside, XXVII), planteose (O- α-D-galactopyranosyl-(1 → 6)- β-D-fructofuranosyl α-D-glucopyranoside, XXVIII) and melezitose (O- α-D-glucopyranosyl-(1 → 3)- β-D-fructofuranosyl α-D-glucopyranoside, XXIX) exerted an enhancement in the rate of glucose release.



XXVII



XXVIII

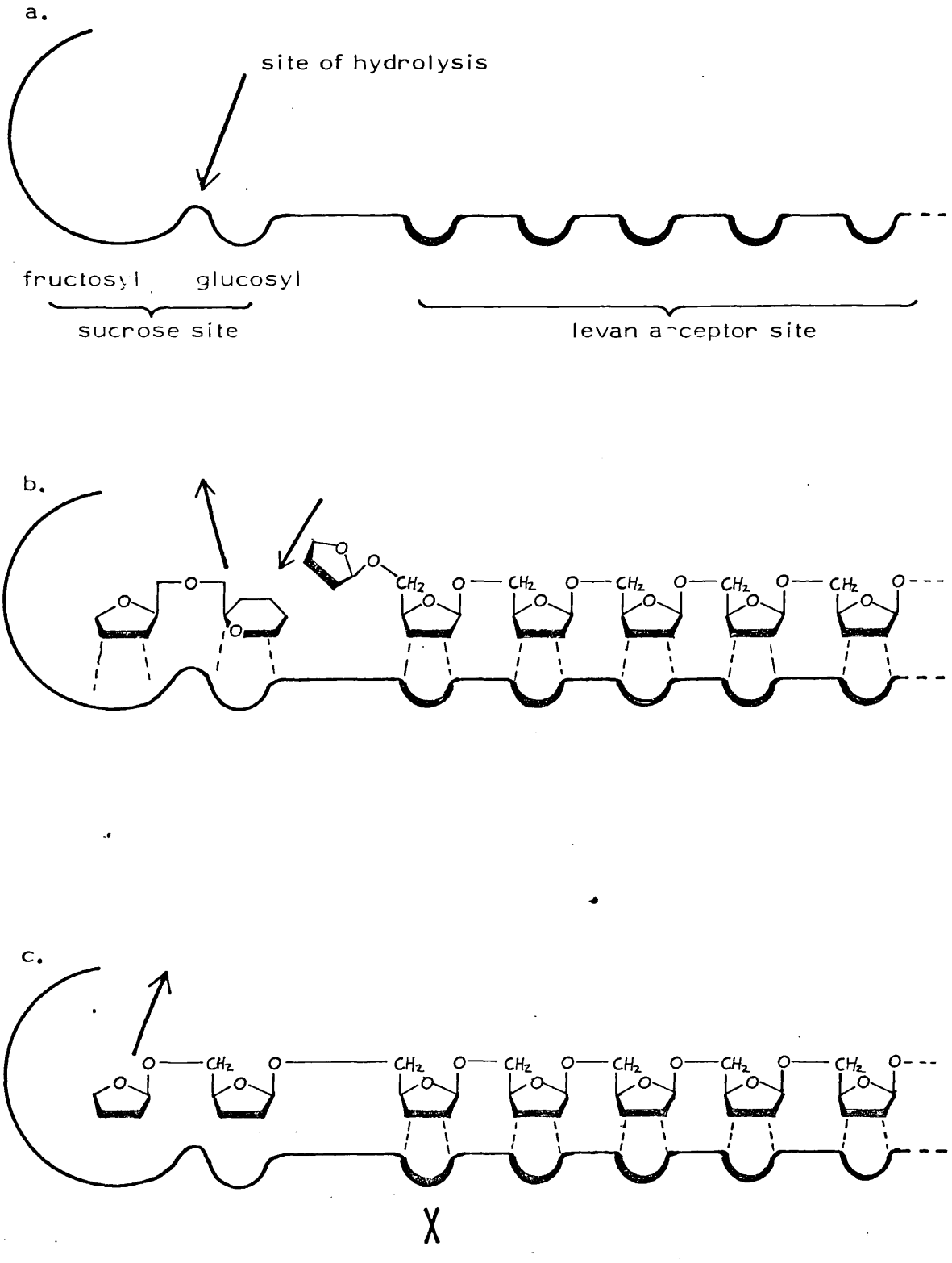


XXIX

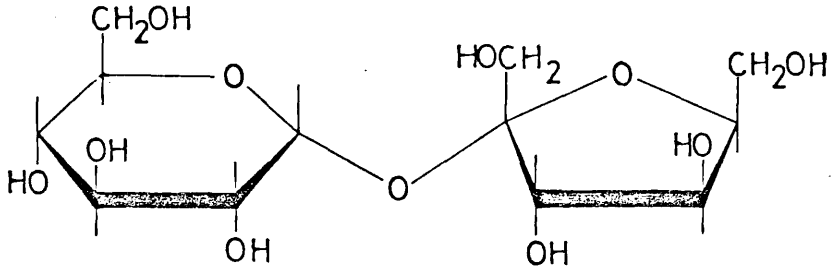
As with yeast invertase[115] and A. levanicum levansucrase[102], methanol was found to possess acceptor activity, the resulting methyl β -D-fructofuranoside not being hydrolysed, however, as in the case of yeast invertase. Higher alcohols did not possess acceptor activity although a slow reaction with glycerol lead to glycoside formation, a reaction independently studied by Ebert and Stricker.[108] By using ^{14}C - labelled glycerol, it was shown to be incorporated in levans, suggesting that glycerol might act as an initiator for levan synthesis. Other polyols such as D-glucitol and myo - inositol (structurally analogous to D-glucopyranose) exhibited no acceptor activity.

To account for the properties of the enzyme, it was proposed that there exist three specific active sites (Fig. 1.3.a.), a site for the donor molecule, subdivided into aldosyl and fructosyl sites, and a site for the levan acceptor. It was suggested that a fold in the polypeptide chain accounts for the high specificity for the transferred group, the only minor modification permitted being inversion

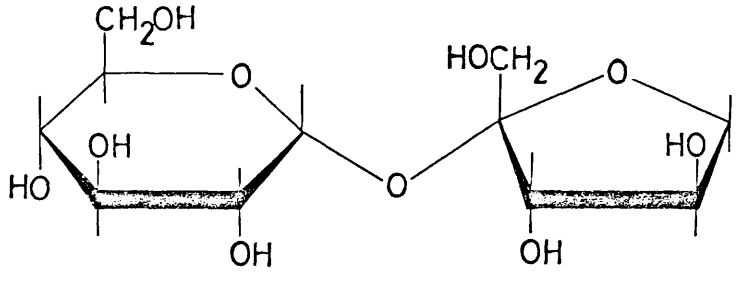
Figure I. 3. Model for levansucrase : Active sites and mechanism of transfructosylation [113]



of configuration at C-5, as in α-D-glucopyranosyl β-L-xylo-2-hexuloside (XXX), or replacement of the C-6 hydroxymethyl group of the keto-hexose by hydrogen as in α-D-glucopyranosyl β-D-threo-2-pentuloside (XXXI).



XXX



XXXI

The levan acceptor site is in the vicinity of the sucrose site, the last residues at the non-reducing end of the levan chain not being in direct contact with the protein surface, allowing a degree of freedom of movement (Fig. I.3.b.). This was concluded from the fact that the smallest levan oligomer to display an affinity for levan in the hydrolysing reaction was levantriose[116], indicating that X is the first site having affinity for fructofuranose residues. (Fig. I.3.c.).

As the affinity increases with DP, the adjacent sites contribute towards the attachment of the levan chain.

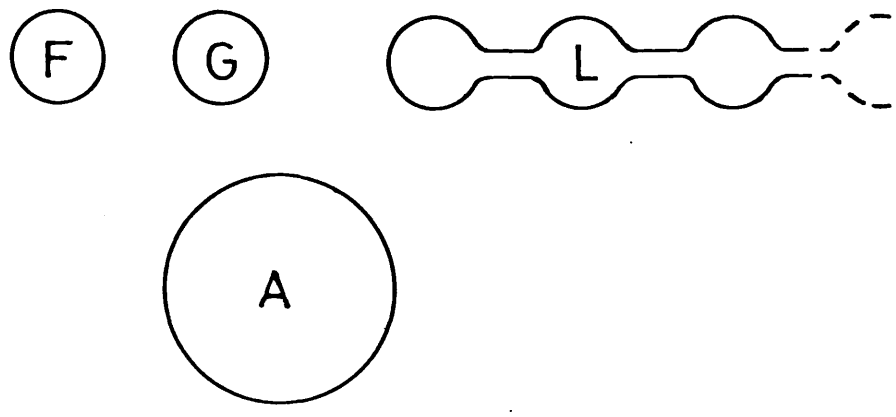
During levan synthesis, a sucrose molecule becomes attached to its specific site (Fig. I.3.b.), the terminal residues of the acceptor being mobile. D - glucose is eliminated, the terminal non-reducing residue of the acceptor then occupying its place, thereby presenting the C - 6 hydroxymethyl group to become linked to the free fructosyl residue (Fig. I.3.c.).

To account for the possible fructosyl transfer to a wide range of acceptors, a non-specific space, A, can be envisaged, in the proximity of both the glucosyl site and the levan acceptor site, which allows acceptor molecules "access" to the transferred fructosyl radical (Fig. I.4.).

The synthesis of 1 - kestose (XXIII) would involve initial fixation of a sucrose molecule at its specific site and a second sucrose molecule with its fructosyl residue attached at A and the glucosyl residue in the glucosyl part of the sucrose site. The transfer of the free fructosyl group, upon elimination of D-glucose, occurs preferentially, for steric reasons, to the C - 1 hydroxymethyl group of the acceptor fructosyl residue (Fig. I.5.).

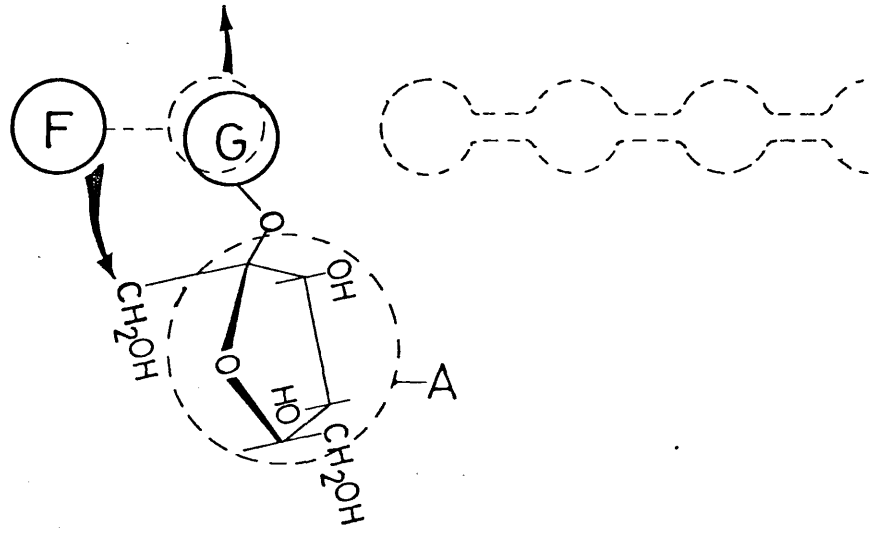
Rapoport et al.[117] established that no primer is necessary for levan synthesis. In the reaction with sucrose, sucrose was found to act as the first acceptor in the synthesis of levan chains and 1-kestose (XXIII) is the first product formed. The reaction was then found to be propagated by the successive addition to 1-kestose of fructosyl residues linked through positions C-2 and C-6. By means of ^{14}C-labelled substrates, D-fructose and D-glucose, at high concentrations, were also found to be capable of initiating levan formation.

Figure I.4. Model for levansucrase : Active sites [113]



Key : F = fructosyl site
G = glucosyl site > sucrose site
L = levan acceptor site
A = non-specific space for acceptor molecules

Figure I.5. Levansucrase : Formation of 1 - kestose (XXIII) [113]

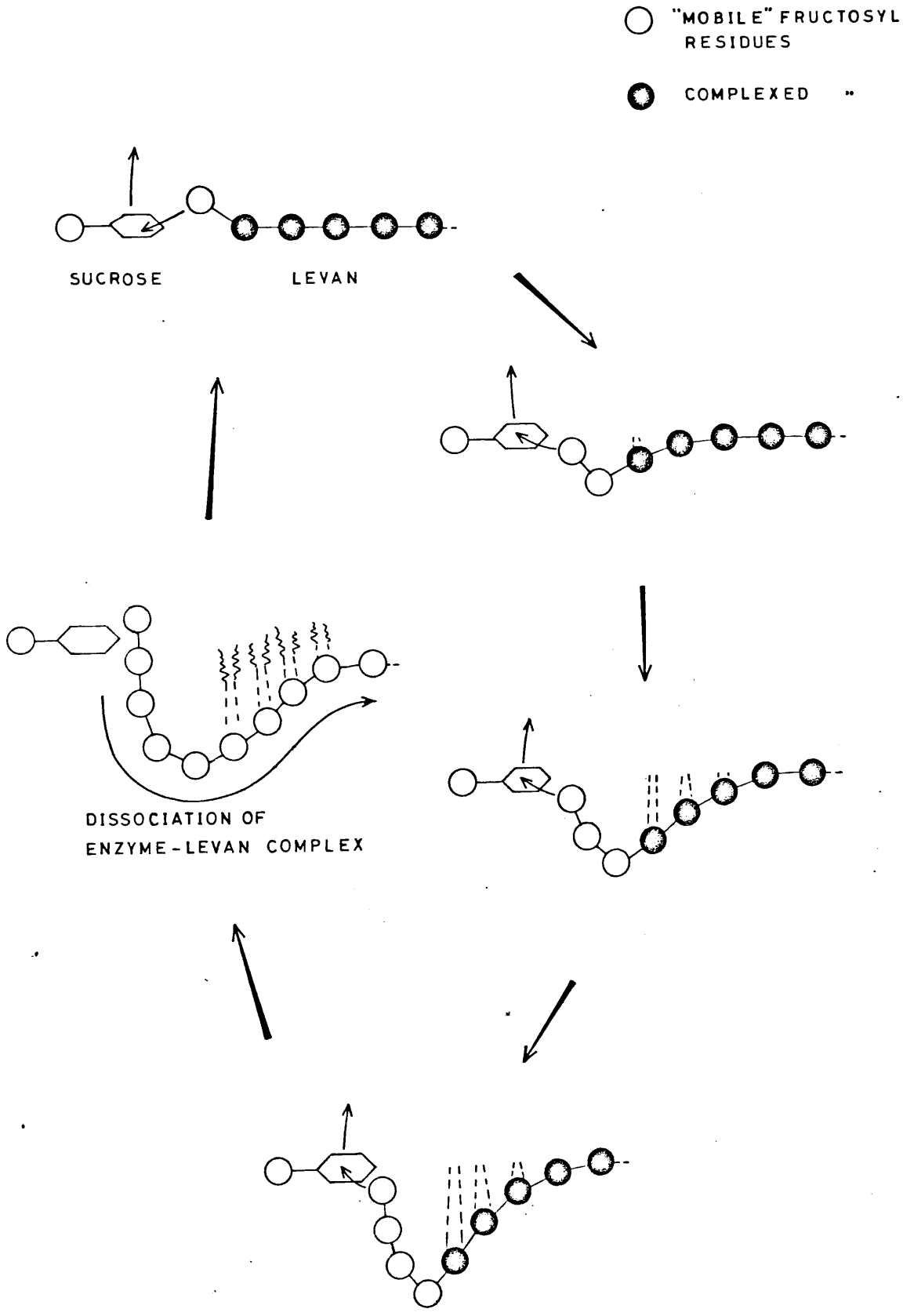


Key : F = fructosyl group at specific site
G = glucosyl residue
A = non-specific space for acceptor molecules

It has previously been mentioned that two mechanisms for enzymatic synthesis have been proposed : the "insertion" or "unimolecular" process and the "stepwise" or "multimolecular" process. In the former an enzyme molecule is bound to an acceptor molecule and totally builds up the polymer molecule before starting a new one. This implies permanent attachment with the increasing chain and would lead to a relatively low number of high molecular weight products. In the latter mechanism, however, all the polymer molecules grow at the same time since no permanent enzyme attachment is involved,leading to a larger number of smaller molecules. In this case, addition of low molecular weight levan initiators would lower the average molecular weight of the levans synthesised.

The observations obtained with B. subtilis levansucrase cannot readily be accounted for by either of these simple theories. For example, Dedonder and Péaud-Lenöel [104] have demonstrated that addition of low molecular weight levan diminishes the average molecular weight of the levans synthesised, a result also obtained by Mattoon et al.[118] , although in both cases very high molecular weight levans were also synthesised to a degree of 10%. Rapoport et al.[117] , as a result of studies on the molecular weight distribution of levans obtained with this enzyme, suggested an intermediate type of mechanism which they termed a "multiaddition" process. This mechanism (Fig. 1.6.) relies upon the presence of the non-specific acceptor space, A , (Fig. 1.4.). Fructosyl residues are successively transferred to the "mobile" end of the levan acceptor molecule, the increasing mobile chain end being accommodated at the non-specific acceptor site, A, until the distortion becomes too great and the levan molecule becomes dissociated from its specific site and slides along to the initial position (Fig. 1.6.). At the moment of dissociation the levan molecule can become completely released from the enzyme thus accounting for the multimolecular characteristics of what would otherwise be a purely unimolecular process.

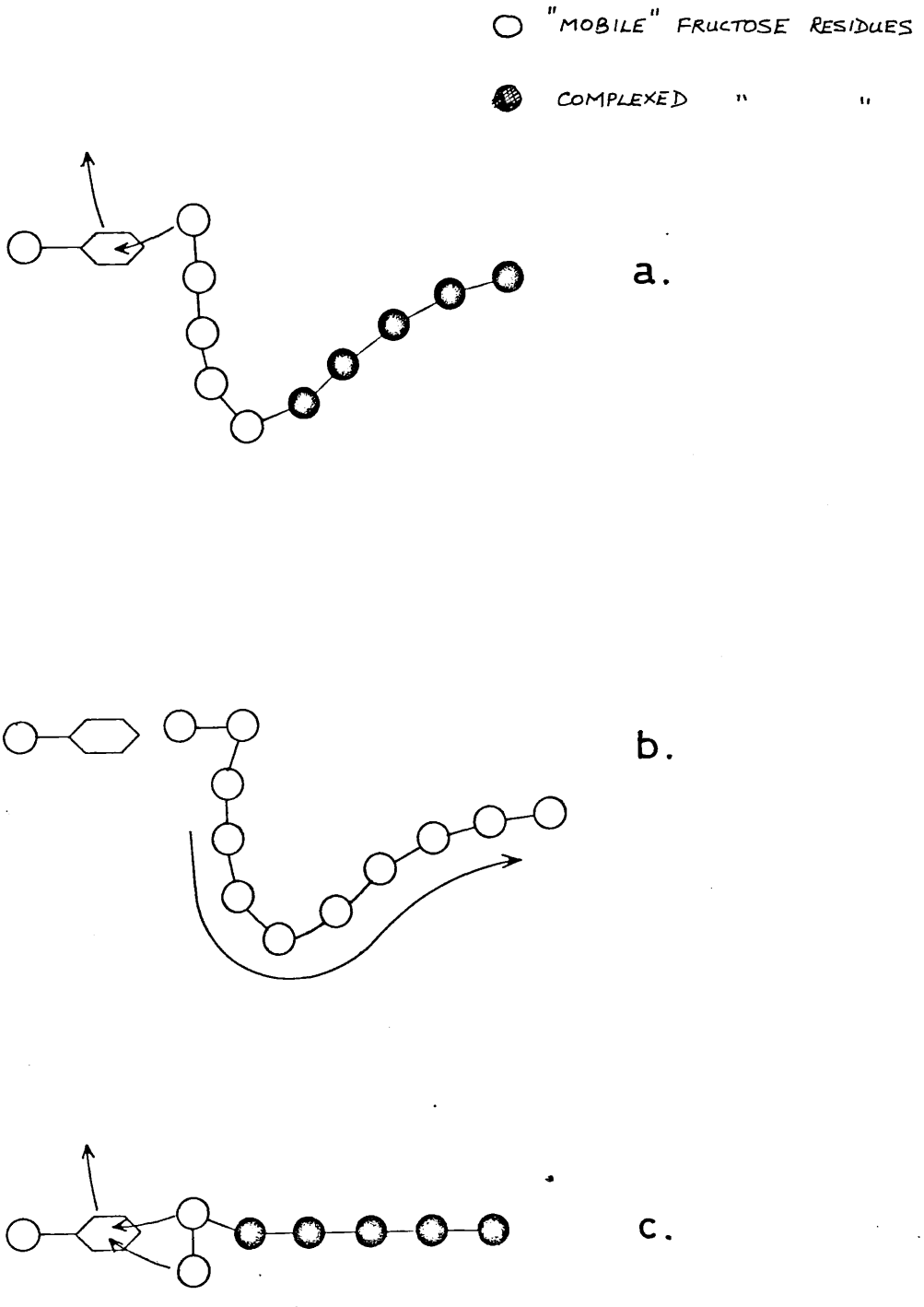
Figure 1.6. Multiaddition mechanism of levan synthesis[117]



Rapoport and Dedonder[119] attempted to explain the origin of branches. They established, by methylation analysis, that the degree of branching in the polymer is dependant upon the experimental conditions employed during the enzymic synthesis. In the presence of a high concentration of enzyme, the degree of branching is increased. The levansucrase of B. subtilis had already been shown to partially hydrolyse levans[112] (p. 38), the extent of hydrolysis being suppressed at high levan concentrations. This was thought to be due to the formation of new branch linkages under these conditions, verified by determination of the degree of branching of a levan before and after enzyme hydrolysis. Thus it seems that the formation of branch points is due to the levansucrase itself.

The mechanism of this branching is not completely clear in the context of the multiaddition mechanism, but it may be that when the "mobile" end of the growing chain becomes sufficiently long, the alignment of the "acceptor residue" may be such that transfer to the C-1 primary alcohol becomes favoured (Fig. I.7.a.,b.). Then, upon dissociation and movement of the chain along the enzyme to the "relaxed" position (Fig. I.7.c.), a choice of two fructosyl non-reducing ends arises for the subsequent propagation reaction (Fig. I.6.).

Figure 1.7. Possible mechanism for the initiation of branch points in levans



I.D. The Role of Bacterial Fructans in Dental Caries

The belief is now widely held that microbial dental plaque is a necessary prerequisite for the initiation of dental caries.[120]

The main characteristics of dental plaque, the ability to form acidic substances from fermentable carbohydrates and the ability to synthesise extracellular substances, were recognised as early as the last decade of the nineteenth century.[121] Black[122], in 1906, described dental plaque as "a substance that is gelatinoid or gelatine - like, but not gelatine, but which has the appearance of the latter. This material is formed by the same microorganisms which produce the acids that cause caries." During the following years, much controversy existed as to the nature of the extracellular plaque material. Kirk[123,124] claimed the gelatinous material to be the result of lactobacillus precipitated salivary mucin. Subsequently, it has been found that saliva on incubation loses sialic acid and fucose[125,126], and Leach[127] postulated that these hydrolytic steps are necessary prerequisites before salivary glycoproteins can contribute to plaque formation. However, there is also evidence[128,129] that native salivary glycoproteins can be absorbed on the tooth surface, a subject reviewed by Critchley *et al.*[130] in 1968.

It is now known that the bulk of extracellular plaque material consists of bacterial polysaccharides. *Streptococcus sanguis*[131] accounts for about half of the streptococcal flora of dental plaque[132] and members of this species were found to produce extracellular dextran.[91,133] Large amounts of extracellular polysaccharides are synthesised by cariogenic strains of *Streptococcus mutans*.[134-137] Further, periodontal plaque has been associated with *Odontomyces viscosus*[70], an organism capable of synthesising an extracellular fructan from sucrose or raffinose containing

media.[71] There is, in fact, a whole gamut of other bacterial strains associated with dental plaque, notably lactobacilli which synthesise hetero- as well as homo-polysaccharides.[138] In addition, plaque streptococci can, under certain dietary circumstances, produce pronounced quantities of capsular material, as shown by electron microscopy.[139]

Actual evidence for the presence of extracellular polysaccharides in dental plaque was provided by McDougall[140], in 1964, who showed that an aqueous extract contained a fructan. Subsequently, Critchley et al.[130] found fructans and glucans and a glucan to be respectively present in aqueous extracts and 0.1 M NaOH extracts of dental plaque. On the basis of modified Smith degradations they concluded these polysaccharides to be levans and dextrans. Because of the difficulty in harvesting sufficient amounts of dental plaque and the pronounced complexity and variability of this substance, many studies on the extracellular polysaccharides of oral streptococci, notably strains of S. mutans[141,142] have been made by in vitro experiments, minor amounts of fructan being present in many cases.

Some studies on dextransucrases isolated from plaque streptococci have been made. Wood[143], in 1967, obtained a crude dextransucrase preparation from an FA strain of S. mutans and Gibbons and Nygaard[144] demonstrated the presence of both dextransucrase and levansucrase in the culture fluids of other strains of this species. Carlsson[145], in 1970, isolated and purified a levansucrase from S. mutans OMZ 176.

A clue to the possible biological function of levan in dental plaque was provided by Manly[146], in 1961, who noted that dental plaque, after a short exposure to sucrose, continually produces acid for up to six hours. A carbohydrate storage mechanism was postulated, although, at that time, the carbohydrate intermediate was not identified. Gibbons and Banghart[147] incubated mixed cultures from dental plaque, saliva and the tongue with the dextran elaborated by

Leuconostoc mesenteroides and the so-called levan of Streptococcus salivarius. The dextran was relatively resistant to microbial attack whereas the levan was found to be completely hydrolysed. In addition, Leach[148] demonstrated that the proportion of "levan" in the plaque increases when plaque is subjected to increasing concentrations of sucrose. It was found to be rapidly formed initially and diminish in quantity upon cessation of the sucrose exposure. Thus it seems that the "levans" in plaque function as a storage polysaccharide and the dextrans probably serve as a binding agent for the plaque matrix. To further support this hypothesis, Da Costa and Gibbons[149], in 1968, isolated plaque streptococci which, in the presence of levan, produced induced levan hydrolases (levanases). Manly and Richardson[150] succeeded in extracting similar enzymes from dental plaque.

Although the factors involved in the establishment of carious lesions are manifold, it is certain that the ingestion of sucrose is a very important one.[151,152] Cariogenic streptococcal strains produce more copious quantities of extracellular polysaccharides when grown in sucrose containing media than in media containing other dietary sugars.[147] The significance of sucrose, in this context, has been further demonstrated by experimentation with animal diets.[153,154]

Polysaccharides elaborated by bacteria from sucrose undoubtedly play a key role in the establishment of caries; indeed attempts aimed at diminishing plaque formation and hence caries incidence have been made by incorporating dextranases in animal diets [155, 156]. Results in this direction have, however, been disappointing.[157]

It is uncertain to what extent the fructans elaborated by strains of S. salivarius contribute towards the initiation of dental caries. According to Snyder et al.[158], these substances do not act as reserve polysaccharides because of their solubility. Newbrun and Baker[159], in 1968, however, showed that the fructan of

S. salivarius ATCC 13419, thought to be a levan on the basis of i.r. spectroscopy[48], possesses a very high molecular weight ($16 - 23 \times 10^6$). It is possible that such large molecules, albeit soluble, if formed within the plaque matrix, would be unable to diffuse out, thus remaining available for subsequent localised metabolism.

I.E. Streptococcus salivarius

Streptococcus salivarius is a chain forming species belonging to the family Lactobacteriaceae, tribe Streptococcae, genus Streptococcus.

Andrews and Horder[160], in 1906, first gave the name Streptococcus salivarius to the streptococci isolated from the human oral cavity that exhibited the ability to curdle milk - a characteristic not possessed by Streptococcus mitis. Oerskov and Paulsen[161], in 1931, described the excessive polysaccharide synthesis, by streptococci isolated from the human pharynx, when grown in sucrose and raffinose containing media. At this time, S. salivarius was not a well defined species; indeed Sherman[162], in 1937, considered all non-hemolytic streptococcal strains of the human throat to belong to this species. Niven et al.[163] added the ability to synthesise large amounts of soluble fructan from sucrose and raffinose to the defining criteria for this species. Indeed, the ability to form large, soft, mucoid colonies on 5% sucrose agar provides a rapid test in the identification procedure.[164,165] To further strengthen the status of S. salivarius as a well defined species, Niven and Smiley[166] formulated a specific medium that supported strains of S. salivarius but not those of other, more heterogeneous, throat streptococci belonging to the S. mitis group. More recently, classifications of greater sophistication of oral streptococci have been made[135,167], the result of Drucker and Melville's work[167] suggesting that S. salivarius

is a single species, apparently intermediate between the well defined S. mutans and the more heterogeneous S. mitis.

The extent of the participation of S. salivarius and the extracellular polysaccharides and enzymes thereof in the establishment of dental caries is uncertain. It is certain, however, that S. salivarius , although not a major plaque microorganism, is a factor in this disease. This statement is supported by the finding that of all the viridans strains of streptococci investigated, those of S. salivarius were found to produce the greatest amounts of lactic acid.[168] Furthermore, the statistical findings of Shiere et al.[169] indicate a correlation between the population density of S. salivarius in the saliva and the incidence of carious lesions in a random selection of people. In addition, they demonstrated that S. salivarius , in in vitro experiments, is capable of decalcifying tooth enamel in glucose containing media, and to a greater extent in sucrose containing media. Sodium fluoride (1 ppm) was found to inhibit the growth of this organism.

I.F. Aim of the Present Work

The aim of the work, documented in this thesis, has been to characterise the extracellular polysaccharides elaborated by some strains of Streptococcus salivarius, to isolate and determine some of the properties of the extracellular hydrolase produced by one of these strains and to develop methods aimed at determining the type structure of fructans in general.

II. GENERAL METHODS

II.A. Common Procedures

II.A.i. Evaporations were carried out under reduced pressure with a "Buchi" rotary film evaporator.

II.A.ii. Water. Deionised, distilled water was used in all cases unless stated otherwise.

II.A.iii. Lyophilisation (Freeze-drying) was carried out on a "Chem-Lab" freeze-drier.

II.A.iv. Ultra-violet and visible absorbances were measured using a "Pye Unicam" SP500 spectrophotometer.

II.A.v. Molecular sieves. 3 A and 4 A molecular sieves (BDH) were activated by heating at 350° for 4 h.

II.A.vi. Cotton wool bungs for stoppering of culture flasks were bandaged to prevent them igniting during "flaming-off".

II.A.vii. Dialysis was performed with "Visking" tubing ($^{18}/_{32}$" size unless stated otherwise). The tubing was initially rendered free of glycerol by boiling in several changes of water.

II.A.viii. Centrifugation was carried out with a Beckman J21 centrifuge, using JA 10 or JA 20 rotors, as stated.

II.B. Purification of Common Solvents and Reagents

II.B.i. Dimethyl sulphoxide was distilled from calcium hydride under reduced pressure so that the boiling point was about 80°. It was stored over activated 4 A molecular sieve.

II.B.ii. Chloroform was distilled from anhydrous sodium sulphate and stored, refridgerated, in brown glass bottles over anhydrous sodium sulphate.

II.B.iii. N,N - dimethyl formamide was distilled from calcium hydride under reduced pressure and stored over activated 4 A molecular sieve.

II.B.iv. Methanol ("Superdry") was prepared as described by Vogel [170], distilled with the exclusion of moisture, and stored over activated 3 A molecular sieve.

II.B.v. Methyl Iodide was distilled from silver oxide and stored in the dark over silver oxide, activated by heating to 110^{o} for 4 h.

II.B.vi. Pyridine was distilled from sodium hydroxide pellets and stored over sodium hydroxide or potassium hydroxide pellets (BDH, Analar).

II.C. Chromatography Solvents

II.C.i. Solvent 1: for paper chromatography

n-butanol	:	40	parts by volume
ethanol	:	11	" " "
water	:	19	" " "

II.C.ii. Solvent 2: for thin-layer chromatography [171]

n-butanol	:	4	parts by volume
acetic acid	:	1	" " "
water	:	5	" " "

The above mixture was shaken together in a separating funnel and the upper layer was used as the solvent.

II.C.iii. Dipping reagent for paper chromatography [172]

a. Silver nitrate

stock solution : saturated $AgNO_3$

dip solution : 5 ml stock solution in acetone (1 litre). Add water to redissolve precipitate.

b. Sodium hydroxide

stock solution: 500 ml H_2O + 250 g NaOH pellets

dip solution : 960 ml ethanol + 40 ml stock solution

c. 10% aqueous sodium thiosulphate

II. D. Buffer Tables

II. D. i. Citrate - phosphate buffers (0.1 M)[173]

Stock solutions:

A : 0.1 M citric acid (BDH, Analar)

B : 0.2 M dibasic sodium phosphate (BDH, Analar)

Preparation : x ml A + y ml B, diluted to 100 ml

x	y	pH	x	y	pH
44.6	5.4	2.6	24.3	25.7	5.0
42.2	7.8	2.8	23.3	26.7	5.2
39.8	10.2	3.0	22.2	27.8	5.4
37.7	12.3	3.2	21.0	29.0	5.6
35.9	14.1	3.4	19.7	30.3	5.8
33.9	16.1	3.6	17.9	32.1	6.0
32.2	17.7	3.8	16.9	33.1	6.2
30.7	19.3	4.0	15.4	34.6	6.4
29.4	20.6	4.2	13.6	36.4	6.6
27.8	22.2	4.4	9.1	40.9	6.8
26.7	23.3	4.6	6.5	43.6	7.0
25.2	24.8	4.8			

II.D.ii. Phosphate buffers (0.1 M)[174]

Stock solutions:

A : 0.2 M monobasic sodium phosphate (BDH, Analar)

B : 0.2 M dibasic sodium phosphate (BDH, Analar)

Preparation : x ml A + y ml B, diluted to 200 ml

x	y	pH	x	y	pH
93.5	6.5	5.7	45.0	55.0	6.9
92.0	8.0	5.8	39.0	61.0	7.0
90.0	10.0	5.9	33.0	67.0	7.1
87.7	12.3	6.0	28.0	72.0	7.2
85.0	15.0	6.1	23.0	77.0	7.3
81.5	18.5	6.2	19.0	81.0	7.4
77.5	22.5	6.3	16.0	84.0	7.5
73.5	26.5	6.4	13.0	87.0	7.6
68.5	31.5	6.5	10.5	90.5	7.7
62.5	37.5	6.6	8.5	91.5	7.8
56.5	43.5	6.7	7.0	93.0	7.9
51.0	49.0	6.8	5.3	94.7	8.0

II.E. Bacteriological Media

II.E.i. Medium I. : To obtain bacteria in a non dormant state.

Todd-Hewitt Broth concentrate (Oxoid)	10 tablets
D-glucose (BDH, Analar)	1 g
distilled water	100 ml

II.E.ii. Medium II. : Maintenance medium for the sub-culturing of streptococci

Brain Heart Infusion concentrate (Oxoid)	5	tablets
Thioglycollate medium (Difco)	2.4	g
D-glucose (BDH, Analar)	0.5	g
distilled water	100	ml

II.E.iii. Medium III : For the synthesis of fructans.[163]

Tryptone (Difco)	1%
sucrose (BDH, Analar)	8%
K_2HPO_4	0.2%

dissolved in distilled water

II.E.iv. Medium IV : For the production of levanase.

Tryptone (Difco)	1%
Yeast extract (Oxoid)	0.5%
K_2HPO_4	0.3%
levan	1.0%
D-glucose (BDH, Analar)	0.1%

II.F. Bacteriological Procedures

II.F.i. Reactivation of lyophilised bacterial cells

A 1 oz McCartney bottle, with a drilled cap, was filled with Medium I (II.E.i.) (10 ml), sterilised by autoclaving at 122° (15 p.s.i.) for 15 min and allowed to cool to room temperature.

The tube of lyophilised material was manipulated in a sterile cabinet which had previously been irradiated for 1 h with a u.v. lamp. The tube was initially tapped to loosen the contents and deposit them at one end of the tube. The tube was then filed at the opposite end and opened in the vicinity of a Bunsen flame, the open end being flamed off. The contents of the tube were then rapidly transferred to the McCartney bottle. The bottle mouth and cap were flamed off and the cap was replaced.

The bottle was maintained at 37° for 24 h the culture being then ready for sub-culturing.

II.F.ii. Sub-culturing of streptococcal strains

Sufficient calcium carbonate (BDH, Analar) to cover the bottom of a 1 oz McCartney bottle was placed into the required number of bottles which were filled with at least 10 ml of Medium II (II.E.ii.). The bottles were sterilised by autoclaving at 122° (15 p.s.i.) for 15 min and allowed to cool to room temperature.

By means of sterile, disposable syringes (5 ml), a suspension of bacterial cells (II.F.i., 1 ml) was introduced into each bottle, several bottles being inoculated with each strain.

The cultures were maintained at 37° for 24 h and then stored at 4° ready for use. The cells remain viable in this medium for at least one month.

II.G. Chromatographic Methods

II.G.i. Paper chromatography (qualitative)

Paper chromatography was performed on Whatman No. 1 paper by the descending solvent method, using solvent 1 (II.C.i.). After development, papers were dried in an oven at 60° and the chromatographed components were rendered visible using the silver nitrate - sodium hydroxide dipping reagents (II.C.iii.), allowing the papers to air dry between dips.

II.G.ii. Paper chromatography (preparative)

Preparative paper chromatography was performed on Whatman Nos. 17 and 3 mm papers. The papers were prepared as described by Frush[175], water washed for 24 h and allowed to air dry thoroughly.

The mixture to be chromatographed was streaked along the starting line, this being repeated until all of the mixture had been applied. After development in solvent 1 (II.C.i.) for the appropriate time, papers were removed from the tank and air-dried. Three strips, about 1.5 cm wide, were cut from the two edges and centre of the paper. The components on these strips were rendered visible with the silver nitrate - sodium hydroxide reagents (II.C.iii.). The papers were then cut into segments, with the aid of the reference strips, corresponding to the components separated. The material on each segment was eluted off with water, the solution obtained being lyophilised.

II.G.iii. Thin-layer chromatography (t.l.c.)

T.l.c. was performed on pre-coated silica-gel plates (Polygram SIL G), development being carried out with solvent 2 (II.C.ii.) until the solvent-front neared the top of the plate. The plates were removed from the development tank, the position of the solvent-front was marked, and the plates were allowed to air-dry. The separated components were rendered visible by spraying the plates with sulphuric acid (5%, in ethanol), and heating in an oven at 120° for 10 min.

II.H. Analytical Determinations

II.H.i. Fructose determination : Alcoholic anthrone method.[176]

Alcoholic anthrone reagent : Absolute ethanol (50 ml) was placed in a cooled B19 conical flask (500 ml) and sulphuric acid (BDH, Analar, 100 ml) was slowly added with cooling and swirling. When the mixture had returned to room temperature, anthrone (200 mg) was added and dissolved by swirling.

The reagent thus prepared was stoppered and stored at 3^{o}. It remains usable for 3 weeks.

Procedure : An aqueous solution of D-fructose, of accurately known concentration (ca. 60 $\mu g\ ml^{-1}$), was prepared and solutions of the substances being analysed, of accurately known concentration, were also prepared so that the concentrations with respect to fructose were ca. 60$\mu g\ ml^{-1}$. Into stoppered boiling tubes was placed 0.5 ml of each solution and 1.5 ml of water. Into a further boiling tube was placed water (2.0 ml) to serve as a reagent blank. The tubes were cooled in ice-water and alcoholic anthrone reagent (8 ml) was added to each tube, with layering. The stoppers were replaced and mixing was accomplished by inversion. The solutions were maintained, in a water bath, at 50^{o} for exactly 20 min and then cooled for 1 min in an ice-water bath. The contents of the tubes were then transferred to 1 cm glass cells and the absorbances at 620 nm were measured against the reagent blank. Readings were taken twice, from tubes 1 to n and then from n to 1, the mean of the two readings being taken.

The quantity of fructose in the test solutions was found by comparison of the absorbances with that of the known standard. In the case of polysaccharides containing anhydro-D-fructose residues, the results were corrected by use of the factor $\frac{162}{180}$ (0.9) $\left(\frac{\text{"molecular" weight anhydro-D-fructose residue}}{\text{molecular weight fructose}}\right)$.

II.H.ii. Carbohydrate determination : Phenol - sulphuric acid method [177]

Note: Because different sugars, in particular ketoses and hexoses, develop different colours in this method, it is necessary to construct appropriate calibration graphs.

Calibration graphs : D-fructose (in the case of fructans, 0 - 100 μg in 10 μg steps) was made up to 1 ml with water in each of 11 boiling tubes. Phenol (4%, 1 ml) was added to each tube followed by 18 M sulphuric acid (BDH, Analar, 5 ml) rapidly added from a dispenser as consistently as possible. The contents of the tubes were allowed to cool to room temperature and the absorbances at 470 nm, relative to the reagent blank, were obtained (II.A.iv.) using 1 cm cells. A calibration graph was constructed plotting carbohydrate (in μg) against absorbance at 470 nm.

Carbohydrate determination: Having established which sugar(s) is (are) present, and having constructed the appropriate calibration graph, the carbohydrate content was determined as follows.

A solution of the dry material under test, of accurately known concentration, was prepared containing ca. 50 $\mu g\ ml^{-1}$ carbohydrate. One ml of this solution and a 1 ml water blank were then treated as above and the carbohydrate content found from the appropriate calibration curve.

In the case of polysaccharides composed of anhydro-hexose units, the result is corrected by the use of the factor $0.9\ (\frac{\text{molecular weight anhydro-hexose unit}}{\text{hexose}})$.

II.H.iii. Reducing sugar determination[178]

Reagent A: Anhydrous sodium carbonate (25 g), sodium potassium tartrate (25 g), sodium bicarbonate (20 g) and anhydrous sodium sulphate (200 g) were dissolved in water (800 ml) and made up to 1 l with water.

Reagent B: Cupric sulphate pentahydrate (30 g) was dissolved in water (200 ml) containing four drops of sulphuric acid (18 M, S.G. 1.84).

Reagent C: Ammonium molybdate tetrahydrate (25 g) was dissolved in water (450 ml) to which sulphuric acid (18 M, 21 ml) was added. Sodium arsenate heptahydrate (3 g) was dissolved separately in water (25 ml) and added slowly to the above solution with stirring. The whole was diluted to 500 ml and warmed for 30 min in a water bath at 30°.

Reagent D: Reagent B (1.0 ml) was added to reagent A (25 ml).

Calibration graph: A solution of D-fructose (or D-glucose), of accurately known concentration, containing ca. 300 $\mu g\ ml^{-1}$ was prepared and aliquots (0.1-1.0 ml, 0.1 ml steps) were placed in boiling tubes and made up to 1.0 ml with water. A further tube contained only water (1.0 ml) to serve as a reagent blank. Reagent D (1.0 ml) was added to each tube which was then placed in a boiling water bath for 10 min and cooled under running cold tap water for 5 min. Reagent C (1.0 ml) was added to each tube, the tubes being shaken to liberate carbon dioxide evolved. Water (22 ml) was added to each tube. The contents were mixed by inversion and allowed to stand for 20 min. The absorbances at 520 nm were measured against the reagent blank using 1 cm glass cells (II.A.iv.) and a graph was constructed plotting absorbance against weight of reducing sugar.

Determination of reducing sugar content: An aqueous solution of the material or solution under test of accurately known concentration, was prepared so that an aliquot of 1 ml (or less) contained up to 250 µg reducing sugar (as hexose). The aliquot was made up to 1.0 ml with water, the procedure being then as above. The weight of reducing sugar (expressed as fructose or glucose) in the aliquot taken was found from the calibration graph.

II.H. iv. Determination of the degree of polymerisation (DP) of reducing oligosaccharides.[179]

The method depends upon measuring the carbohydrate content of a solution of the oligosaccharide before and after reduction with a solution of potassium (or sodium) borohydride.

A solution of 4% potassium borohydride (25 ml) was prepared and diluted as follows :

a) 10 ml was made up to 20 ml with water (active borohydride solution)

b) 10 ml was made up to 20 ml with 1M-sulphuric acid (inactive borohydride solution)

Procedure : An aqueous solution of the oligosaccharide was prepared, containing between 30 and 60 µg ml^{-1}. Aliquots (1 ml) of this solution were treated, in duplicate, with active borohydride solution (1 ml) and inactive borohydride solution (1 ml) and left to stand in capped tubes for 24 h. The carbohydrate content of each tube was determined by the phenol-sulphuric acid method (II.H. ii.).

Calculation :

Degree of polymerisation, $DP = \frac{A}{A - A\,red}$

where A = absorbance at 487 nm of unchanged oligosaccharide

A red = absorbance at 487 nm of reduced oligosaccharide

II. J. Specific Preparations and Methods

II. J. i. Preparation of methylsulphinylmethylsodium ("dimsyl" carbanion)[180]

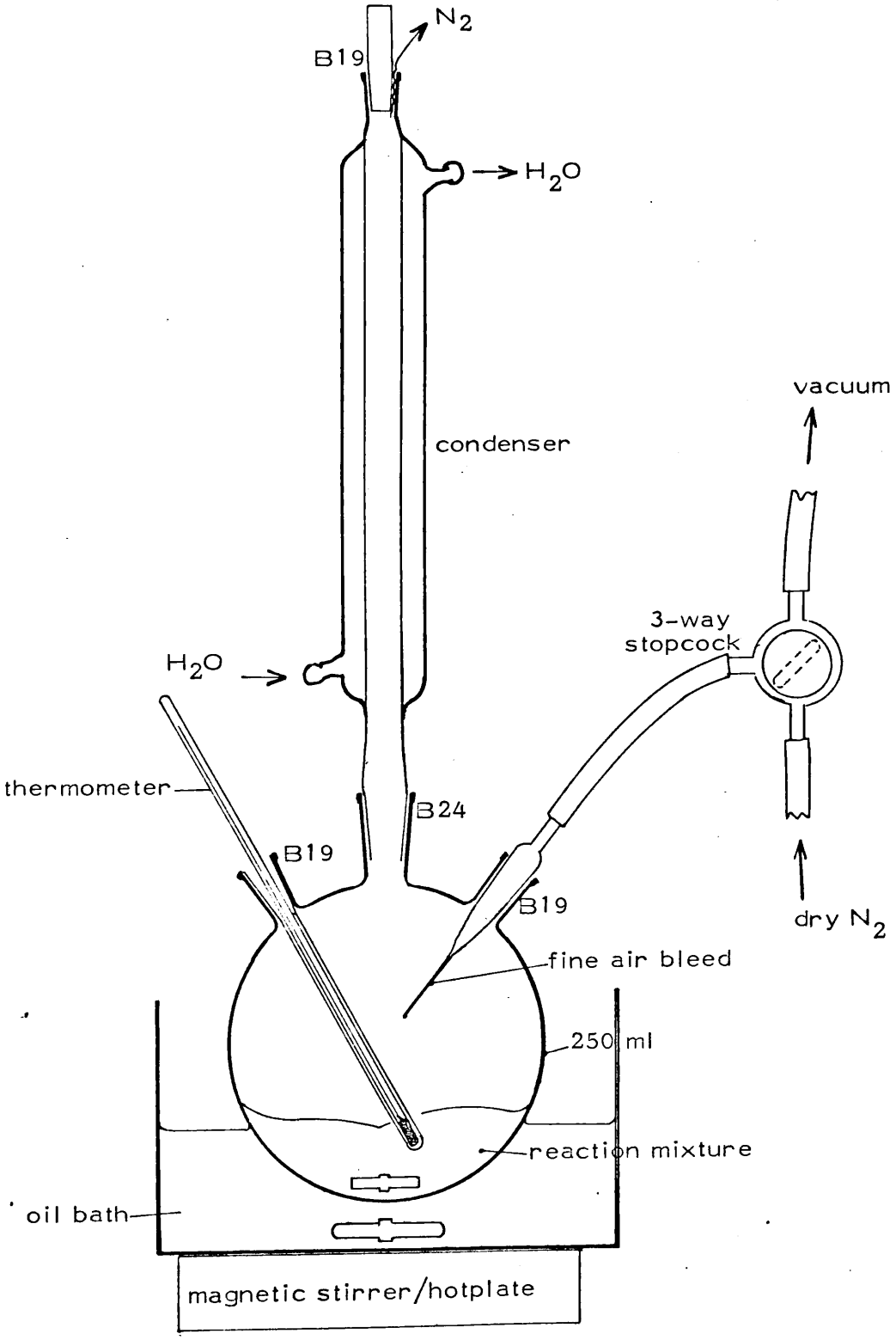
Reaction: Methylsulphinylmethylsodium is formed by the reaction between dry sodium hydride and dimethyl sulphoxide in the absence of air, the resultant carbanion being resonance stabilised.

$$NaH + (CH_3)_2S{=}O \xrightarrow[N_2]{} CH_3{-}S(=O)^{-}{-}CH_2Na^{+} + H_2$$

Method: Sodium hydride (50%, coated with mineral oil, 3g) was weighed into a three-necked 250 ml round bottom flask (B19, B24, B19) and sodium-dried n-pentane (60ml) was added. The mixture was swirled and allowed to stand, the clear solution of oil in pentane was decanted off and the process was repeated twice more. The flask containing the sodium hydride was set up as in the diagram (Fig. II. 1.) and evacuated for 15 min, the magnetic stirrer being in operation. The apparatus was then flushed with dry nitrogen, excess pressure being released by loosening the top B19 stopper. The flask was then alternatively evacuated and flushed with nitrogen until the sodium hydride was completely dry.

Redistilled, dry dimethyl sulphoxide (II. B. i.) was introduced, under a flow of nitrogen, via the condenser. The mixture was stirred, at 45°, for several hours until the solution became almost clear and evolution of hydrogen had ceased. The carbanion solution, thus prepared, was transferred to

Figure II.1. Apparatus for the preparation of "dimsyl" carbanion



clean, dry McCartney bottles which were tightly capped and refridgerated ready for use. Dimsyl carbanion prepared in this way remains usable for several weeks.

Titration: Dimsyl carbanion solution (1 ml) was placed in a conical flask (100 ml), with a little water (in a fume cupboard !) and phenol-phthalein indicator (1 - 2 drops), and titrated against 0.1 M-hydrochloric acid.

N.b. For methylation, according to the method of Hakomori[181], at least 2M dimsyl carbanion solution should be used.

II. J. ii. Gas chromatography-mass spectrometry (gc-ms) of O-acetyl-O-methyl-alditols

Instruments:
Perkin Elmer gas chromatograph, model F11, (with modified oven head)
Hitachi Perkin Elmer RMS4 mass spectrometer
Hitachi Perkin Elmer 196 twin-pen recorder
SE model 3006 ultra-violet recorder

Experimental conditions:

column	: 12 foot, narrow bore, packed with OV255, 3%, on Chromasorb Q, 80 - 100 mesh	
oven temperature	: 190° (unless otherwise stated)	
carrier gas	: helium	10 p.s.i.
detector gases	: hydrogen	30 p.s.i.
	air	20 p.s.i.
mass range	: 0 - 600	

Procedure: The alditol acetate mixture was dissolved in redistilled chloroform (2-3 drops, II.B.ii.) and 1 μl was injected into the gas chromatograph. When the total ion monitor registered the presence of each chromatographed peak in the ionisation chamber of the mass spectrometer, the mass spectrum of the component(s) giving rise to the peak was recorded.

II.J.iii. Measurement of retention time and peak area by gas chromatography - peak integration

Instruments:

Pye 104 gas chromatograph

Hewlett-Packard series 3370 B integrator

Experimental conditions:

columns	: 9 foot, glass, packed with OV225 3% on Chromasorb Q, 80-100 mesh
injection volume	: 1 μl in chloroform solvent
oven temperature	: 175^{o} (unless otherwise stated)
carrier gas	: nitrogen 40 ml min^{-1}
detector gases	: hydrogen 17 p.s.i. air 12 p.s.i.
amplifier	: 20 x 10^{2} attenuation
integrator	: full scale deflection at 10 mV input
slope sensitivity	: 0.03 mV min^{-1} up 0.03 mV min^{-1} down
noise suppression	: 3
base line reset delay	: 0.1 min
area threshold	: 100
shoulder control	: front ON rear 10 mV

Procedure: The chromatograph was used with two identical columns with equalised carrier gas flow rate to ensure a stable base line. Retention times (in seconds) and peak areas were automatically recorded by the integrator.

II. J. iv. Preparation of a charcoal - celite column (44 x 7 cm) for the separation of oligosaccharides

A mixture of activated charcoal (BDH, 400 g) and celite (545, Koch-Light, 400 g) was treated with concentrated hydrochloric acid (2.5 l) for 24 h. The supernatent liquid was decanted off, and the pH of the mixture was raised by successively adding water, mixing, allowing to settle and decanting off the supernatent liquid. The solid was then filtered off in a Buchner funnel, under vacuum, and washed with water until the filtrate was neutral. The filter cake was treated with ethanol (2.5 l) for 24 h, with occasional stirring. It was then filtered off, as before, and water washed extensively to free it of ethanol. The filter cake was then slurried with water to give a smooth, pourable mixture.

A 2 inch thick layer of acid washed celite (545) was introduced into the bottom of a glass column (44 x 7 cm), fitted with a stop-cock and plug of glass wool in the constriction above the stop-cock. The charcoal - celite slurry was carefully added above the celite layer. The stop-cock was opened and further additions of slurry were made with stirring up of the bed surface. After all the slurry had been added and the bed had packed down, a further layer of acid-washed celite was added. The column was washed by eluting through several litres of distilled water.

II. J. v. Preparation of a charcoal - celite column (70 x 7.5 cm) for the separation of oligosaccharides

The procedure was the same as in II. J. iv. except that double quantities of charcoal, celite (800 g of each), hydrochloric acid and ethanol (5 litres of each) were required.

II. J. vi. Methylation of oligosaccharides

The oligosaccharide (0.5 - 2.0 mg) was dried for 24 h at 60° under vacuum and then shaken with methyl iodide (0.2 ml), N,N - dimethylformamide (0.2 ml) and silver oxide (0.2 g) at room temperature, for 18 h, in the dark. The mixture was filtered through a Hirsch funnel, under vacuum, containing a glass filter paper, the residue being rinsed with a little dry redistilled chloroform. The combined filtrate and rinsings were evaporated to dryness, at room temperature, at a pressure of 0.3 mm Hg.

II. J. vii. Hydrolysis of methylated oligosaccharides

The methylated oligosaccharide (II. J. vi) was dissolved in methanol (2 ml), oxalic acid (0.5 M, 0.5 ml) was added and the mixture was refluxed for 18 h. Excess methanol was removed by evaporation under reduced pressure, at 35°, and replaced with water (2 ml). Refluxing was continued for 3 h, the reaction mixture then being neutralised with calcium carbonate (BDH, Analar). Excess calcium carbonate and insoluble calcium oxalate formed were removed by filtration under vacuum. The precipitate was rinsed with a little cold water, the rinsings and filtrate being combined.

II. J. viii. Borodeuteride reduction of hydrolysed, methylated oligosaccharides

To the combined filtrate and rinsings from II. J. vii. was added excess (ca.2 mg) sodium borodeuteride (Merck). The mixture was allowed to stand at room temperature for 2 h, and was then neutralised with washed Dowex 50W-X8 resin (BDH). The resin was removed by filtration and the filtrate was evaporated under reduced pressure to dryness. The residue was thrice co-distilled with methanol (BDH, Analar, 5ml), the final time being to dryness.

II. J. ix. Acetylation of partially methylated alditols

The partially methylated alditols, deuterium labelled at position C-2 (II. J. viii.), were treated with pyridine (0.5 ml) and acetic anhydride (BDH, Analar, 0.5 ml). The mixture was allowed to stand at room temperature for 10 min before heating on a steam-bath for 20 min, the reaction flask being fitted with a reflux condenser with a calcium chloride drying tube. Excess acetylating agents were removed by evaporation under reduced pressure, the final traces by co-distillation with toluene or water.

III. EXPERIMENTAL

III.A. Preparation of Polysaccharides Elaborated by *Streptococcus salivarius* from a Sucrose-Containing Medium

III.A.i. Optimum culture time determinations

Medium III (II.E.iii., 100 ml) was placed in each of five 250 ml conical flasks. Calcium carbonate (BDH, Analar, 5 g) was added to each flask, these being stoppered with cotton wool plugs (II.A.vi.) and sterilised by autoclaving at 122° (15 p.s.i.) for 15 min.

Each flask was inoculated with a freshly sub-cultured suspension of bacterial cells (II.F.ii., 5 ml), in the vicinity of a flame, with a sterile disposable syringe, the cotton wool stopper being flamed off before replacement. The cultures were maintained at 37°, removed at time intervals of 24 h and subjected to the following procedure.

The culture pH was measured and the culture fluid was centrifuged (II.A.viii.), for 20 min, at 3000 rpm (980 g, JA10 rotor) and 0°. The volume of the supernatent was measured and ethanol (2.3 volumes) was slowly added, with stirring, to render the concentration with respect to ethanol, 70%. The insoluble polysaccharide was precipitated by centrifugation at 980 g (JA10 rotor) for 20 min, the supernatent being discarded. The centrifugate was dissolved in water (100 ml), the water being added in small portions, reprecipitated with ethanol (2.3 volumes) and again centrifuged. The re-dissolution,

re-precipitation procedure was repeated a further 3 times. Finally the product was dissolved in water (100 ml), dialysed (II.A.vii.) for 48 h and freeze dried. The weight of freeze dried product was measured.

III.A.ii. Preparation of a large batch of polysaccharide from 8% sucrose broth

Medium III (II.E.iii., 1.25 l) was placed in each of four 2 litre conical flasks together with calcium carbonate (BDH, Analar, 30 g). The flasks were equipped as in Fig. III.1., sterilised by autoclaving at 122° (15 p.s.i.) for 15 min and allowed to cool to room temperature.

Each flask was inoculated, via the Pasteur pipette, with a freshly sub-cultured suspension of bacterial cells (II.F.ii., 5 ml) from a sterile disposable syringe. The cultures were maintained at 37° for the optimum time. The culture fluid was then further treated as in III.A.i.

III.A.iii. Preparation of a large batch of polysaccharide from 8% sucrose broth (dialysis tube method)

Six flasks were equipped as in Fig. III.2., and sterilised by autoclaving at 122° (15 p.s.i.) for 15 min. Each flask was inoculated, via the Pasteur pipette, with a freshly sub-cultured suspension of bacterial cells (II.F.ii., 5 ml) care being taken to avoid contamination. The cultures were maintained at 37° for the optimum time. The culture fluids contained in the dialysis tubes were then further treated as in III.A.i.

III.B. Specific Rotation of Polysaccharides

A solution, of accurately known concentration (ca. 1 mg ml^{-1}), of the dried polysaccharide was prepared. The rotation of this solution was measured in a cell of

Figure III.1. Culture flask for polysaccharide production

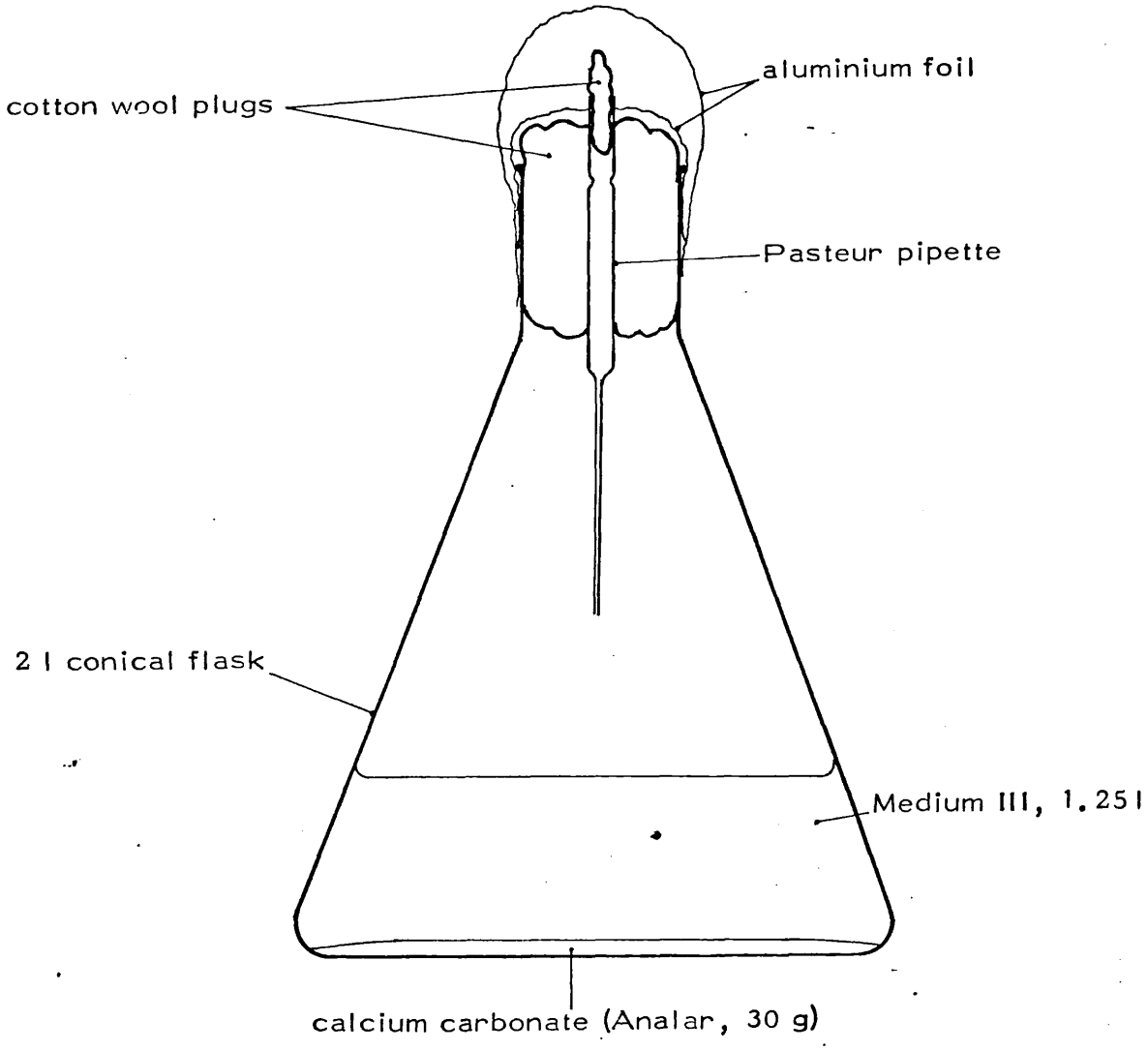
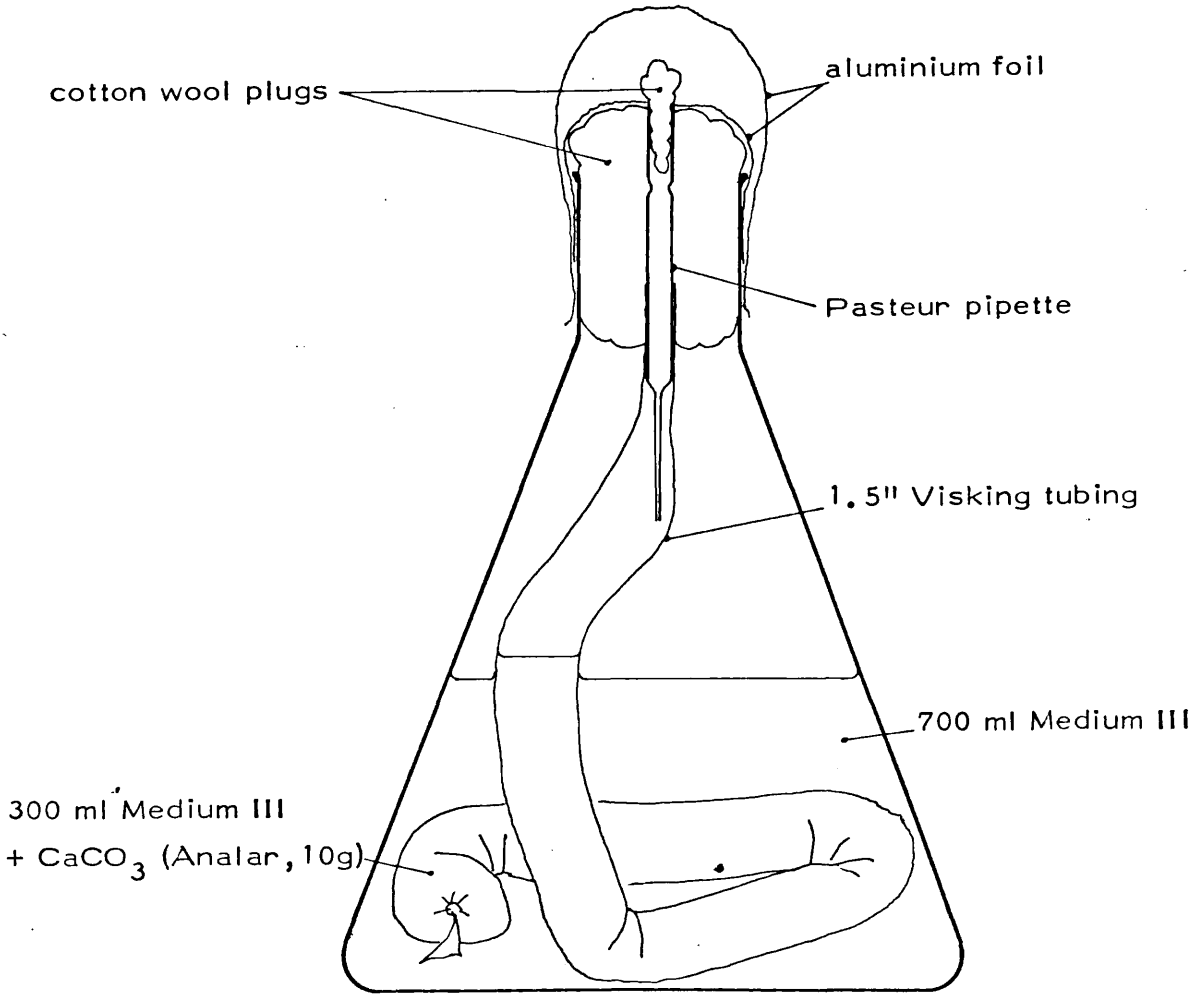


Figure III. 2. Culture flask for polysaccharide production (dialysis tube method)



1 dm path length and 1 ml capacity, at ambient temperature, using a Perkin Elmer 141 polarimeter, the instrument being initially set on zero with the cell, in position, containing deionised water. Negative rotations were found by subtracting the readings obtained from 100.000.

Calculation:

Specific rotation at temperature, t, $[\alpha]_D^t = \frac{\alpha \times 100}{cl}$

where α = measured rotation

c = concentration in g 100 ml^{-1}

l = path length in dm

D refers to measurement being made at the sodium-D-line (589 nm)

III.C. Partial Acid Hydrolysis Experiments

III.C.i. Preliminary partial acid hydrolysis of the fructan elaborated by Streptococcus salivarius strain 51

The polysaccharide (10 mg) was placed in each of two 25 ml round bottom flasks (B 14). Sulphuric acid (0.005 M, 1 ml) was added to one flask which was left at room temperature for 15 h, the contents then being neutralised with barium carbonate. Oxalic acid (0.005 M, 1 ml) was added to the second flask which was heated on a water bath at 70° for 3 h, the contents being neutralised with calcium carbonate. The resulting mixtures were filtered and the filtrates were subjected to paper chromatography (II.G.i.) for 24 h, D-fructose being used as a reference.

III.C.ii. Determination of the optimum time of partial hydrolysis with 0.005 M oxalic acid at 70°

The polysaccharide (10 mg) was placed in each of five 25 ml B 14 round bottom flasks and oxalic acid (0.005 M, 1.0 ml) was added to each flask. The flasks were

placed in a water bath at 70° for periods of 0.5, 1.0, 2.0, 3.0 and 4.5 h, the contents then being neutralised with calcium carbonate and filtered. The filtrates were partially concentrated under reduced pressure and examined by paper chromatography (II.G.i.) for 24 h with D-fructose as reference.

III.C.iii. Determination of R_f and R_m values of the products of the partial acid hydrolysis of the fructan elaborated by Streptococcus salivarius strain 51

The polysaccharide (20 mg) was hydrolysed for 2 h with 0.01 N oxalic acid (2 ml) at 70°. The hydrolysate was neutralised with calcium carbonate (BDH, Analar) and filtered. The filtrate was concentrated under reduced pressure and subjected to paper chromatography (II.G.i.), with D-fructose as a reference, for 15, 48, 72 and 144 h. From the 15 h chromatogram were obtained the distances moved, from the starting line, of the solvent front and fructose. The distances moved relative to fructose and disaccharide were measured, for all other components, from the 48, 72 and 144 h chromatograms.

III.D. Linkage Analysis of Fructans : Preparation of O-Acetyl-O-Methyl-Alditols

III.D.i. Methylation. (adapted from the method of Hakomori[181])

The fructan (20 mg) was weighed into a 1 oz. McCartney bottle, containing a small teflon-covered magnetic follower, and dried under vacuum at 60° for 24 h. Dry dimethyl sulphoxide (II.B.i., 4 ml) was added, the bottle being flushed with dry nitrogen prior to a drilled cap with a rubber seal being rapidly screwed on. The flask was warmed at 40°, with stirring, to ensure complete dissolution of the polysaccharide.

'Dimsyl' carbanion solution (II.J.i., 1 ml) was added dropwise from a syringeand the mixture was stirred for 6 h, with warming at 40° for the first hour. The bottle was then cooled in ice-water and redistilled methyl iodide (II.B.v., 0.1 ml) was added by syringe. The mixture was stirred, at room temperature, overnight and further dimsyl carbanion solution (1 ml) was added, the mixture being stirred for a further 6 h. An excess (1.5 ml) of methyl iodide was added, after cooling in ice-water, and the mixture was again stirred overnight. The resulting clear, straw coloured, solution was poured into a small quantity of water (ca.5 ml) and dialysed (II.A.vii.) against deionised water until only the methylated polysaccharide remained as a white solid.

The methylated polysaccharide was extracted with chloroform (BDH, Analar, 2x10 ml) in a separating funnel. The chloroform layers were run off, combined and evaporated to dryness under reduced pressure.

III.D.ii. Hydrolysis. (adapted from the method of Lewis et al.[69])

Methanol (BDH, Analar, 10 ml) was added to the methylated polysaccharide contained in a B19 25 ml round bottom flask. The flask was heated, under reflux, on an oil bath, until the methylated polysaccharide had dissolved. Oxalic acid (2.5 M, Analar, 3 ml) was added to the solution, the mixture being refluxed for 18 h. The methanol was evaporated off, under reduced pressure at 35° and replaced with water (10 ml). Refluxing was continued for a further 4 h. The solution was neutralised with calcium carbonate (Analar), cooled, and then filtered through a glass fibre filter paper, under vacuum, the residue being washed with small quantities of cold water.

III.D.iii. Reduction. To the combined filtrate and washings from III.D.ii. was added excess (10 mg) sodium borohydride. The mixture was maintained at room temperature for 4 h and neutralised with water-washed Dowex 50W-X8 (H) resin (BDH). The resin was removed by filtration and the filtrate was evaporated to dryness under reduced pressure. The residue was thrice co-distilled with methanol (BDH, Analar, 20 ml), the final time being to dryness.

III.D.iv. Acetylation. The partially methylated alditols, obtained in III.D.iii, were treated with redistilled, dry pyridine (II.B.vi., 1.0 ml) and acetic anhydride (BDH, Analar, 1.0 ml). The mixture was heated on a steam bath for 15 min, the flask being fitted with a condenser with calcium chloride drying tube. The acetylating reagents were removed by evaporation under reduced pressure, the final traces being removed by co-distillation with water.

III.E. Linkage Analysis of Fructans : Preparation of O-Acetyl-O-methyl-alditols, Deuterium-labelled at C-2

As III.D. except that sodium borodeuteride (Merck, 98% D) replaced sodium borohydride in the reduction step (III.D.iii.).

III.F. Oligosaccharide Preparations

III.F.i. Oligosaccharides from the partial acid hydrolysate of the levan elaborated by Streptococcus salivarius strain 51

S. salivarius 51 levan (15 g) was hydrolysed with oxalic acid (0.005 M, 1.5 l) at 70° for 2 h. The mixture was neutralised with calcium carbonate (BDH, Analar) and filtered. The filtrate was concentrated, under reduced pressure, to a volume of about 100 ml and again filtered.

The clear liquid was examined by paper chromatography (II.G.i.). A charcoal-celite column,(44x7 cm), was prepared (II.J.iv.), and the partial hydrolysate was introduced thereon. The column was successively eluted with 1%, 5%, 10% and 20% ethanol, elution being continued, at each concentration, until no further material in the eluate could be revealed by t.l.c. (II.G.iii.).

Fractions (35 ml) were collected with a Gilson "Escargot" fraction collector and every fifth fraction was sampled (1 ml). The samples were concentrated under reduced pressure to a small volume and examined by t.l.c. (II.G.iii.). Fractions containing similar components were pooled, filtered and concentrated to a small volume under reduced pressure at 40°. Each pooled fraction was examined by paper chromatography (II.G.i.). The components in each pooled fraction were then separated by preparative paper chromatography on Whatman no. 17 papers (II.G.ii.). The material corresponding to each DP was combined, freeze-dried and weighed.

III.F.ii. Oligosaccharides from the partial acid hydrolysate of inulin

Inulin (Sigma, 32 g) was hydrolysed at 70° for 1 h with oxalic acid (0.005 M, 1.5 l). The mixture was neutralised with calcium carbonate (BDH, Analar) and filtered. The filtrate was concentrated to about 100 ml under reduced pressure and again filtered, through a glass filter paper. The clear filtrate was examined by paper chromatography (II.G.i.). A charcoal-celite column (70x7.5 cm) was prepared (II.J.v.) and the concentrated solution of the oligosaccharides was applied to the column. The column was initially eluted with distilled water (10 l) to remove most of the

monosaccharide. A 5% ethanol solution (5 l) was then eluted through, fractions (35 ml) being collected with a Gilson Escargot fraction collector. Gradient elution was then carried out, over the following 10 l, to bring the final ethanol concentration of the eluant to 20%. Every fifth fraction was sampled (1 ml), the samples being concentrated under reduced pressure and examined by t.l.c. (II.G.iii.). Fractions containing similar components were pooled, filtered and concentrated under reduced pressure to a small volume. Each concentrated pooled fraction was subjected to preparative paper chromatography on Whatman no. 17 papers (II.G.ii.), and the purity of the components obtained was checked by paper chromatography (II.G.i.). To obtain components of DP-3 and higher, homogeneous to paper chromatography, it was necessary to re-chromatograph them on Whatman 3 mm papers. Finally, all components of similar DP were combined and freeze-dried.

III.G. Molecular Weight Determinations

III.G.i. Attempted molecular weight determinations of *Streptococcus salivarius* levans by gel filtration on Sepharose 4B

Sepharose 4B (Pharmacia Fine Chemicals AB) was made into a pourable slurry with water and deaerated under vacuum. A Pharmacia K25/40 column was filled with the slurry and the bed was allowed to settle under elution with 1% sodium chloride solution. When the bed had substantially settled, the clear eluant above the bed surface was removed with a pipette, the surface of the gel was stirred up with a glass rod and more of the slurry was added. The procedure was repeated until the surface of the settled bed was 2 - 3 cm from the top of the column. The column was left to equilibrate

overnight with an eluant of 1% sodium chloride, the operating head being about 40 cm.

The eluant above the gel was removed and Blue Dextran 2000 (Pharmacia AB, 10 mg in 1 ml 1% NaCl) was carefully introduced onto the column with a Pasteur pipette and allowed to absorb onto the gel. Eluant (1 ml) was twice applied and allowed to absorb onto the gel. Elution was then continued with an operating head of about 40 cm and flow rate of about 10 ml h^{-1}. The eluate was fractionated (2 ml), with a Gilson Escargot fraction collector, commencing with the application of the Blue Dextran. The absorbance, at 600 nm, of each fraction was determined with an EEL 197 colorimeter. A histogram was constructed plotting absorbance against elution volume. From this the void volume of the column was found. Each of the bacterial levans was subjected to chromatography by the same procedure. A solution, containing 25 mg in eluant (1 ml), was applied in each case. Aliquots (0.2 ml) were removed from the fractions collected and subjected to the phenol - sulphuric acid method for carbohydrate content (II.H.ii.). Absorbances at 487 nm were measured with the EEL colorimeter and histograms were constructed, plotting absorbance against elution volume.

III.G.ii. Molecular weight determinations of *Streptococcus salivarius* levans by gel filtration on Sepharose 2B

The procedure was as in III.G.i. except that a Pharmacia K15/90 column was employed and Sepharose 2B was the packing material. Also the eluate fraction size was 2.5 ml (75 drops) collected with a Gilson MTDC drop counter - fraction collector.

Blue Dextran (20 mg in 1 ml), polysaccharide (25 mg in 1 ml) and D - glucose (10 mg in 1 ml) were subjected to chromatography on the above column. For the phenol - sulphuric acid determinations, 0.5 ml aliquots were taken from the polysaccharide fractions, and 0.1 ml aliquots in the D - glucose case.

III. G. iii. Sedimentation constant and molecular weight determinations on the levans elaborated by strains of Streptococcus salivarius

A 0.5% solution of each polysaccharide (in 0.1 M - potassium chloride, 5 ml) was prepared.

Sedimentation runs were performed, using a Spinco Model E ultracentrifuge, at 21,740 r.p.m. and 20°. Two of the polysaccharide solutions were run simultaneously, one in a cell with plain windows, the other in a cell with wedge windows, this causing a relative displacement of the two optical images obtained. When the rotor had accelerated to the operating speed, the clock was started and photographs of the Schlieren image were taken at appropriate time intervals, as the peaks obtained migrated between the boundaries of the cell. The remaining polysaccharide was run in a cell counterbalanced by a blank cell with a reference hole.

The photographic plates were developed and the migration of the peaks relative to one or other of the reference lines was measured for each exposure. With a knowledge of the dimensions of the rotor and cells and the magnification factor of the image, the distance of the migrating peak from the rotor axis was calculated in each case.

III.H. Reducing-End Linkage Analysis of Oligosaccharides by Initial Borodeuteride Reduction and Methylation Analysis

The oligosaccharide (10 mg) was dissolved in water (10 ml) and excess sodium borodeuteride (5 mg) was added, the mixture being allowed to stand at room temperature for 6 h. The mixture was neutralised with washed Dowex 50W-X8 resin. The resin was filtered off and the filtrate was evaporated to dryness under reduced pressure, the residue being thrice co-distilled with methanol. The product was transferred to a 10 ml B14 round bottom flask, with a little methanol, which was then removed by evaporation under reduced pressure. The resulting oligosaccharide alditol was dessicated.

The product was methylated, hydrolysed, reduced with sodium borodeuteride and acetylated (II.J.vi., vii., viii., ix.). The resulting mixture of O-acetyl-O-methyl alditols was separated by gas chromatography and analysed by mass spectrometry (II.J.ii.).

III. J. Preparation of the Extracellular Levanase of Streptococcus salivarius strain 51 (Adapted from method of Mezner[188])

Medium IV (II.E.iv., 1 l) and calcium carbonate (BDH, Analar, 10 g) were placed in a 2 l conical flask, which was fitted with a cotton wool bung (II.A.vi.) and sterilised by autoclaving for 15 min at 122° (15 p.s.i.). A freshly sub-cultured suspension of S. salivarius strain 51 (II.F.ii., 5 ml) was poured into the flask, in the vicinity of a flame, the mouth of the flask and cotton wool bung being flamed off before replacement. The culture was maintained at 37° for 48 h. The culture fluid was centrifuged for 20 min at 8000 rpm (7020 g, JA10 rotor, II.A.viii.) and 4°. The supernatent was dialysed (II.A.vii., 1.5" tubing) against an equal volume of saturated ammonium sulphate (BDH, Analar) for 48 h, at 4°, and then sufficient solid ammonium sulphate was slowly added, with stirring, to render the concentration 60% w.r.t. ammonium sulphate. The mixture was allowed to stand at 4° for 24 h and was then centrifuged at 8000 rpm (7020 g, JA10 rotor) for 20 min, the centrifugate being dissolved in water (100 ml) and dialysed against deionised water for 48 h at 4°. The resulting solution was concentrated in an Amicon Model 52 concentration cell (UM 10 membrane, molecular weight cut-off 10,000) to a volume of ca. 5 ml. It was then subjected to gel filtration on Sephadex G200 (Pharmacia AB) using a Pharmacia K15/90 column, at 4°, the eluant employed being phosphate buffer (0.1 M, pH 6.6, II.D.ii.). The eluate was fractionated (3 ml) with an LKB fraction collector, the absorbance at 280 nm being automatically measured and recorded with an LKB Autoanalyser.

Aliquots (0.2 ml) were removed from the fractions containing high molecular weight material with an absorbance at 280 nm and tested for levanase activity by preparing the following digest in each case.

levan (51, 5 mg ml^{-1})	0.5 ml
fraction sample	0.2 ml
phosphate buffer (0.1 M, pH 6.6)	2.0 ml

The digests were maintained at 37° and aliquots (0.2 ml) were removed initially and after 3 h and 7 h, deactivated by immersing in boiling water for 15 s, and subjected to the Nelson method for estimation of reducing sugar content (II.H.iii.).

Fractions containing levanase activity were pooled and stored under a toluene layer at 4° or lyophilised.

III.K. Determination of the Optimum pH for the Hydrolysis of Levan by the Extracellular Levanase of Streptococcus salivarius strain 51

Citrate-phosphate buffers (0.1 M, II.D.i.) and phosphate buffers (0.1 M, II.D.ii.) covering the pH ranges 4.4 - 6.8 and 5.7 - 8.0 respectively, in 0.2 pH unit steps, were prepared. Freeze-dried levanase preparation (II.J., 30 mg) was dissolved in water (30 ml) and dialysed (II.A.vii.) against deionised water at 4° for 24 h. The following digests were prepared:

levan (51, 2 mg ml^{-1})	0.5 ml
enzyme solution	0.5 ml
butter	1.0 ml

An aliquot (0.4 ml) was initially removed from one of the digests, to serve as a blank, and deactivated by immersion in boiling water for 15 s. The digests were maintained at

37° for 15 h, aliquots (0.4 ml) were then removed, and together with the blank made up to 1 ml with water and subjected to the Nelson method for estimation of reducing sugar content (II.H.iii.). The absorbances, at 520 nm, for each pH value were measured and plotted on a graph against pH.

III.L. Action of the Extracellular Levanase of *Streptococcus salivarius* strain 51 on Levan

III.L.i. Qualitative digest

The following digest was prepared:

levan (51 , 20 mg ml^{-1})	5.0 ml
levanase preparation (III.J.)	1.0 ml
phosphate buffer (0.1 M, pH 6.75, II.D.ii.)	4.0 ml

An aliquot (1.0 ml) was initially removed and deactivated by immersing in a boiling water bath for 30 s. The digest was maintained at 37° and aliquots (1.0 ml) were removed after 1, 2, 3, 4, 5 and 22 h, and deactivated. The aliquots were deionised by shaking with Biodemineralite resin (BDH) for 1 h. The resin was removed by filtration and the filtrates were subjected to paper chromatography (II.G.i.) for 24 h, D-fructose being a reference compound.

III.L.ii. Quantitative digest

The freeze-dried levanase preparation (III.J., 15 mg) was dissolved in water (3 ml) and dialysed (II.A.vii., 0.25" tubing) against deionised water at 4° for 24 h. The following digest was prepared:

levan (51 , 5 mg ml^{-1})	0.5 ml
levanase solution (5 mg ml^{-1})	1.0 ml
phosphate buffer (0.1 M, pH 6.75)	1.5 ml
toluene	2 drops

Initially an aliquot (0.2 ml) was removed, to serve as a reagent blank, and deactivated by immersion in boiling water for 15 s. The digest was maintained at 37° and aliquots (0.2 ml) were removed at time intervals and deactivated. The aliquots were made up to 1 ml with water and the reducing sugar contents (as fructose) were determined by the Nelson method (II.H. iii.).

III.M. Determination of Protein Content of Freeze-Dried Levanase Preparation of Streptococcus salivarius strain 51

Reagents :

Lowry A :	2% sodium carbonate in 0.1 M sodium hydroxide	1 l
" B1 :	1% aqueous cupric sulphate pentahydrate (BDH, Analar)	50 ml
" B2 :	2% aqueous sodium tartrate	50 ml
" C :	A (50 ml) B1 (0.5 ml)) discard after 1 day B2 (0.5 ml))	
" D :	Folin and Ciocalteau's Reagent (BDH)	5 ml
	deionised water	5 ml

Calibration graph : A solution of bovine serum albumin (Sigma), of accurately known concentration (ca. 15 mg ml^{-1}) was prepared. Aliquots of this solution (0.1 – 1.0 ml in 0.1 ml steps) were taken and made up to 1.0 ml with water. In addition, water (1.0 ml) served as a reagent blank. To each solution (0.8 ml) was added Lowry C (4 ml), the mixtures being allowed to stand at room temperature for 10 min. Lowry D (0.4 ml) was added to each tube, the contents being mixed immediately and allowed to stand for at least 30 min. Absorbances of

750 nm were measured, relative to the reagent blank, and a graph was constructed, plotting absorbance against weight of protein (in μg).

Protein determination : Freeze-dried enzyme preparation (III. J., 5 mg) was dissolved in water (0.8 ml) and treated, as above, together with a reagent blank. The absorbance at 750 nm, was measured and the weight of protein in the enzyme sample was found from the calibration graph.

III.N. Discontinuous Gel Electrophoresis of the Levanase of Streptococcus salivarius strain 51

Apparatus : Shandon Southern Analytical Gel Electrophoresis Outfit SAE 2717

The freeze-dried levanase preparation (III. J., 15 mg) was dissolved in water (3 ml), dialysed (II. A. vii., 0.25" tubing) against deionised water at 4^{o} for 48 h and re-freeze-dried. It was subjected to disc electrophoresis, together with bovine serum albumin (BDH, 100 μg), as a reference, following the methods given in the Shandon operating instructions and by Williams and Reisfeld.[182] A 7.5% polyacrylamide gel, which has a molecular weight cut off of ca. 3×10^{5} was employed. The protein bands were rendered visible with the combined stain-fixative of Malik and Berrie.[183]

III.O. Determination of Action-Type (exo or endo) of the Levanase of Streptococcus salivarius strain 51 on Levan

A Pharmacia K9/30 column, having a bed volume of ca. 19 ml, was packed with Sepharose 2B (Pharmacia AB). Blue Dextran 2000 (Pharmacia AB, 10 mg in 0.3 ml 1% sodium chloride solution) was subjected to gel filtration on this column, with 0.1% sodium chloride eluant, the eluate

being fractionated (25 drops, 0.6 ml) with a Gilson MTDC fraction collector. The fractions were diluted by additon of water (1.2 ml) and the absorbances, at 620 nm, were determined using an EEL 197 colorimeter.

D-fructose (2 - 3 mg in 0.3 ml eluant) was subjected to gel filtration, as above, aliquots (100 µg) of the fractions obtained being subjected to the phenol - sulphuric method (II.H. ii.) for carbohydrate content, the absorbances, at 487 nm, being measured with the EEL colorimeter.

A solution of 51 levan (25 mg ml^{-1}) was prepared. Of this solution, 0.15 ml was diluted to 0.3 ml with the eluant used and subjected to gel filtration, as above, aliquots (0.5 ml) of the fractions obtained being determined by the phenol-sulphuric acid method, as above.

The following digest was prepared:

levan (25 mg ml^{-1})	1.0 ml
levanase preparation (III. J. ,freeze-dried)	5 mg
phosphate buffer (0.1 M, pH 6.4, II. D. ii.)	1.0 ml
toluene	2 drops

The digest was maintained at 37° and portions (0.3 ml) were removed after 24 h and 96 h, and deactivated by immersing in a boiling water bath for 15 s. Each of these partial hydrolysates was, in turn, subjected to gel filtration, as above, aliquots (0.5 ml) being taken from the fractions obtained for estimation of carbohydrate content by the phenol - sulphuric acid method (II.H. ii.).

Histograms were constructed plotting absorbances at 600 nm (in the case of Blue Dextran) and 487 nm against elution volume (expressed as fraction number).

III.P. Preparation of Methyl β-D-fructofuranoside (adapted from the method of Horvath and Metzenberg[171])

The following ion-exchange resins were employed :
Dowex-50 H^+ (AG-50W-X8, 200-400 mesh)
Dowex-1 Cl^- (AGI-X8, 200-400 mesh)

Sucrose (6 g) was dessicated, under vacuum, over phosphorous pentoxide and refluxed together with dry Dowex-50W H^+ (10 g) in anhydrous methanol (II.B.iv.) with constant stirring for 1 h. The reaction mixture was cooled and the resin removed by filtration. The pH of the filtrate was adjusted to 7.0 with ammonium hydroxide (0.1 M) and evaporated to a thick syrup under reduced pressure. The syrup was dissolved in water (1 l) and the pH was again adjusted to 7.0 with ammonium hydroxide (0.1 M).

The reaction mixture was examined by t.l.c. (II.G.iii.), sucrose, D-fructose and D-glucose being spotted as references.

A glass chromatography column (40 x 3 cm) was prepared containing Dowex-1 Cl^-. The latter was converted to the borate form by the method of Khym and Zill[184]. The aqueous reaction mixture was introduced at the top of this column and fractions (20 ml) of the self-eluate were collected with a Gilson Escargot fraction collector. Aliquots (1 ml) of alternate fractions were evaporated down to a small volume and examined by t.l.c. (II.G.iii.). Fractions containing only the component with R_f 0.42 were pooled and evaporated down, under reduced pressure, in a tared flask. The resulting yellow syrup was dried, in a dessicator, over phosphorous pentoxide. The yield was then measured. A solution of the syrup, of accurately known concentration (ca. 1%), was prepared and its rotation at ambient temperature, at the sodium D-line, was measured, the specific rotation being calculated.

III.Q. Action of Streptococcus salivarius 51 Levanase and Yeast Invertase on Methyl β-D-fructofuranoside

Yeast invertase (β-D-fructofuranosidase, E.C. 3.2.1.26), grade X, from Candida utilis (activity: 535 Units mg^{-1}) was obtained from Sigma.

A solution of methyl β-D-fructofuranoside (III.P.) was prepared containing about 10 mg ml^{-1}. The following digests were then prepared:

a) methyl β-D-fructofuranoside (9.2 mg ml^{-1})	0.8	ml
invertase	1.0	mg
phosphate-citrate buffer (0.1 M, pH 5.6)	4.2	ml
b) as a), except invertase	20	mg
c) methyl β-D-fructofuranoside (9.2 mg ml^{-1})	0.8	ml
freeze-dried levanase (III.J.)	20	mg
phosphate buffer (0.1 M, pH 6.6)	4.2	ml

Aliquots (200 µl) were initially removed and deactivated by immersion in boiling water for 15 s. The digests were maintained at 37° and aliquots (200 µl) were removed at time intervals (up to 45 min in the case of a) and 5 h for b) and c) and deactivated. The reducing sugar content of the aliquots was determined by the Nelson method (II.H. iii.) and graphs were constructed plotting % hydrolysis of the substrate against digest time.

III.R. Action of Candida utilis Invertase on Levan

III.R.i. Qualitative digest

The following digest was prepared:

levan (51, 5 mg ml^{-1})	1	ml
water	3	ml
invertase (Grade X, 1 mg ml^{-1})	1	ml
toluene	3	drops

An aliquot (0.5 ml) was initially removed and deactivated by immersion in boiling water for 15 s. The digest was maintained at 37° and further aliquots (0.5 ml) were removed at time intervals up to 50 h and deactivated. The aliquots were concentrated to a small volume, under reduced pressure, and subjected to paper chromatography for 24 h (II.G.i.) with D-fructose as a reference.

III.R.ii. Determination of the optimum pH for the action of *C. utilis* invertase on levan

Citrate-phosphate buffers (0.1 M, II.D.i.) and phosphate buffers (0.1 M, II.D.ii.), covering the respective pH ranges 3.1 - 5.6 and 5.6 - 8.0, were prepared.

The following digest was prepared with each buffer:

levan (51 , 5 mg ml^{-1})	1	ml
invertase (grade X, 1 mg ml^{-1})	1	ml
buffer	3	ml
toluene	3	drops

The digests were maintained at 37° and aliquots (200 µl) were removed at time intervals and deactivated by immersing in boiling water for 15 s. The reducing sugar content of each aliquot was estimated by the Nelson method (II.H.iii.).

III.R.iii. Quantitative digest

The following digest was prepared:

levan (51 , 5 mg ml^{-1})	1	ml
invertase (grade X, 4 mg ml^{-1})	1	ml
phosphate buffer (0.1 M, pH 6.6)	3	ml
toluene	3	drops

An aliquot (200 µl) was initially removed and deactivated by immersing in a boiling water bath for 15 s. The digest was maintained at 37° and aliquots (200 µl)

were removed at time intervals up to 500 h and deactivated. The inactive aliquots were stored at 4° until the digest was complete. The reducing sugar content, as D-fructose, was determined by the Nelson method (II.H.iii.).

III.S. Action of *Streptococcus salivarius* 51 Levanase and *Candida utilis* Invertase on Inulin

Freeze-dried levanase preparation (III.J., 10 mg) was dissolved in water (2 ml) and dialysed (II.A.vii., 0.25" tubing) against deionised water, at 4°, for 24 h.

The following digests were prepared :

a)	inulin (Sigma, 5 mg ml^{-1})	1 ml
	invertase (grade X, 1 mg ml^{-1})	1 ml
	phosphate buffer (0.1 M, pH 6.6, II.D.ii.)	3 ml
	toluene	3 drops
b)	inulin (5 mg ml^{-1})	1 ml
	dialysed levanase (5 mg ml^{-1})	2 ml
	phosphate buffer (0.1 M, pH 6.6)	2 ml
	toluene	3 drops

Aliquots (200 µl) were removed initially and deactivated by immersion in a boiling water bath for 15 s. The digest was maintained at 37° and further aliquots (200 µl) were removed at time intervals up to 45 h, deactivated and stored at 4°. The reducing sugar contents of the aliquots were then determined by the Nelson method (II.H.iii.).

III.T. Action of *Streptococcus salivarius* 51 Levanase on Oligosaccharides Obtained from Inulin

Levanase preparation (III.J., 2 ml) was dialysed (II.A.vii., 0.25" tubing) against deionised water, at 4°, for 24 h. The following digests were prepared, in

which the oligosaccharides obtained from inulin, inulobiose (I_2) through to inuloheptaose (I_7) were prepared as in II.F.ii.

oligosaccharide	2 mg
dialysed levanase	0.2 mg
water	1.3 ml

Two aliquots (100 µl) were initially removed from each digest and deactivated by immersing in boiling water for 15 s. The digests were maintained at 37° and 2 aliquots (100 µl) were removed from each, at time intervals up to 17.5 h, and deactivated. One series of aliquots was subjected to the Nelson method (II.H.iii.) to determine the reducing sugar content. Absorbances at 520 nm being plotted graphically against digest time. The other series of aliquots was concentrated under reduced pressure and examined by paper chromatography (II.G.i.) with D-fructose and D-glucose references for 24 h.

III.U. Preparation of Levans Partially Hydrolysed by *S. salivarius* 51 Levanase

III.U.i. Small scale quantitative digest

The following digest was prepared:

levan (51, 15 mg ml^{-1})	1 ml
levanase preparation (III.J.)	0.5 ml
phosphate buffer (0.1M, pH 6.6, II.D.ii.)	4.0 ml

An aliquot (50 µl) was initially removed and deactivated by immersing in boiling water for 15 s. The digest was maintained at 37° and further aliquots (50 µl) were removed at time intervals up to 10 h and deactivated. The aliquots were subjected to the Nelson method (II.H.iii.) for estimation of reducing sugar

content and, with a knowledge of the maximum available fructose in a 50 µl aliquot, the degree of hydrolysis was calculated in each case and plotted against digest time.

From the graph the digest times required for any degree of hydrolysis could be found.

III.U.ii. Large scale digest

A digest was prepared, as in III.U.i., but with a 15-fold increase in quantities :

levan (51 , 15 mg ml^{-1})	15	ml
levanase preparation (III.J.)	7.5	ml
phosphate buffer (0.1 M, pH 6.6)	60	ml

Initially an aliquot (50 µl) was removed and deactivated by immersion in a boiling water bath. The digest was maintained at 37°, and , at the times required for the desired degrees of hydrolysis (from III.U.i.), which are 10, 20, 40 and 60%, appropriate large aliquots, in addition to those of 50 µl, were removed and deactivated by immersion in a boiling water bath for 1 min and 15 s respectively. Further aliquots (50 µl) were removed and deactivated at time intervals up to 22 h.

The reducing sugar content, and hence degree of hydrolysis, was determined, for each 50 µl aliquot, by the Nelson method (II.H.iii.). The large aliquots were dialysed (II.A.vii.) extensively against deionised water and freeze-dried, the yield of each partially hydrolysed product being recorded.

IV. RESULTS AND DISCUSSION

IV.A. Preparation and Analysis of the Polysaccharides Elaborated by Streptococcus salivarius strain 51, ATCC 13419 and NCTC 8606

IV.A.i. Optimum culture times for the preparation of the polysaccharides

It has been shown[149] that strains of oral streptococci often possess the ability to induce enzymes capable of hydrolysing the polysaccharide products obtained when cultured in sucrose containing media. In order to obtain optimum yields of polysaccharides from the strains of S. salivarius employed it was considered necessary to initially study, on a small scale, the dependence of polysaccharide yield on culture time.

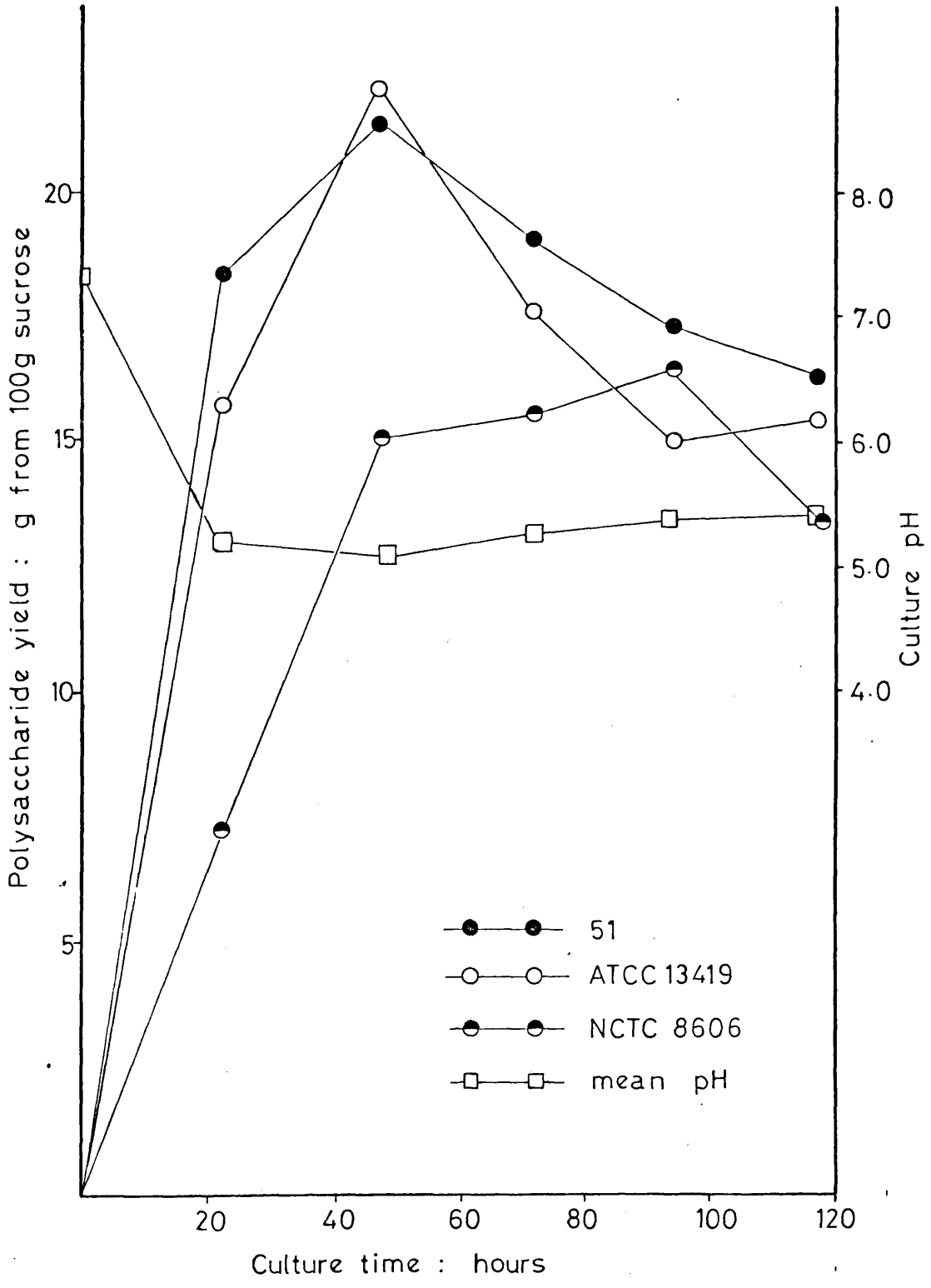
Small scale preparations were carried out as described in III.A.i. and the results (Fig. IV.1.) show that polysaccharide yields are indeed culture time dependant, passing through maxima. This was considered to be evidence for the induction of hydrolysing enzymes.

The strain NCTC 8606 differs from the other two in that its maximum polysaccharide yield was reached in a longer time (ca. 90 h compared with ca. 50 h for strains ATCC 13419 and 51). The yield was also smaller (ca. 17 g from 100 g sucrose compared with ca. 22g). The presence of calcium carbonate in the cultures effectively neutralised acid metabolites as evidenced by the maintenance of a steady pH (ca. 5.5) after the first 25 h.

IV.A.ii. Large scale preparations and analysis of the polysaccharides

In order to have sufficient of the polysaccharides to perform all the subsequent investigations, large scale

Figure IV. 1. Dependence of polysaccharide yield and culture pH on culture time



preparations were carried out as described in III.A.ii. (for strains 51 and ATCC 13419) and III.A.iii. (for strain NCTC 8606). Culture times actually employed were ca. 80% of the maximum yield times arrived at in IV.A.i. (to ensure that the products would be relatively undegraded by induced hydrolase systems) and were, respectively, 40 h, 40 h and 80 h for strains 51, ATCC 13419 and NCTC 8606.

Samples of the lyophilised polysaccharides were analysed for ash, moisture and nitrogen contents. Fructose and carbohydrate contents and specific rotations were also determined (as described in II.H.i., II.H.ii., and III.B.), and the results are shown in Table IV.1. The high fructose contents suggest that all three polysaccharides are fructans, this being confirmed by acid hydrolysis of these materials with 0.005 M sulphuric acid as described in III.C.i. Paper chromatography of the hydrolysates revealed fructose to be the only component (see IV.E.i., p. 162). The negative specific rotations suggest the anomeric configurations to be of the β -type.

IV.B. Characterisation of the Fructans Elaborated by Streptococcus salivarius strain 51, ATCC 13419 and NCTC 8606

IV.B.i. Methylation studies (gas chromatography - electron impact mass spectrometry)

It has been established in section IV.A. that the polysaccharides elaborated by the three strains of S. salivarius under consideration are composed of fructose residues, the interfructosidic linkages, on the basis of rotational data (Table IV.1.), being probably of the β -type. In order to further characterise them it was necessary to determine the positions of the interfructosidic linkages, the degree of branching (if any) and the ring size of the fructosyl residues. Such determinations are frequently most easily carried out by the application of

Table IV. 1. Analysis of the extracellular polysaccharides of S. salivarius strains 51, ATCC 13419 and NCTC 8606

	51	ATCC 13419	NCTC 8606
polysaccharide yield (g from 100 g sucrose)	17.5	13.8	4.1
carbohydrate content %*	87.8	87.2	88.9
fructose content %*	93.8	87.6	85.4
ash content** %*	4.69	3.47	8.34
moisture content %**	9.67	8.08	13.95
nitrogen content** %*	0.53	0.65	0.74
protein content*** %*	3.32	4.08	4.65
$[\alpha]_D^{20}$	-59.3	-52.6	-61.4

* On dry basis

** analyses performed by A. Bernhardt, Elbach über Engelskirchen, Germany

*** nitrogen x 6.25

methylation analysis. Exhaustive methylation of a polysaccharide results in the free hydroxyl positions becoming methylated. Upon subsequent hydrolysis, the partially methylated constituent monosaccharides are generated. Determination of the non methylated positions, in the partially methylated monosaccharides, often yields information concerning the positions of interglycosidic linkages and ring sizes of the constituent sugars. Classically such determinations involved separation of mixtures of partially methylated sugars by column chromatography [34,53] or distillation in quantities sufficient for subsequent chemical characterisation and quantisation. Such procedures were not only arduous but required large quantities of methylated polysaccharides. In recent years the application of combined gas chromatography - electron impact mass spectrometry (gc-ms) has greatly facilitated such determinations with only very small quantities of methylated polysaccharide being required [75,76]. It is usual practice to transform the methylated sugars obtained by hydrolysis of the methylated polymer into corresponding O-acetyl-O-methyl-alditols by reduction and acetylation. This results in the elimination of possible $\alpha\beta$ isomerism (hence giving rise to less complicated chromatograms) and provides for sufficient volatility and suitable substituents for the facilitation of mass spectrometric analysis. The positions of acetate and methoxyl groups in the resulting O-acetyl-O-methyl-alditols, ascertained by analysis of the resulting mass spectra, indicate which positions are involved in ring formation and through which positions linkages occur in the constituent residues of the parent polymer although it is not always possible to differentiate between the possibilities.

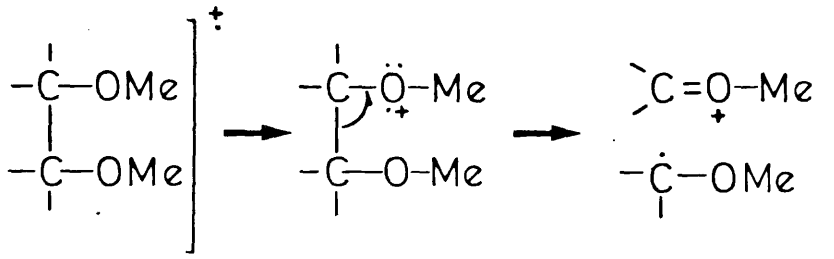
The success of this method relies upon the simple fact that the substitutional pattern in an O-acetyl-O-methyl-alditol dictates its modes of fragmentation during electron impact ms. The charge-radical generated by electron impact can reside on the ether oxygen atom of a methoxyl group and be relatively stabilised by the inductive effect of the methyl group. Under the conditions of electron impact normally employed (ca. 80 eV) excess energy tends to promote fragmentation of the molecular ion-radical in

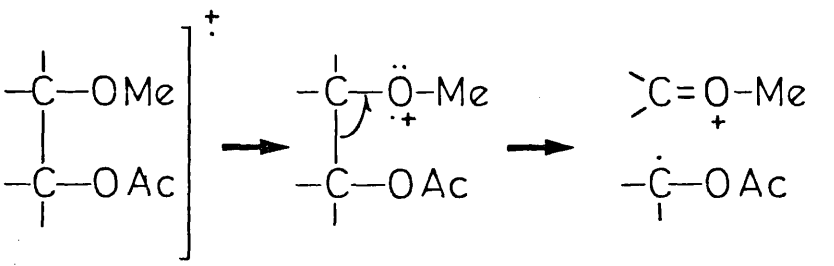
the case of O - acetyl - O - methyl alditols, normally by fission of the C - C bond between the C atom carrying the charged ether O atom and an adjacent C atom. This results in the formation of a charged fragment (detected according to its $^{m}/e$ ratio) and a free radical. It follows that fragmentation between adjacent C atoms that both carry a methoxyl group occurs most readily (Fig. IV. 2. a.). Because of the electron-withdrawing effect of the carbonyl group adjacent to the ester oxygen atom in an acetate group, the charge-radical is not so readily accommodated at an ester O atom. Consequently fission between adjacent C atoms carrying methoxyl and acetate groups invariably results in the fragment carrying the methoxyl group being detected as the charged species (Fig. IV. 2. b.). Where adjacent C atoms both carry acetate groups, it follows that formation of the ion-radical and subsequent C - C bond fission rarely occurs (Fig. IV. 2. c.).

Charged species that result from fragmentations of molecular ion-radicals are termed primary fragments and are characteristic of a particular substitutional pattern in the O - acetyl - O - methyl - alditol, although little or no indication of stereochemistry can be gained by this method. In addition, secondary fragments can arise from primary fragments by the single or consecutive loss of neutral molecules. Examples of commonly encountered primary and secondary fragment formation are given in Fig. IV. 3.

It was decided, in this study, to attempt the characterisation of the fructans elaborated by S. salivarius strains 51, ATCC 13419 and NCTC 8606 by gc - ms of the O - acetyl - O - methyl - hexitols derived from the methylated fructoses, the hydrolysis products of the methylated fructans. Samples of the polysaccharides were, therefore, converted to O - acetyl - O - methyl - hexitol mixtures as described in section III. D. The mixtures were subjected to gc - ms as described in II. J. ii., the resulting gas chromatogram and mass spectra being shown in Figs. IV. 4. and IV. 5. respectively.

Figure IV. 2. Ion-radical stabilisation and modes of primary fragmentation in O-acetyl-O-methyl-alditols.





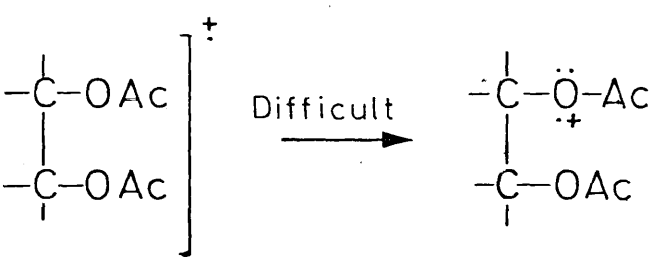


Figure IV. 3. Primary and secondary fragmentation modes in O – acetyl – O methyl – alditols

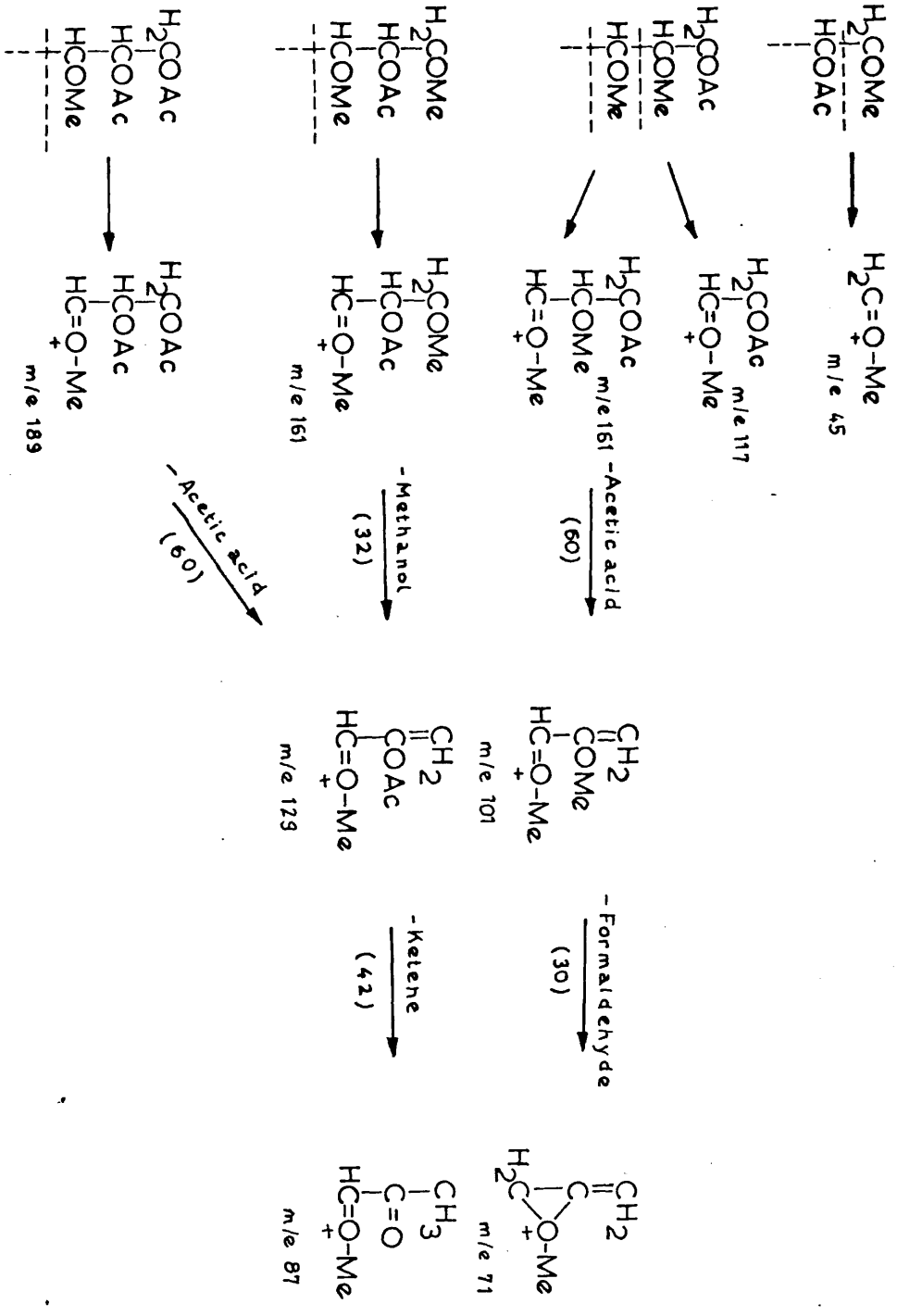


Figure IV. 4. Gas chromatogram of O - acetyl - O - methyl - hexitol mixture derived from the fructan of S. salivarius strain 51

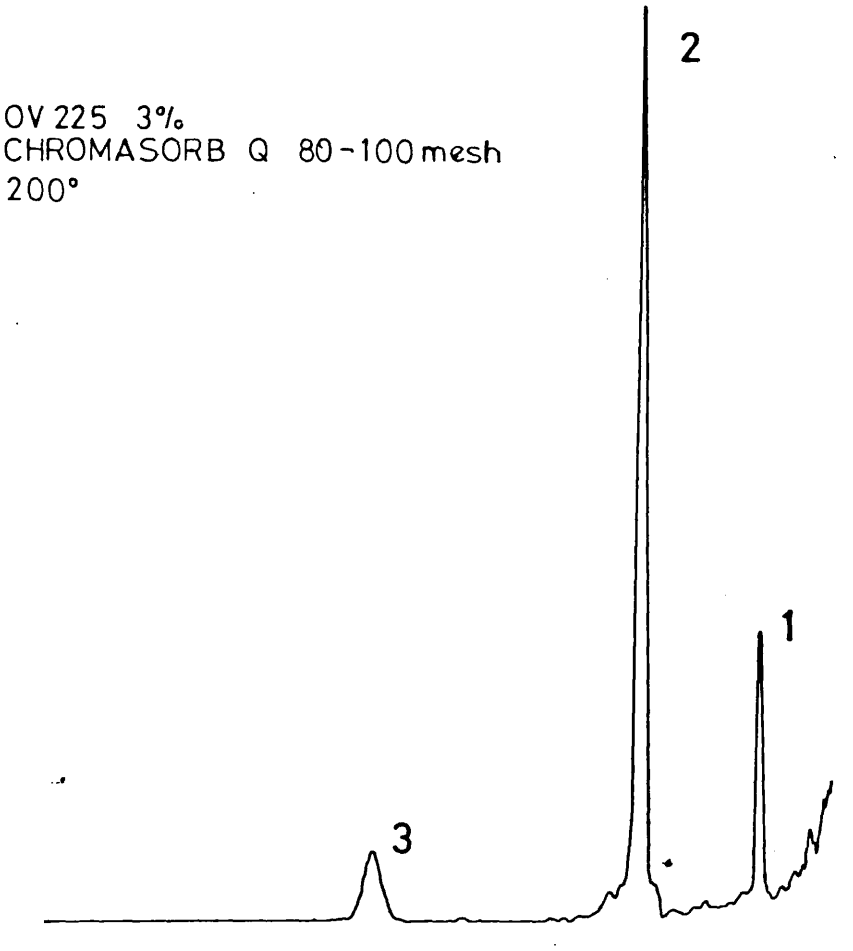


Figure IV. 5. Electron impact mass spectra of O - acetyl - O - methyl - hexitols derived from S. salivarius fructans

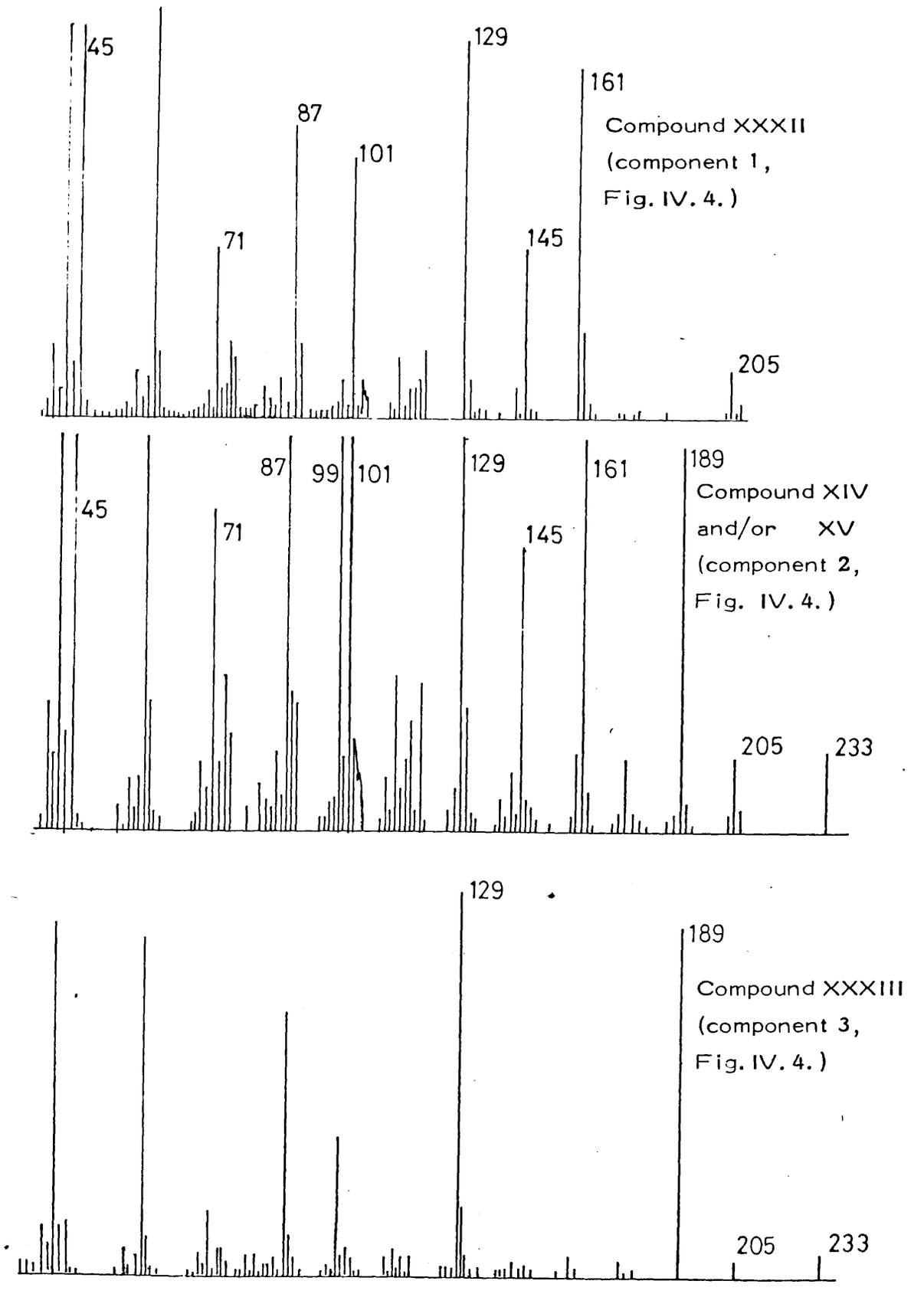


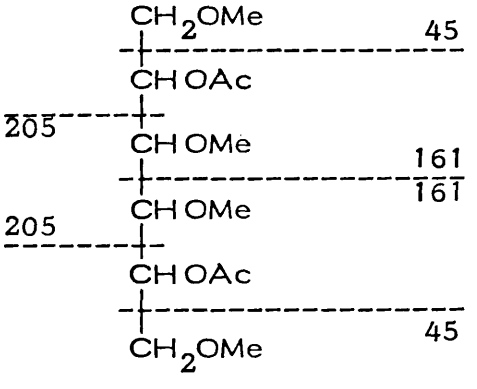
Table IV. 2. Chromatographic properties and electron impact mass spectrometric analysis of O-acetyl-O-methyl-hexitols derived from the fructans of S. salivarius

Component	R_{tmg}	Mass spectrometry		Parent structural unit
		primary* and secondary fragments (m/e)	positions of methoxyl groups in O-acetyl-O-methyl-hexitol	
1	0.79	45, 71, 87, 101, 129, 145, 161, 205	1,3,4,6 (XXXII)	non-reducing D-fructofuranosyl terminus (XXXIV)
2	1.70	45, 71, 87, 99, 101, 129, 145, 161, 173, 189, 205, 233	1,3,4 (XIV) or 3,4,6 (XV)	D-fructofuranose linked through positions 2 and 6 (V) or D-fructopyranose linked through positions 2 and 5 (VI) D-fructofuranose linked through positions 1 and 2 (XXXV)
3	3.95	87, 99, 129, 189, 233	3,4 (XXXIII)	D-fructofuranose linked through positions 1, 2 and 6 (XXXVI) or D-fructopyranose linked through positions 1, 2 and 5 (XXXVII)

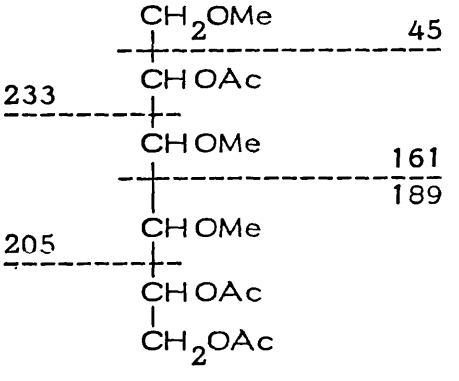
* Primary fragments underlined

The mass spectral interpretations and retention times of the components relative to the reference compound 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-glucitol (measured as described in II.J.iii.) are given in Table IV.2.

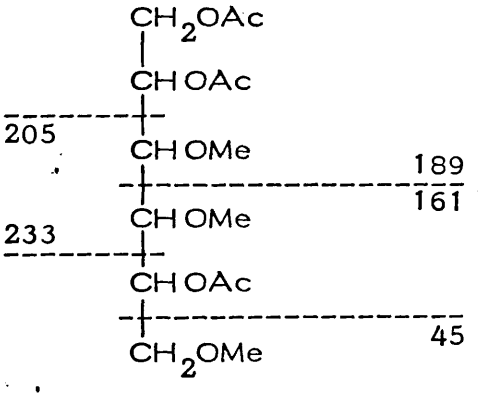
It is evident that upon subjecting the three fructans to the sequence of reactions described in III.D., 2,5-di-O-acetyl-1,3,4,6-tetra-O-methyl hexitol (XXXII), 2,5,6-tri-O-acetyl-1,3,4-tri-O-methyl hexitol (XIV) and/or 1,2,5-tri-O-acetyl-3,4,6-tri-O-methyl hexitol (XV) and 1,2,5,6-tetra-O-acetyl-3,4-di-O-methyl hexitol (XXXIII) are produced.



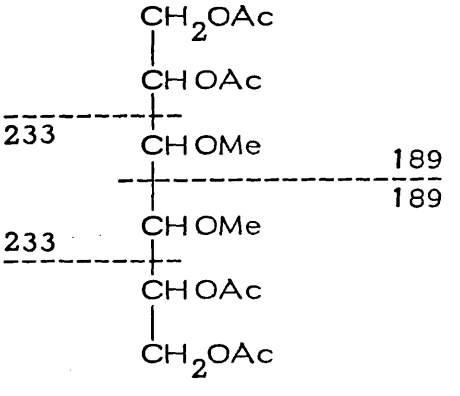
XXXII



XIV

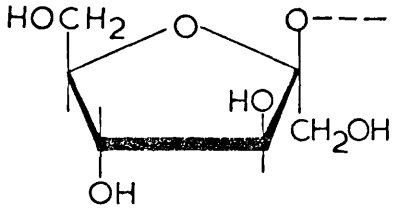


XV

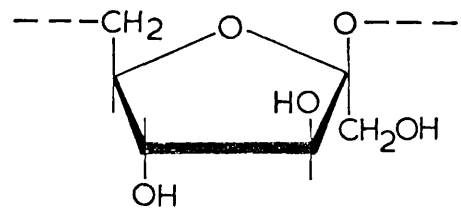


XXXIII

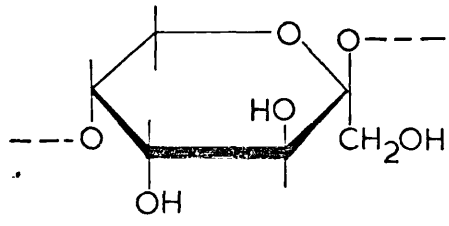
Compound XXXII must have originated from D-fructofuranosyl non-reducing terminal units (XXXIV) in the parent polysaccharide. Compounds XIV and XV, which are indistinguishable by mass spectrometric analysis since they would both give rise to the same fragments, are respectively indicative of D-fructofuranose residues linked through positions 2 and 6 (V) [or pyranose residues linked through positions 2 and 5 (VI)] and D-fructofuranose residues linked through positions 2 and 1 (XXXV). Compound XXXIII is evidence of branching D-fructofuranose residues linked through positions 2, 6 and 1 (XXXVI) or D-fructopyranose residues linked through positions 2, 5 and 1 (XXXVII).



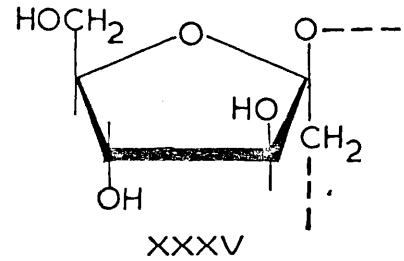
XXXIV



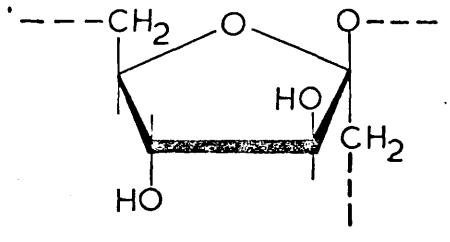
V



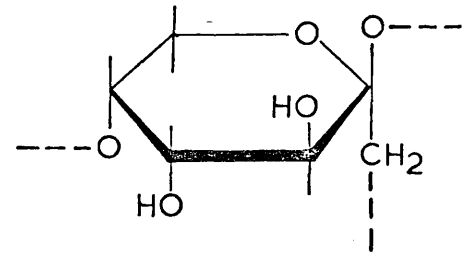
VI



XXXV



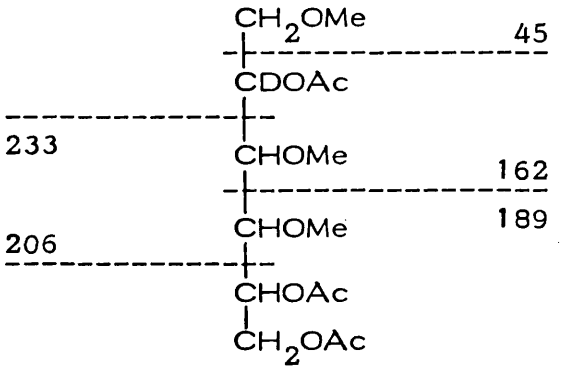
XXXVI



XXXVII

It might have been expected that each hexitol derivative would be present in the epimeric gluco- and manno-configurations since reduction of a ketose generates a new chiral centre at C-2. However, under the experimental conditions employed, the two forms of each hexitol derivative were not separated, in agreement with the findings of Kubberød.[77]

The characterisation of the fructans depended, then, upon determining whether the tri-O-acetyl-tri-O-methyl-hexitol derivative was XIV or XV. A problem of this nature was resolved by Lindberg and co-workers[74] by the introduction of a deuterium label at C-2 during the reduction step of the methylation analysis using sodium borodeuteride (see Section I.B., p. 28). Upon adopting this modification (III.E.) for all three fructans, the tri-O-acetyl-tri-O-methyl-hexitol was established as being 2-d-2,5,6-tri-O-acetyl-1,3,4-tri-O-methyl-hexitol (XVI), the partial mass spectrum of this compound being

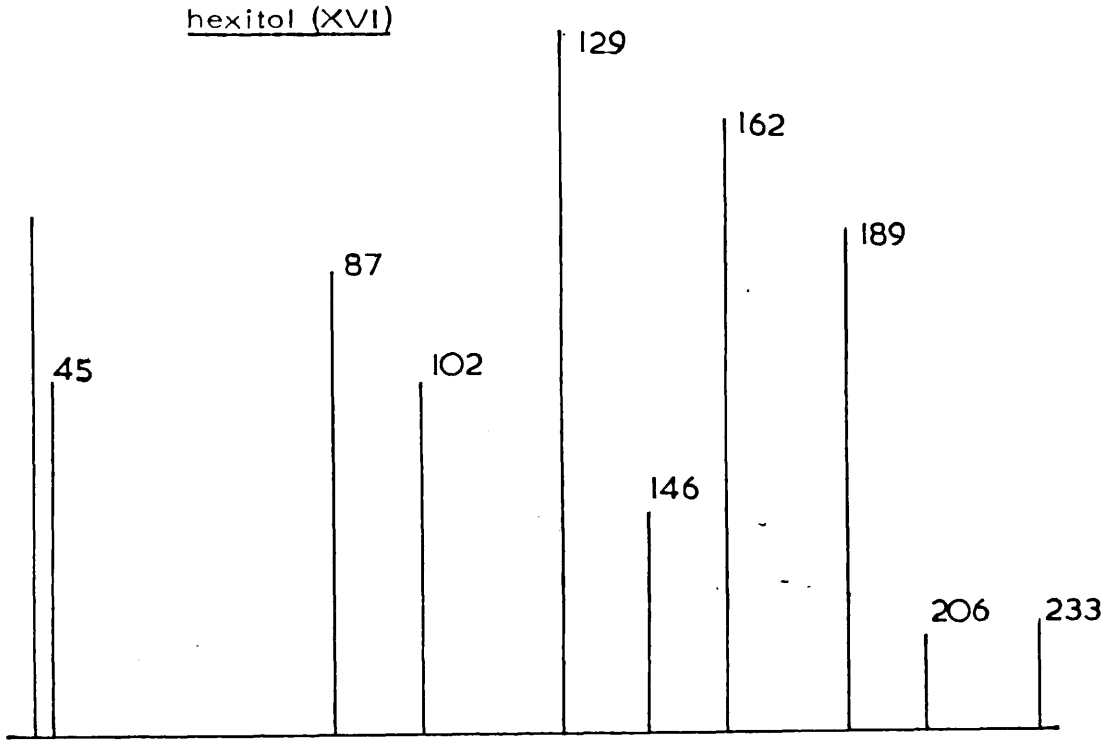


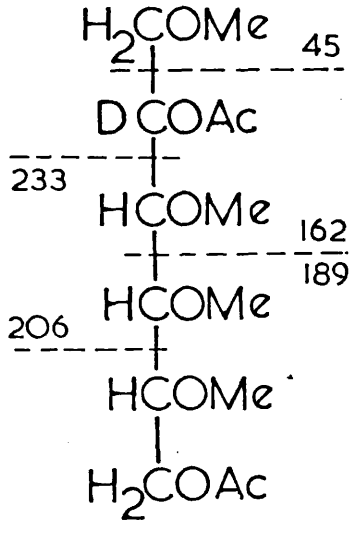
XVI

shown in Fig, IV.6. The non-terminal, non-branching residues in the three polysaccharides are, therefore, D-fructofuranose residues linked through positions 2 and 6 (V) or D-fructopyranose residues linked through positions 2 and 5 (VI).

The determination of ring size (and hence linkage position) of such residues is not possible by the above procedure and the resolution of this problem is dealt with after a discussion of the degree of branching.

Figure IV. 6. Partial electron impact mass spectrum of 2-d-2,5,6-tri-O-acetyl-1,3,4-tri-O-methyl-hexitol (XVI)





XVI

IV.B.ii. Degree of branching

It has been established that the fructans of this investigation are branched polymers. The "degree of branching" (proportion of all residues that are branching residues) in a polysaccharide composed of hexosyl residues can be determined by measurement of the proportion of derived O-acetyl-O-methyl-hexitols that are tetra-O-acetyl-di-O-methyl-hexitols (derived from branching residues). Such determinations were carried out for the three fructans of the investigation by electronic integration of the peaks in the chromatograms of the hexitol derivatives as described in II.J.iii. An equal weight-response theory was assumed: i.e. measured peak areas were taken to be proportional to the weights of components as detected by flame ionisation, thus allowing the molar ratios of the three O-acetyl-O-methyl-hexitols to be calculated. Such an assumption has been experimentally shown to yield results of reasonable accuracy (perhaps 5%) for such derivatives whereas for other derivatives e.g. analogous partially ethylated hexitol acetates the Effective Carbon Response theory gives a higher degree of accuracy.[185]

From the results, which are given in Table IV.3., it is evident that the molar ratio of tetra-O-acetyl-di-O-methyl-hexitol to di-O-acetyl-tetra-O-methyl-hexitol is not in every case unity. These departures from the expected ratio of the two components were due to the interference of spurious peaks, derived from the reagents employed, in the region of the di-O-acetyl-tetra-O-methyl-hexitol peak. Remethylation of portions of the methylated fructans followed by the same sequence of reactions resulted, however, in no change in the molar ratio of the tetra-O-acetyl-di-O-methyl-hexitol to the tri-O-acetyl-tri-O-methyl-hexitol. Therefore the methylations were concluded to be complete. Degrees of branching and numbers of residues in average repeating units were calculated, the results appearing in Table IV.3.

Table IV. 3. Molar ratios of O - acetyl - O - methyl - hexitols ; degree of branching and average repeating unit size of the fructans elaborated by strains of S. salivarius

Strain	O - acetyl - O - methyl - hexitol					Degree of branching % *	Number of residues in average repeating unit ** (to nearest whole number)
	gc-relative peak areas			molar ratio			
	tetra-O-methyl	tri-O-methyl	di-O-methyl	tetra-O-methyl	tri-O-methyl	di-O-methyl	
51	1.07	6.18	1.00	1.26	6.73	1.00	11.5	9
ATCC 13419	1.18	8.18	1.00	1.39	8.88	1.00	9.2	11
NCTC 8606	0.86	5.64	1.00	1.01	6.14	1.00	12.3	8

* Degree of branching, % = $\frac{\text{moles tetra - O - acetyl - di - O - methyl - hexitol} \times 100}{\text{moles (2x tetra - O - acetyl - di - O - methyl - + tri - O - acetyl - tri - O - methyl -) hexitols}}$

** Number of residues in average repeating unit = $\frac{100}{\text{Degree of branching}}$

The fructan elaborated by NCTC 8606 [the strain which displayed the slowest rate of levan synthesis (see Fig. IV. 1.)] , which was synthesised under conditions of higher substrate, enzyme and product concentration (in a dialysis tube as described in III. A. iii.) is branched to the greatest extent (12. 3 %). This finding supports that of Rapoport and Dedonder[119], who noted that high enzyme concentrations cause an increase in degree of branching during their work on the levansucrase of Bacillus subtilis (see section I. C., p. 48).

IV. B. iii. Ring size of non-terminal D-fructose residues

Having established that the fructans elaborated by strains of S. salivarius are branched polymers possessing D-fructofuranosyl units at chain ends (XXXIV), the problem of ring size of non-terminal residues remained to be solved. Circumstantial evidence, viz. the fact that these polymers are enzymatically synthesised ab initio sucrose (β-D-fructofuranosyl-α-D-glucopyranoside, XXV) and the further fact that Candida utilis invertase (a hydrolase specific for β-D-fructofuranosidic linkages) hydrolysed the fructan of S. salivarius strain 51 (see later, IV. D. iv. b.), suggest that they possess D-fructofuranose residues. Prior to this present investigation there has been, to the author's knowledge, no unambiguous chemical proof of ring size in bacterial fructans (a discussion of the work of Hibbert et al.[23], in this context, is given in Section I. B., p. 17). It was considered therefore essential, as part of this investigation, to determine, by chemical means, the ring size of non-terminal residues.

To accomplish this it was necessary to obtain a disaccharide preparation, as described in III. F. i., from a partial acid hydrolysate of one of the fructans (the most abundant, that of strain 51) and to determine the position of linkage to the reducing group. Since it is most likely

that a random degradative process such as acid hydrolysis would liberate oligosaccharides from all parts of the polysaccharide molecule, the results would reflect positions of linkages and hence ring size of the constituent D-fructose residues.

It was conceived that the following sequence of reactions : -

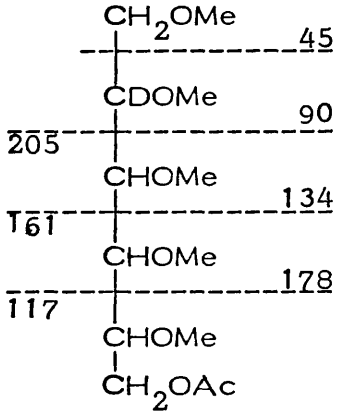
Disaccharide preparation

1) reduction with $NaBD_4$
2) methylation
3) hydrolysis
4) reduction with $NaBD_4$
5) acetylation

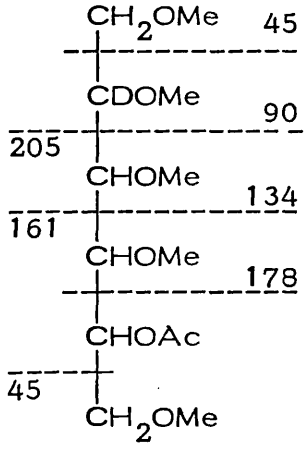
↓

2-d-O-acetyl-O-methyl-hexitols

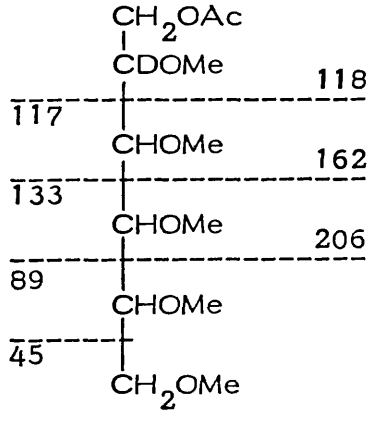
would yield, in addition to 2-d-2,5-di-O-acetyl-1,3,4,6-tetra-O-methyl-hexitol (XXXII) from non-reducing fructofuranosyl groups (XXXIV), a 2-d-O-acetyl-penta-O-methyl-hexitol with the acetate group at C-6 (XXXVIII) or C-5 (XXXIX) depending upon the position of the linkage in the disaccharide.



XXXVIII



XXXIX



XL

It is evident that compounds XXXVIII and XXXIX could be differentiated by mass spectrometry since the fragment $^{m}/_{e}$ 117 can only originate from the former.

The disaccharide preparation was subjected to the sequence of reactions above as described in III.H. The resulting mixture of 2-d-O-acetyl-O-methyl-hexitols was analysed by gc-ms, as described in II.J.ii., the resulting gas chromatogram and partial mass spectrum of component 1 (R_{tmg} = 0.50) being shown in Figs. IV.7 and IV.8. respectively. Chromatographic and mass spectrometric data is given in Table IV.4.

Table IV.4. Chromatographic properties, mass spectrometric analysis and structural significance of 2-d-O-acetyl-O-methyl-hexitols derived from the borodeuteride reduced disaccharide preparation.

Component	R_{tmg}	Mass spectrometry		Structural unit in disaccharide
		primary fragments m/e	positions of methoxyl groups	
1	0.50	45,90,117, 134,161,178, 205	1,2,3,4,5 (XXXVIII)	D-fructofuranose linked through C-6
		45,89,118, 133,162,177, 206	2,3,4,5,6 (XL)	D-fructose linked through C-1
2	0.79	45,161,162, 205,206	1,3,4,6	non-reducing D-fructofuranosyl group (XXXIV)

Figure IV. 7. Gas chromatogram of the 2-d-O-acetyl-O-methyl-hexitol mixture derived from the disaccharide preparation obtained from the fructan of S. salivarius strain 51

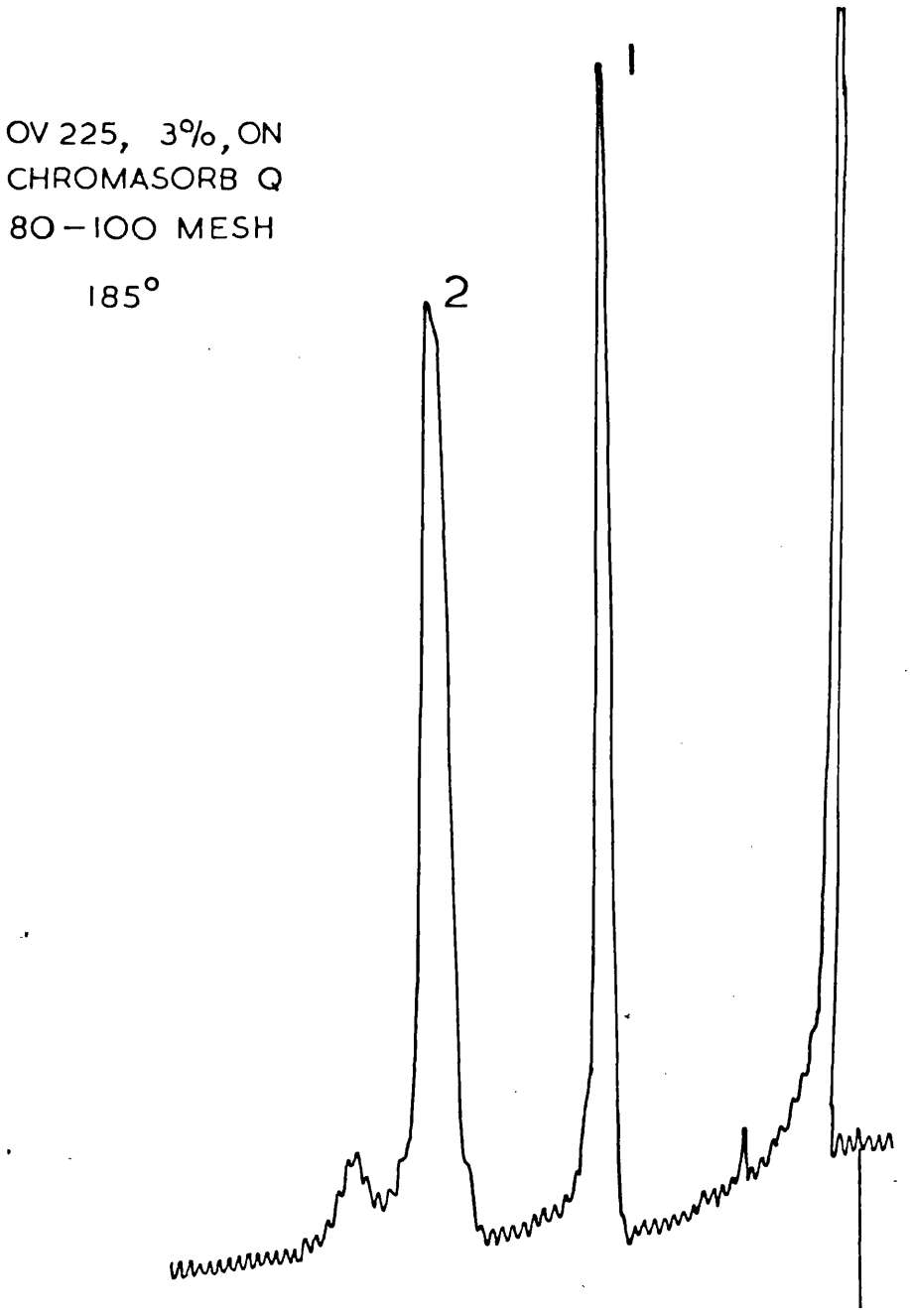
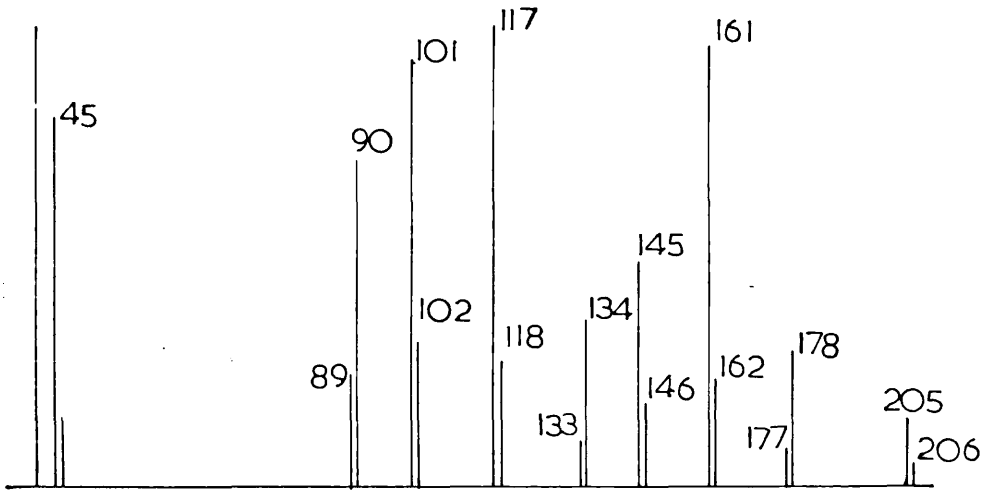
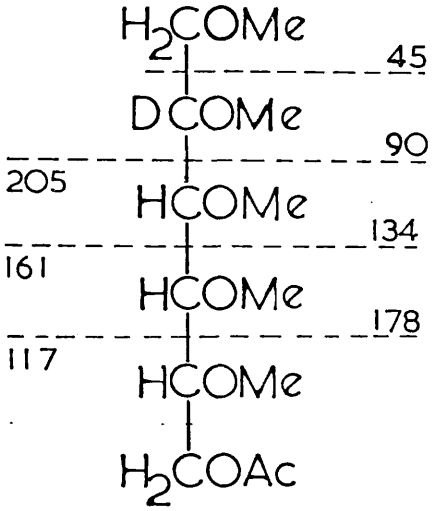
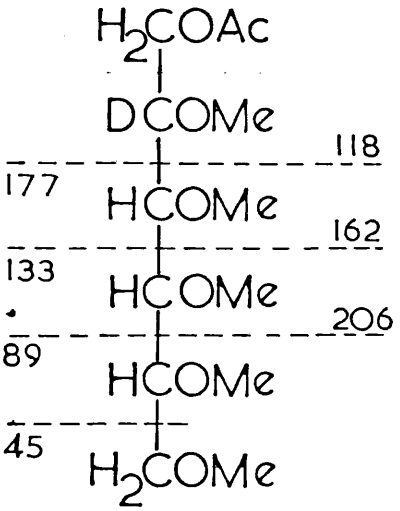


Figure IV.8. Partial electron impact mass spectrum of 2-d-6-O-acetyl-1,2,3,4,5-penta-O-methyl-hexitol (XXXVIII) and 2-d-1-O-acetyl-2,3,4,5,6-penta-O-methyl-hexitol (XL) (ratio ca. 4:1)





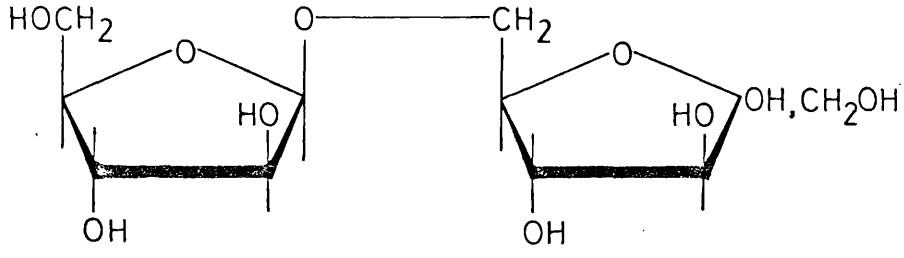
XXXVIII



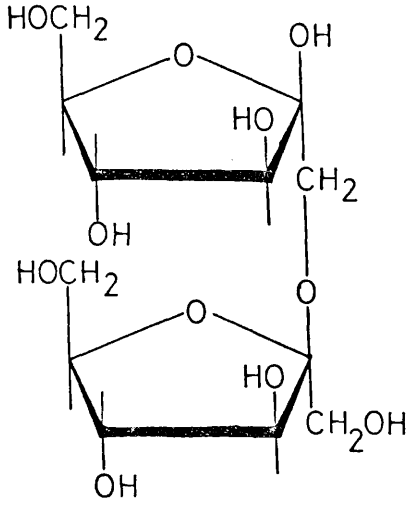
XL

The results obtained indicate component 2, R_{tmg}= 0.79, to be the expected compound XXXII, derived from terminal non-reducing D-fructofuranosyl units (XXXIV). Component 1, R_{tmg}= 0.50, appears to be a mixture of 2-d-6-O-acetyl-1,2,3,4,5-penta-O-methyl-hexitol (XXXVIII) and 2-d-1-O-acetyl-2,3,4,5,6-penta-O-methyl-hexitol (XL), these compounds being derived from the reducing groups of 6-O-β-D-fructofuranosyl-D-fructofuranose (levanbiose, XLI) and 1-O-β-D-fructofuranosyl-D-fructose (inulobiose, XLII).

Since, in the mixed mass spectrum of compounds XXXVIII and XL (Fig. IV. 8.) abundances of fragments derived from each (respectively m/e 90 and 89, 117 and 118, 134 and 133, 161 and 162, 178 and 177) are in the approximate ratio 4 : 1 respectively, it follows that levanbiose (XLI) and inulobiose (XLII) are present in the disaccharide preparation in similar proportions. Inulobiose is undoubtedly present as a result of hydrolysis of the 2 ⟶ 6 linkages on either side of a branching residue.



XLI

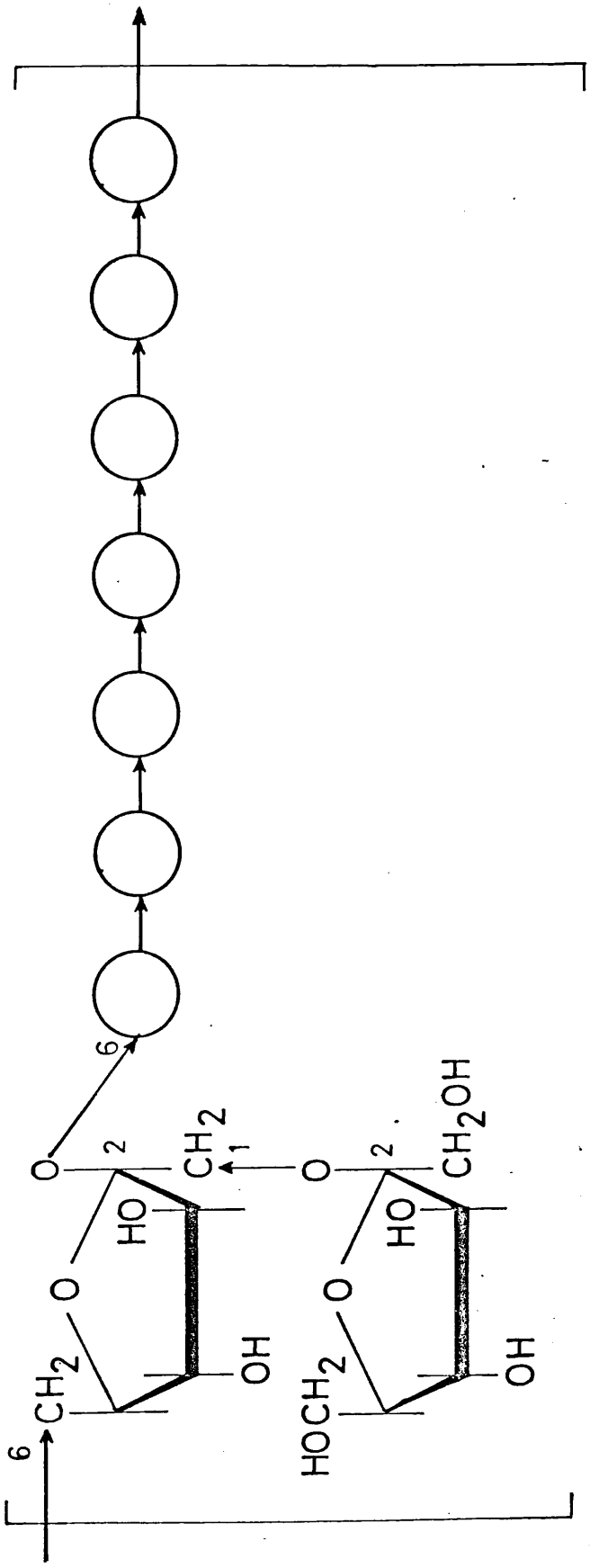


XLII

It can be concluded that the fructan is composed of D-fructofuranosyl units at chain ends, and D-fructofuranose residues linked through positions 2 and 6 and 2,6 and 1. In addition to rotational evidence (Table IV.1., p. 101), hydrolysis by invertase (see section IV.D. iv.b.) suggests the anomeric configuration to be of the β - type. The fructan elaborated by S. salivarius strain 51 has, therefore, been shown to comply with the definition of a levan given in I.A.

It is believed that these results constitute the first chemical characterisation of a levan. An average repeating unit of the levan elaborated by S. salivarius strain 51 is shown in Fig. IV.9. As the other fructans of this investigation are synthesised by strains of the same species from the same substrate and, in addition to yielding the same O-acetyl-O-methyl-hexitols upon methylation analysis, possess similar specific rotations, it was thought reasonable to conclude that they, likewise, are true bacterial levans.

Figure IV.9. Levan of S. salivarius strain 51 : Average repeating unit



IV.C. Molecular Weight Determinations

Having established that the polysaccharides of this investigation are levans, it was considered necessary to obtain information relating to their molecular weights. Previous studies [32,46,51,56,59] (see Section I.B.) have suggested that molecular weights of bacterial fructans can be extremely high.

In general, enzymically synthesised polysaccharides are polydispersed materials with molecular sizes and weights being distributed over a range of values. In addition, there can often be some doubt as to whether the extremely high values for molecular weights, sometimes obtained by physical methods, represent true molecular weights or, in fact, particle weights of aggregations of molecules. Two physical methods, viz. gel filtration and sedimentation, were used to determine the molecular weights of the levans of this investigation. Because of possible doubt mentioned above, it was felt justified to make use of certain assumptions and approximations leading to approximate values for average molecular weights.

IV.C.i. Gel filtration

Gel filtration is a technique that separates molecules essentially according to physical size. Suitable gels contain interstices or pores of varying sizes that tend to hinder the passage of molecules through the gel. Thus large molecules, above a certain size, may pass through the gel unhindered and, if applied to a gel-filtration column, be excluded from the column, emerging in the void volume. Smaller molecules will be retarded by the gel more or less according to the fraction of pores that can accommodate them. Consequently the smallest molecules will be the last to emerge from the gel. Gel filtration media are available in a large number of grades each being able to separate components within a certain molecular

weight range. The exclusion limit of a medium (the largest size molecule that is retarded) depends upon the type (and shape) of molecules being separated. When the technique is employed for molecular weight determinations of poly- or mono-dispersed substances it is therefore usual to calibrate the gel filtration column with reference compounds of known molecular weight. If suitable reference compounds are available the method is capable of yielding fairly accurate results for molecular weights of unknown substances, these being "weight average" results in the case of poly-dispersed materials.

It was thought likely that Sepharose gels (Pharmacia AB), which consist of spherical beads of modified agarose, and which possess extremely high exclusion limits for polysaccharides, might prove to be suitable for fractionating the levans of this investigation.

The levans were subjected to gel filtration on Sepharose 4B and 2B, as described in III. G. i. and III. G. ii., the resulting elution profiles being shown in Figs. IV. 10. and IV. 11. respectively. It is evident that all three levans are excluded from the column containing Sepharose 4B, which has a stated exclusion limit for polysaccharides of 5×10^6. In the case of Sepharose 2B, however, only partial exclusion occurs, this gel having a stated exclusion limit for polysaccharides of 20×10^6.

If the parameters V_o, V_t and V_e are considered,

where V_o = void volume of the column
V_t = total volume of the gel bed
V_e = elution volume of the solute

then $K_{av} = \frac{V_e - V_o}{V_t - V_o}$ [186]

where Kav = the fraction of the volume of the gel available to the solute

Figure IV. 10. Gel filtration elution profile of an S. salivarius levan (strain 51) on Sepharose 4B

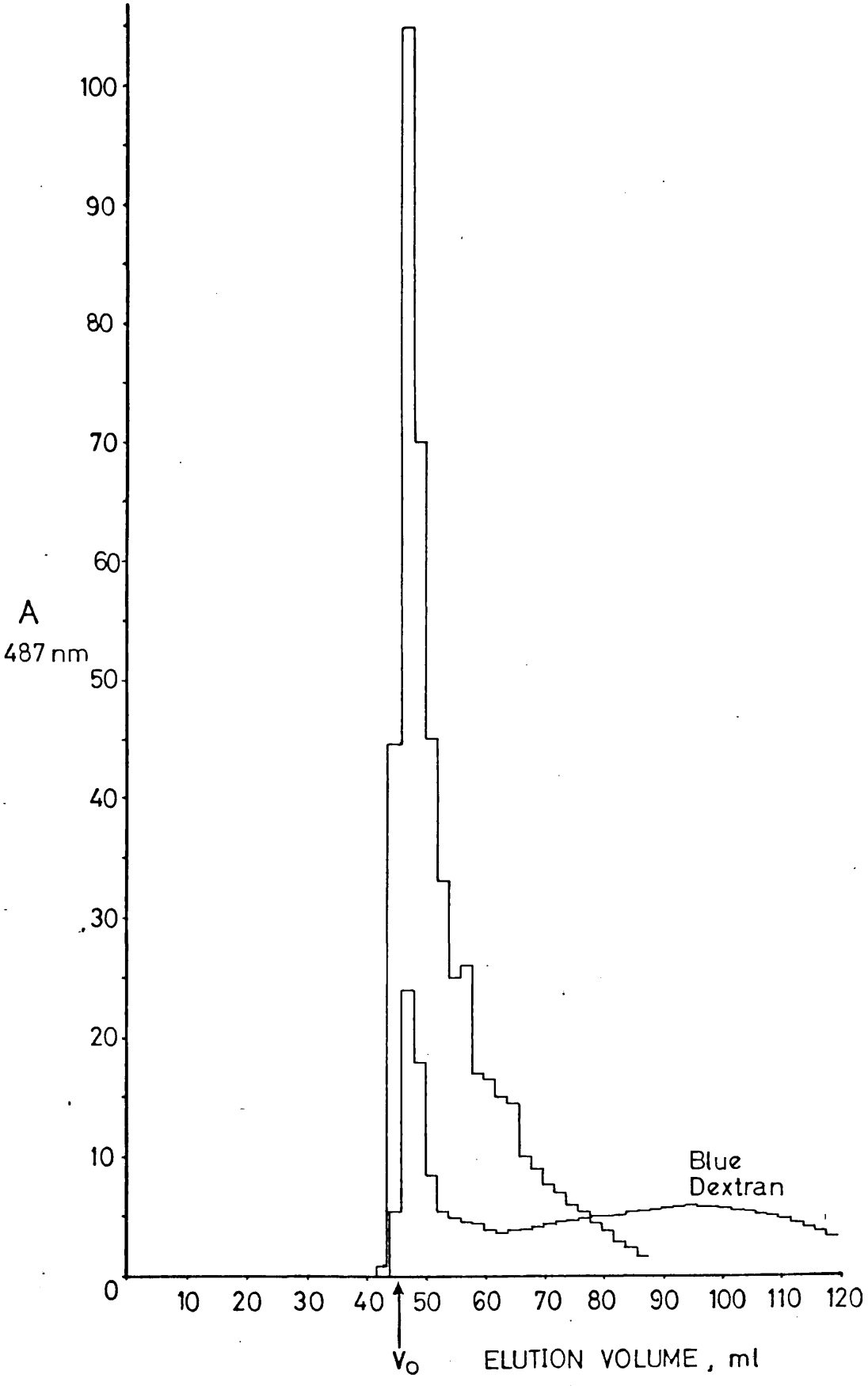
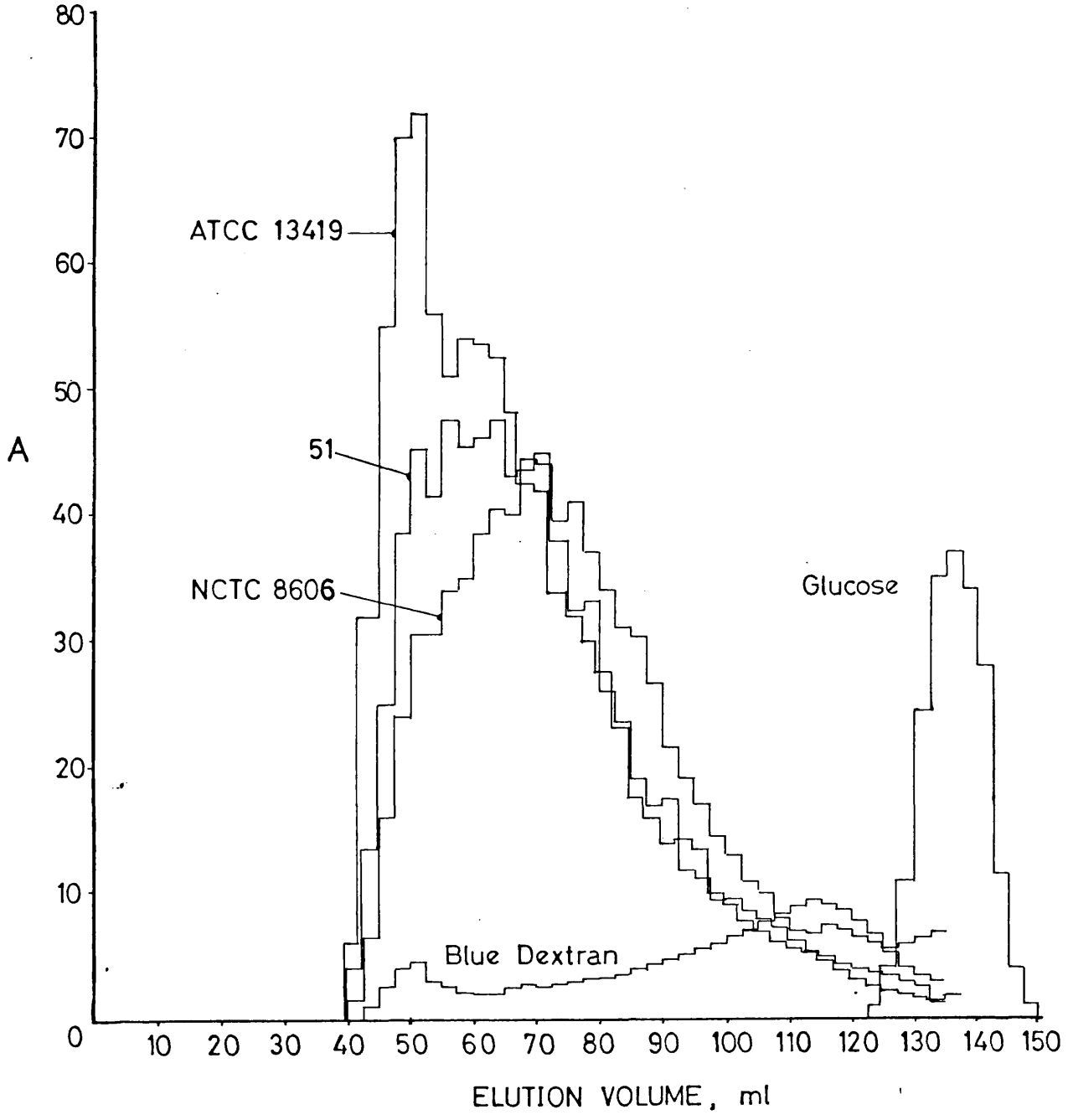


Figure IV.11. Gel filtration elution profiles of S. salivarius levans on Sepharose 2B



Because suitable reference compounds were not available for the calibration of a column with such a high exclusion limit, an inverse linear relationship was assumed[187] between K_{av} and $\log_{10}$ of an average molecular weight, $\overline{MW}$.

Then, $1 - K_{av} \propto \log_{10} \overline{MW}$

Assuming $K_{av} = 0$ at the stated exclusion limit (20×10^6),

then $1 - K_{av} = 1.0$ when $\log_{10} \overline{MW} = 7.3$

Experimental values of K_{av} (Table IV.5.) were found for the three polysaccharides and approximate average molecular weights, $\overline{MW}$, were calculated with the expression

$$\log_{10} \overline{MW} = (1 - K_{av}) \times 7.3$$

Table IV.5. Gel filtration properties of the levans elaborated by strains of S. salivarius : approximate molecular weights

Strain	V_e, ml	K_{av}*	$1 - K_{av}$	$\log_{10} \overline{MW}$	$\overline{MW}$
ATCC 13419	~ 50	0 (excluded)	1.0	7.30	20×10^6
51	~ 55	0.942	0.058	6.88	7.6×10^6
NCTC 8606	~ 65	0.827	0.173	6.04	1.2×10^6

* K_{av} calculated from measured parameters for column (using Blue Dextran 2000 and D-glucose) : -

$V_t = 136.5$ ml

$V_o = 50.0$ ml

IV. C. ii. Sedimentation

The analytical ultracentrifuge enables the sedimentation of solutes, under the influence of a gravitational field, to be monitored. This is accomplished by passing light through windows in the rotor cell(s) and observing the refractive boundary created by the migrating solute. Differentiation of the refractive boundary by Schlieren optics results in a migrating peak being observable, the shape of which being determined by the homogeneity of the solute. Thus highly homogeneous, high molecular weight materials such as crystalline enzymes will produce symmetrical sharp peaks whereas polydispersed materials can be expected to produce somewhat broader, less well defined peaks, the shape of which depending upon the molecular weight distribution of the solute in question.

Usually when a polydispersed material is being investigated, data derived from the (photographically) recorded Schlieren images can be used to calculate a sedimentation constant, S, which can, in turn, be used in the calculation of an average molecular weight if measurements are taken to the apex of the observed migrating peak.

The sedimentation constant S_c, at a solute concentration of c, is expressed in Svedberg units : -

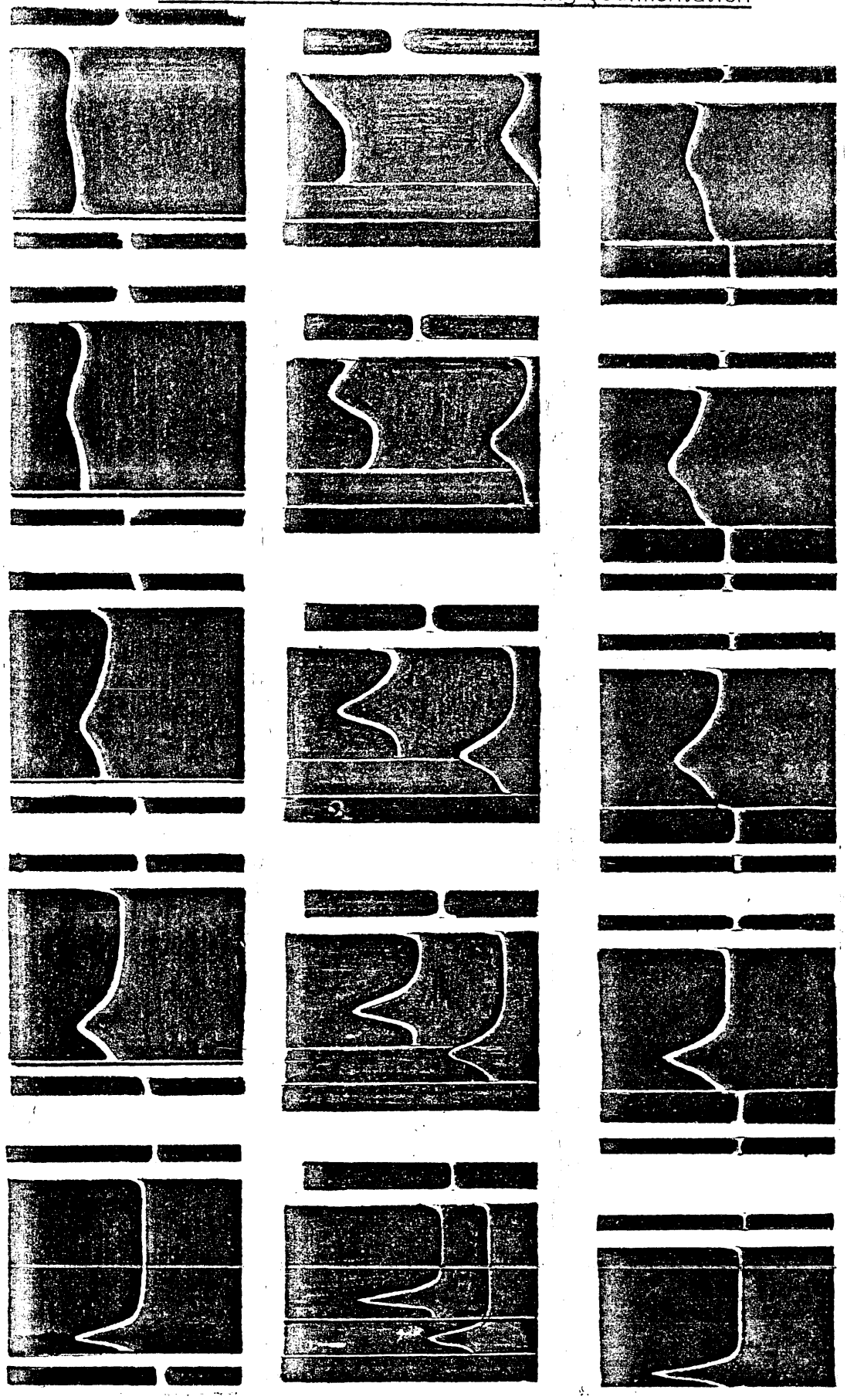
$$S_c = \frac{2.303}{\omega^2} \cdot \frac{d \log_{10} x}{dt} \cdot 10^3$$

where ω = angular velocity of rotor in radians s^{-1}

x = distance (cm) of solute peak from rotor axis at time t (secs., from commencement of run)

The three levans were subjected to ultracentrifugal sedimentation, as described in III. G. iii., the resultant Schlieren images being depicted in Fig. IV. 12. It is evident that a single symmetrical peak is produced by each

Figure IV. 12. Schlieren images of levans during sedimentation



NCTC 8606 ATCC 13419 51 51

21,740 rpm 21,740 rpm 14,290 rpm

polysaccharide, although that of strain 51 exhibits a slight shoulder on the trailing edge (i.e. the low molecular weight side). With a knowledge of the rotor and cell employed (Fig. IV.13.) the distance (x cm) of the peak apex from the rotor axis could be determined in each case. The results, shown graphically in Fig. IV.14., demonstrate that a linear relationship exists between $\log_{10} x$ and t. Sedimentation constants were calculated for each polysaccharide using the above expression, the concentration, (c), in each case being 5 mg ml^{-1} . The values for S_c thus obtained are given in Table IV.6.

Since polysaccharides are non-ideal solutes, S_c values vary with concentration. It is usual practice to obtain S_c values over a range of concentrations and, by extrapolation, obtain a value for S_o , the sedimentation constant at zero concentration.

Newbrun and Baker[159] obtained S_c values for the levan elaborated by S. salivarius ATCC 13419 (one of the strains of this present investigation) over a range of concentrations and found that a linear relationship existed between the reciprocal of S_c and concentration, c : -

$$\frac{d\left(\frac{1}{S_c}\right)}{d\,c} = 2.91 \times 10^{-4}$$

where concentration, c , is in mg ml^{-1}

Assuming the same relationship to hold for the levans of the present investigation, values of S_o were calculated and recorded in Table IV.6. The same authors obtained an average value for the molecular weight ($\overline{MW}$) of ATCC 13419 levan of 23.3×10^6 when $S_o = 217.4$. On the basis of this determination and on the assumption that $\overline{MW}^{2/3}$ is proportional to S_o (if the range is not too great)[159] , the derived values of S_o were used to determine approximate values of $\overline{MW}$ for each of the levans of this investigation, the results being given in Table IV.6.

Figure IV. 13. Ultracentrifuge rotor and cell dimensions

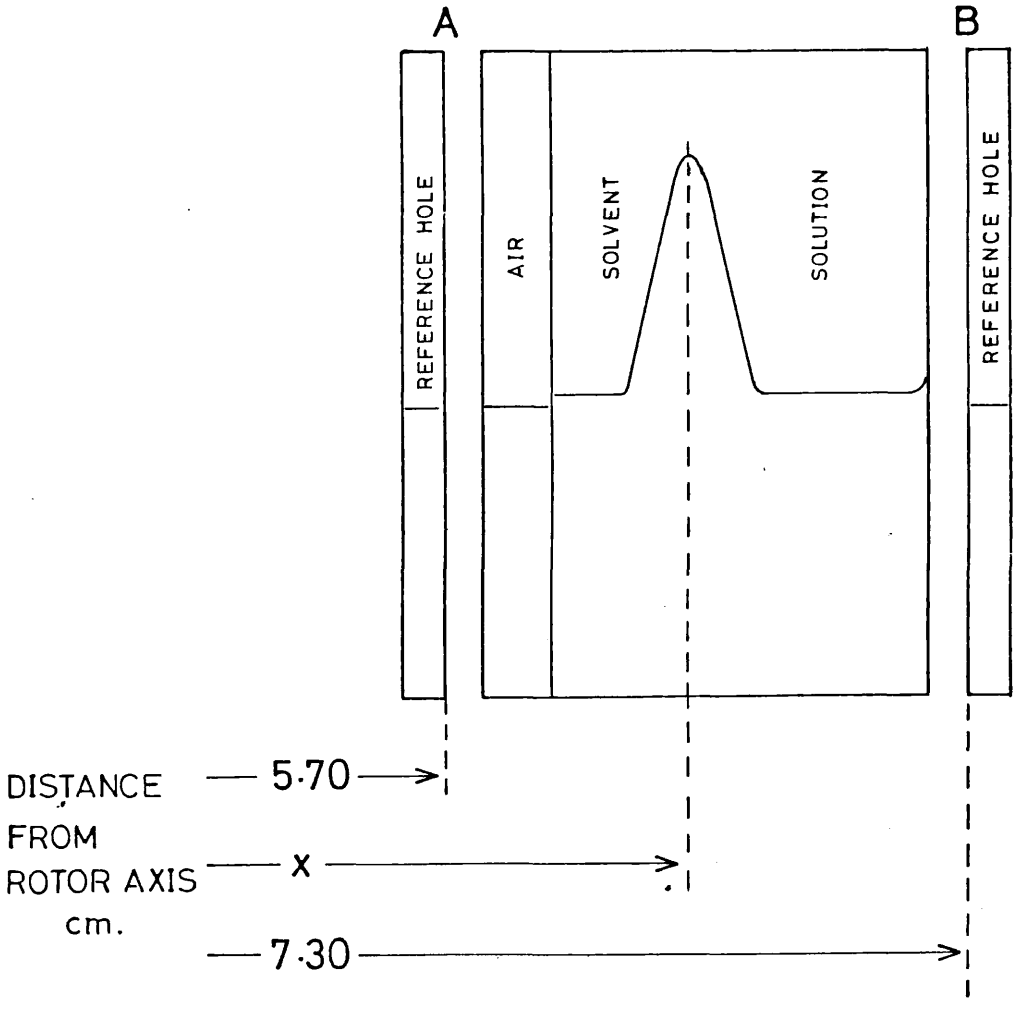


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Figure IV. 14. Sedimentation curves of S. salivarius levans

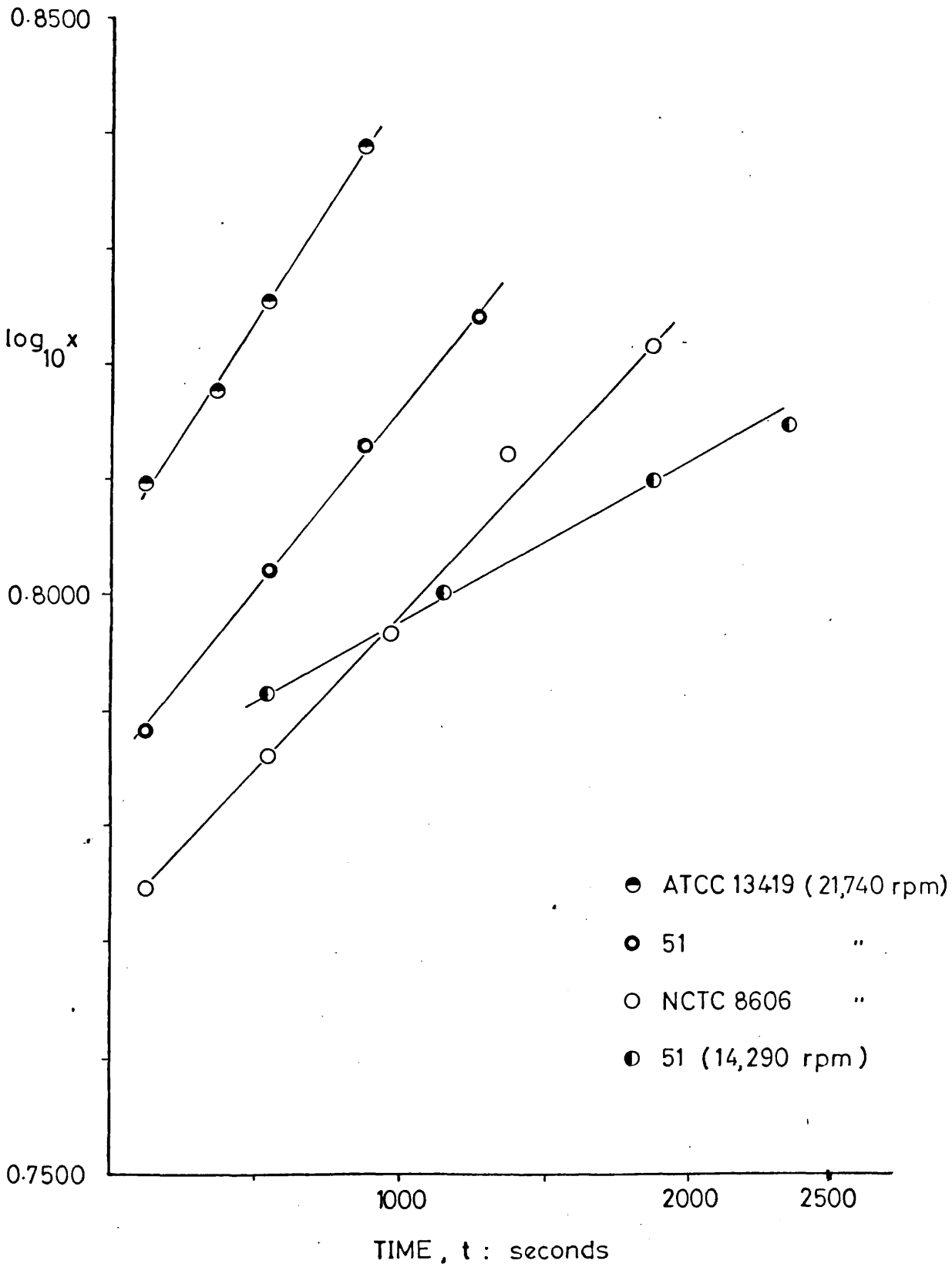


Table IV.6. Sedimentation constants and approximate average molecular weights of S. salivarius levans

Strain	Rotor Speed r.p.m.	* $S_c \cdot 10^{13}$	$\frac{1}{S_c \cdot 10^{13}}$	$\frac{1}{S_o \cdot 10^{13}}$	** $S_o \cdot 10^{13}$	$\overline{MW}$ (approx.)	$\overline{MW}$ (approx.) (by gel filtration)
ATCC 13419	21,740	172.4	0.0058	0.00435	230.0	25.4×10^6	20×10^6
51	21,740	140.2	0.00713	0.00568	170.6	16.2×10^6	7.6×10^6
	14,290	142.7	0.00701	0.00556	179.9	17.5×10^6	
NCTC 8606	21,740	121.1	0.00826	0.00681	146.8	12.9×10^6	1.2×10^6

* S_c = sedimentation constant at concentration, c , of 5 mg ml

** S_o = " " zero concentration

Comparison of the results obtained by the two methods (Table IV. 6.) shows reasonable agreement for the levans of ATCC 13419 and 51. The two figures for NCTC 8606 levan, however, differ by a factor of about ten, no doubt due to the errors involved in the various assumptions made becoming greater at a lower molecular weight. It is evident, however, that the latter levan is of considerably smaller molecular size than the other two. This levan was synthesised by the dialysis tube procedure (III. A. iii.), under conditions which probably involve higher enzyme and substrate concentrations, conditions that would be expected to modify the molecular weight of the polysaccharide produced.

It should be questioned as to whether the results obtained truly represent molecular weights or rather the weights of aggregations of molecules. The only unambiguous solution of this problem would be to determine the number of reducing terminii or initiator molecules in a given amount of polysaccharide, since each molecule can possess only one such terminus. However, with such large molecules, or aggregations of molecules, there exists no suitable method for assaying the reducing terminii. It has been shown that levan molecules are formed ab initio sucrose[113], thus any methods would have to rely essentially upon detection and estimation of the terminal non-reducing D-glucose residues. Obviously any such method would be inherently unreliable because of the low abundance of D-glucose residues, and in addition, the possibility of D-glucose, liberated in the enzymic synthesis of the polymer, being trapped or occluded in the polymer structure and co-determined, could not be eliminated.

IV.D. The Extracellular Levanase Elaborated by *Streptococcus salivarius* strain 51

It was shown in IV.A.i. that strains of *S. salivarius* apparently exhibit the ability to induce the formation of hydrolases capable of degrading the polymeric products of the enzymic levan synthesis, a behaviour previously associated with other strains of oral streptococci [149] and *Odontomyces viscosus*. [70,71]

Since little is known about the levanases elaborated by oral streptococci it was thought desirable to attempt to extract, partially purify and conduct a preliminary study on the induced levanase of a strain of *S. salivarius*. Strain 51 was selected for the preparation of the enzyme and was cultured in a medium containing a levan elaborated by the same strain, as this was available in the greatest supply.

In this section the preparation, pH optimum and assay of a partially purified levanase is described, followed by an attempted determination of homogeneity, its action on the substrate levan, a comparative study with an invertase and finally its action on oligosaccharides obtained from inulin.

IV.D.i. Preparation, optimum pH and assay

IV.D.i.a. *Preparation.* In order to induce levanase activity *S. salivarius* strain 51 was cultured in a medium containing levan as the main source of carbohydrate essentially in the manner described by Mezner [188] who obtained a crude levanase preparation from *O. viscosus*. Experimental details for the culture and partial purification of an extracellular preparation are given in III.J. The final step in the purification procedure, viz. gel filtration on

Sephadex G200 produced the elution profile shown in Fig. IV. 15. Testing the fractions for levanase activity as described in III. J. yielded a positive result for the higher molecular weight fraction only (fractions 12-18). This active fraction was either stored at 4° under toluene or co-lyophilised with its buffer. Lyophilisation caused negligible loss of activity upon subsequent redissolution in water.

IV. D. i. b. Optimum pH. The optimum pH, at 37°, for the hydrolysis of levan was determined as described in III. K., the results being graphically represented in Fig. IV. 16. It is evident that the enzyme preparation is active in the range pH 4. 5 to 8. 0, the activity being maximal between pH 6. 6 and 6. 8.

IV. D. i. c. Assay of activity. The activity of a hydrolase is usually expressed as the number of μmoles of product liberated from excess substrate by a fixed amount of enzyme at a certain pH (usually the optimum) in a certain time (usually 1 min). In order to assay the levanase preparation for activity towards levan as substrate, it was, of course, necessary to know what the product(s) of the degradation was (were). The result obtained, by paper chromatography of a levanase digest of levan (III. L. i.), suggested that D-fructose is the sole product. Using the lyophilised enzyme preparation, a quantitative digest was prepared as described in III. L. ii. The results obtained from this digest are given in Table IV. 7. and a graph was constructed (Fig. IV. 17.) plotting D-fructose liberated against digest time, the rate of D-fructose liberation being determined from the initial linear part of the curve.

It is also evident from this hydrolysis curve that the degradation of levan by the levanase preparation smoothly proceeds to completion, a finding that suggests that the levanase preparation is capable of cleaving the 2⟶1

Figure IV. 15. Sephadex G200 gel filtration of crude levanase preparation

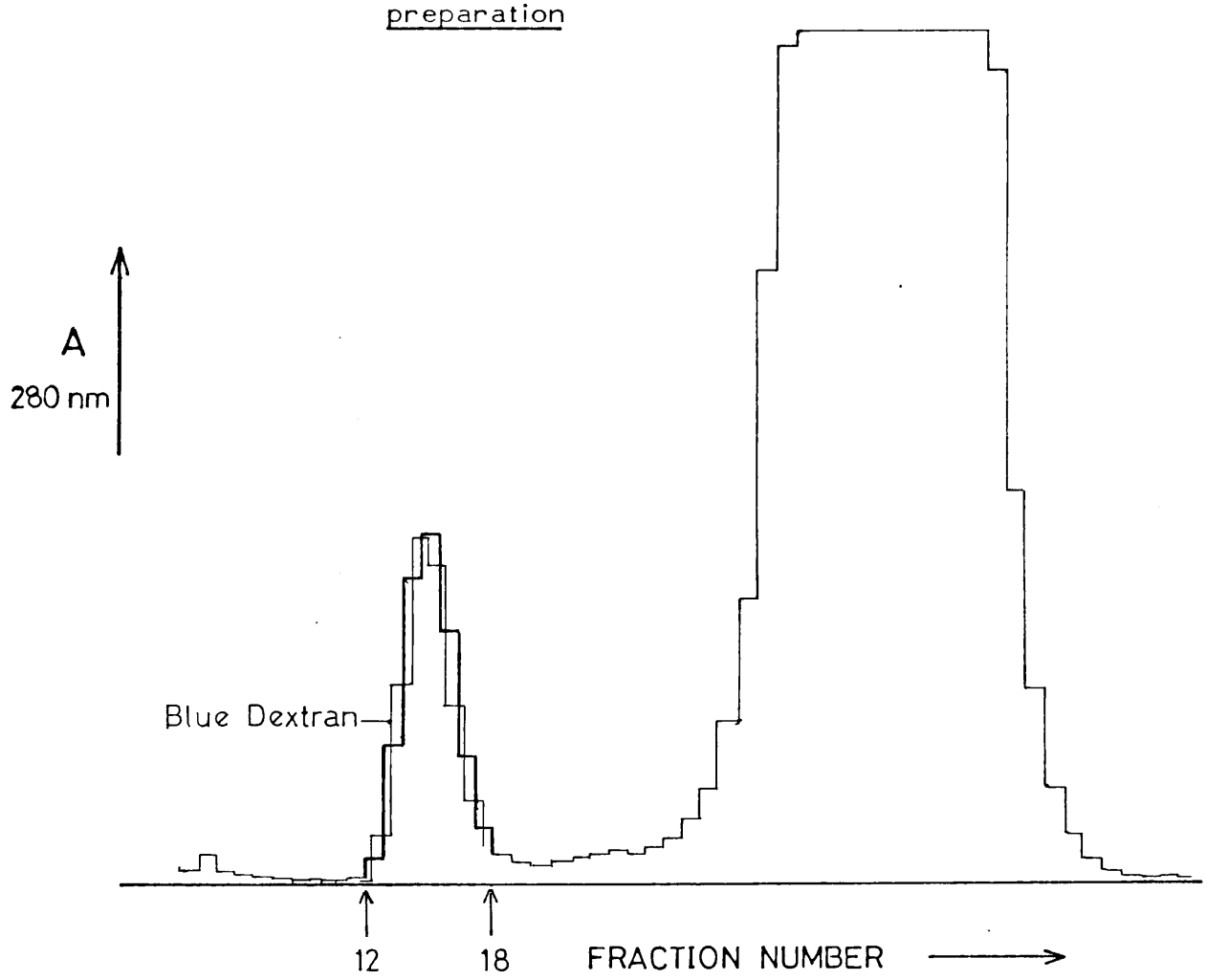


Figure IV. 16. pH optimum of the extracellular levanase of S. salivarius strain 51

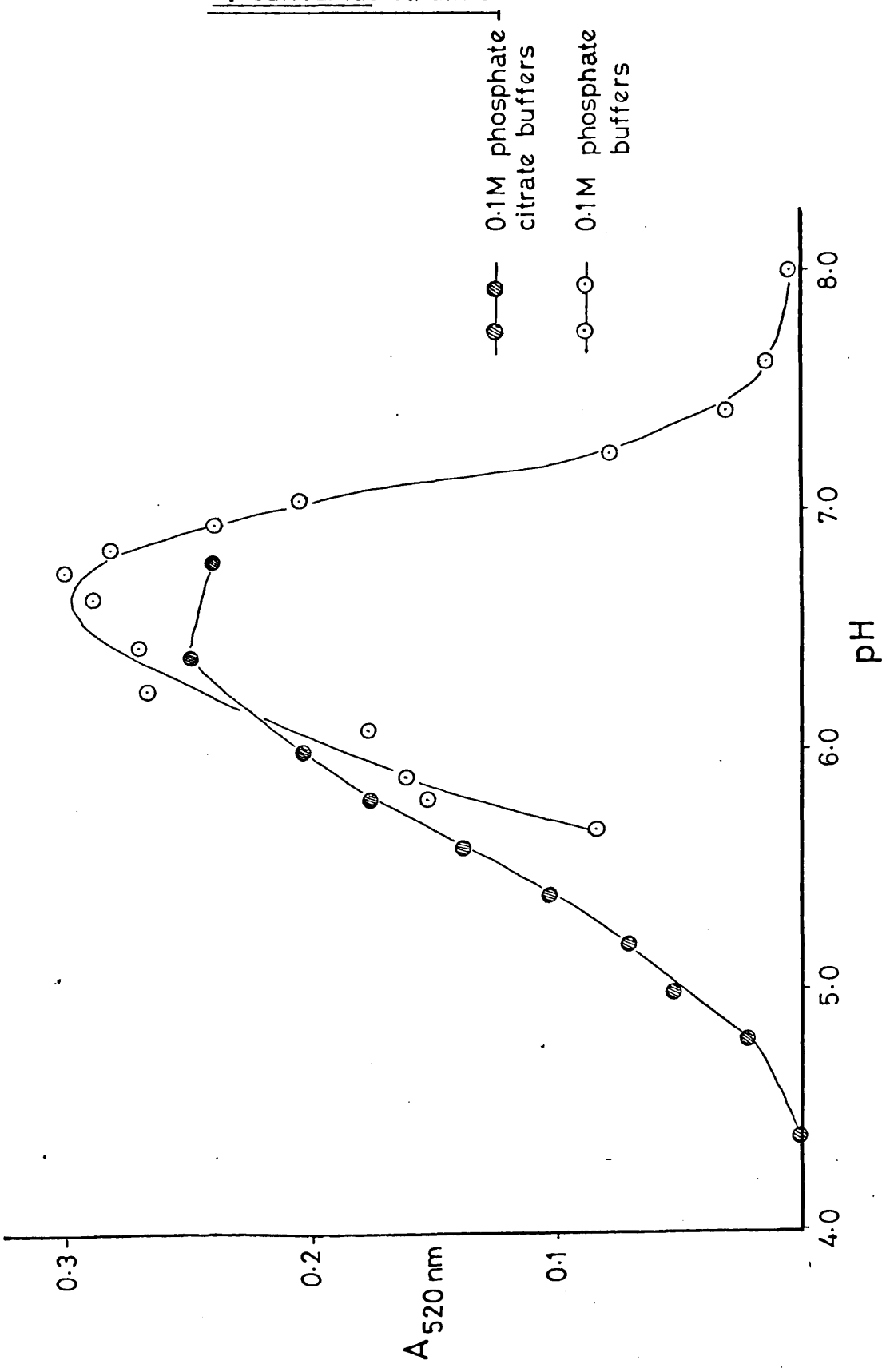


Figure IV. 17. Quantitative levanase - levan digest

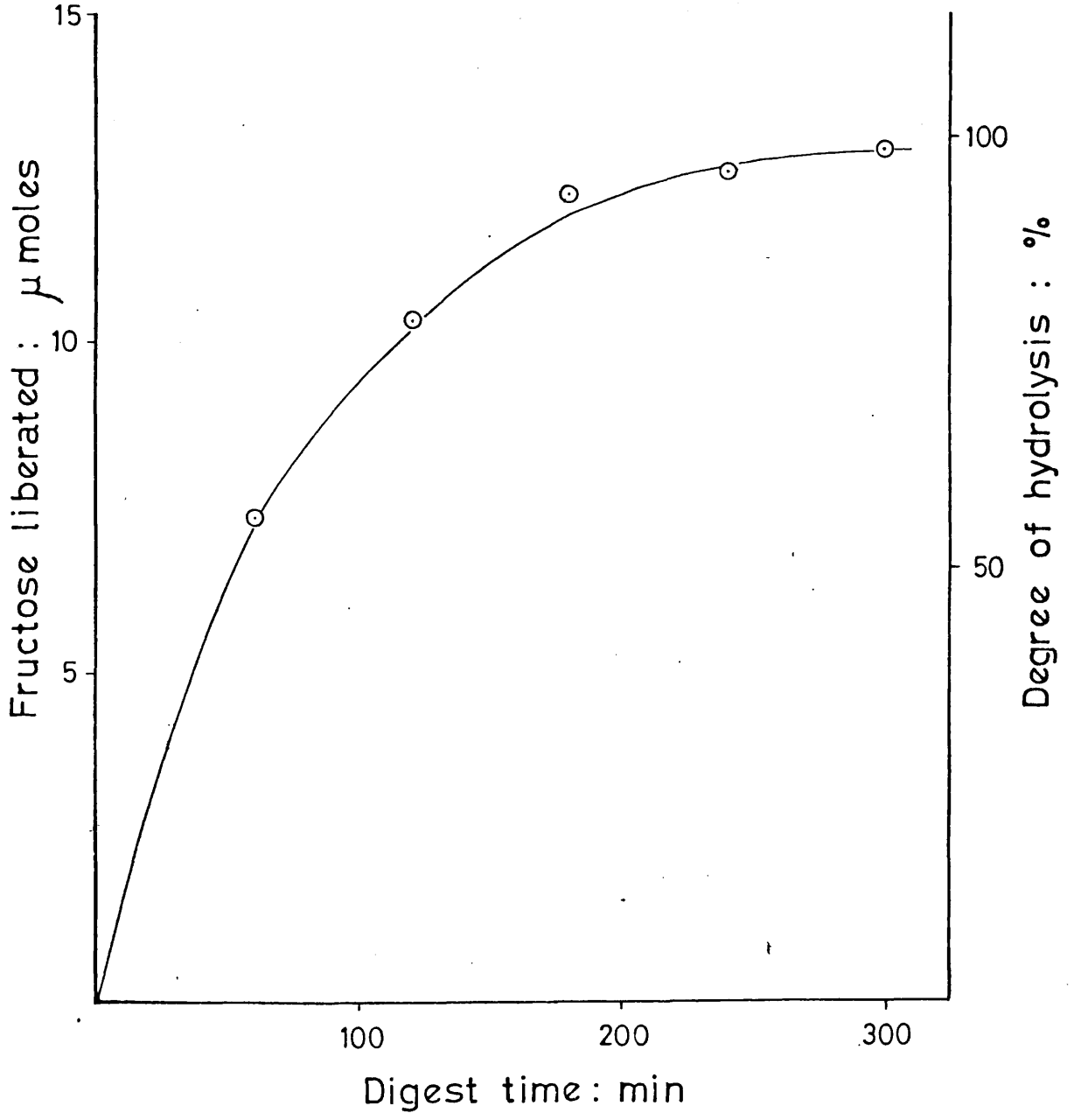


Table IV.7. Quantitative levanase-levan digest : Levanase activity

Digest time min	D-fructose liberated		Degree of hydrolysis %	Activity * U. mg^{-1}
	µg	µmoles		
0	0	0	0	0.024
60	1320	7.33	56.0	
120	1860	10.33	79.0	
180	2205	12.25	93.6	
240	2265	12.58	96.2	
300	2325	12.92	98.7	

* Weight lyophilised enzyme preparation used = 5 mg.

Table IV.8. Protein content of levanase (lyophilised) : Specific activity

Wt. lyophilised enzyme used, mg	Protein content µg mg^{-1}	Specific activity U mg^{-1} protein
5.0	7.4	3.3

interfructosidic linkages occurring at branching residues in addition to the 2 ⟶ 6 interfructosidic linkages.

The protein content of the lyophilised preparation was determined as described in III.M., the result being given in Table IV.8. A unit, U, of levanase activity may be defined as "the amount of enzyme required to liberate 1 μmole of D-fructose per min from levan at 37° and pH 6.6 - 6.8". The Specific activity, defined as the number of units of activity per mg of enzyme protein, was then calculated (Table IV.9.). The value obtained, viz. 3.3, does not appear to be very high compared with, say, the stated activity of 535 (μmoles sucrose hydrolysed min^{-1} mg^{-1}) for the invertase preparation used later in this study (see p. 146). In the case of the levanase preparation, however, the substrate (levan) is of a very high molecular weight and if the enzyme preparation should prove to have an exo action (see later, p. 143) a specific activity value of 3.3 is not unreasonable. In addition, the purity of the preparation has not been established. An improvement in Specific activity may well be possible should the enzyme preparation be subjected to further purification operations.

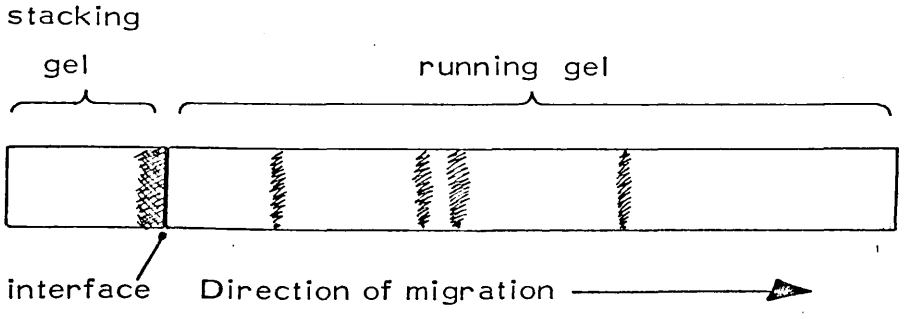
IV.D.ii. Homogeneity of the levanase preparation

During the purification of the enzyme preparation (III.J.), the active material was eluted as a single peak, after the void volume, upon Sephadex G200 gel filtration, as established by the use of Blue Dextran 2000 (Pharmacia AB, Fig. IV.15.). As G200 has stated molecular weight exclusion limits of 8×10^5 for proteins and 2×10^5 for dextrans, the levanase preparation is of high molecular weight, possibly within this range. Because the active material was excluded, however, it is not possible to use the single peak as a criterion of homogeneity. Other methods available for the establishment of homo- or

heterogeneity of biological macromolecules include discontinuous gel electrophoresis which separates components on the basis of their mobility in a potential gradient, and isoelectric focussing in which components of dissimilar isoelectric point are brought to a 'focus' at different points in a pH gradient. Both methods were available since they both use the same basic apparatus but it was decided to use the latter (which requires very expensive ampholytes to set up the pH gradient) only if the electrophoresis experiment suggested the preparation to be homogeneous.

The enzyme preparation was subjected to discontinuous gel electrophoresis, as described in III.N., using a 7.5% polyacrylamide gel which has a stated molecular weight cut-off of ca. 3×10^5. Examination of the resulting stained gel (shown diagramatically in Fig. IV.18.) revealed an intense band at the stacking gel-running gel interface and a number of additional well defined bands, of varying migration rates, along the running gel. These results indicate that a large amount of proteinaceous material is of sufficiently high molecular weight to exclude it from the running gel. The presence of the additional bands clearly indicate that the preparation is heterogeneous and can be concluded to be a mixture of components. Isoelectric focussing of the levanase preparation was, therefore, not attempted.

Fig. IV.18. Gel electrophoresis of levanase preparation



Although of doubtful composition, the levanase preparation was found to be essentially free of levansucrase (synthesising) activity since no levan formation occurred with sucrose as substrate. Conversely, this finding rules out the possibility that the hydrolytic activity is associated with the enzyme(s) responsible for the levan synthesis. The hypotheses that the synthetic reaction is irreversible and that the hydrolase is a separate enzyme, induced when the availability of simple sugars falls below a certain level in the in vivo situation, are therefore supported[148,149] (see IV.A.i., p. 98).

IV.D.iii. Exo action of S. salivarius strain 51 levanase on levan

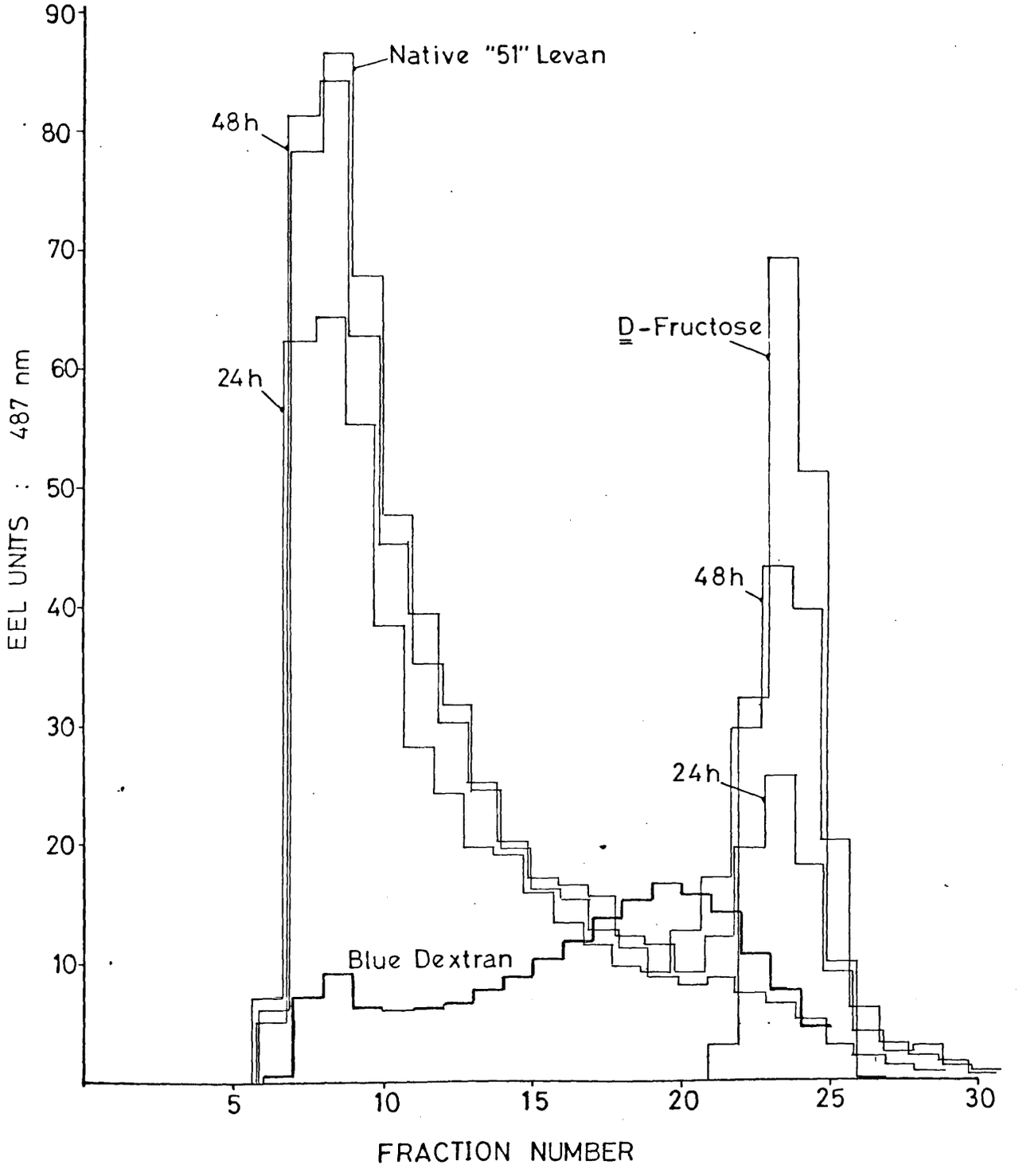
Generally speaking, a hydrolase, acting upon a polysaccharide substrate, possesses either an exo or endo action. In the former case, residues, or groups of two or more residues, are sequentially split off from the non reducing terminus (or terminii in the case of a branched polysaccharide). Endo enzymes, on the other hand, act in a more random fashion, being able to hydrolyse linkages throughout the polymer other than terminal ones, thereby generating products or intermediate products of variable size. An example of a hydrolytic enzyme possessing an exo action is the starch degrading β - amylase[189,190] which promotes a rapid hydrolysis of the outer chains of amylopectin molecules to maltose. Being unable to progress beyond the α-(1$\longrightarrow$6) linkages, which occur at branching positions, a so-called "β - limit dextrin" remains. A further example of an exo-enzyme is the group of amyloglucosidases[191,192] (glucoamylases) which bring about complete degradation of starch, D-glucose being the sole product. Examples of endo - hydrolases are the α - amylases[193], which are found in virtually every type of living cell and which bring about a rapid fragmentation of starch molecules by cleaving α - (1$\longrightarrow$4) - linkages at random, and the amylopectin debranching enzymes isoamylase[194] and pullulanase[195] which act by hydrolysis of the α-(1$\longrightarrow$6) - linkages at branching positions.

It was considered important to establish the action type of the levanase under investigation, not only because action type is a fundamental property of an enzyme, but also because it was realised that a knowledge of the action type might permit the later use of the levanase preparation as a tool in structural studies of levans.

It was established, by paper chromatography, in IV.D.i.c., that D-fructose is most probably the sole product of the enzymic hydrolysis of levan by the levanase under consideration, a result which suggested the enzyme to possess an exo action. It was recognised that an exo action would initially cause little change in the molecular weight distribution of a macromolecular substrate, whereas endo activity would cause drastic changes during the initial stages of the degradation. It was therefore thought that the action type of the levanase could be confirmed by comparing the molecular weight distribution after partial degradation with that of the undegraded levan.

Preparations of the levan of S. salivarius strain 51, partially degraded by levanase, the undegraded levan and D-fructose were separately subjected to gel-filtration on Sepharose 2B as described in III.O. The elution profiles obtained, shown in Fig. IV.19, demonstrate clearly that the molecular weight distribution of the polysaccharide remained essentially unaltered upon partial degradation by levanase, D-fructose being the only product. This result confirms that the levanase of S. salivarius strain 51 degrades levan in an exo fashion.

Figure IV. 19. Exo action of levanase : Gel filtration of partial hydrolysates of levan



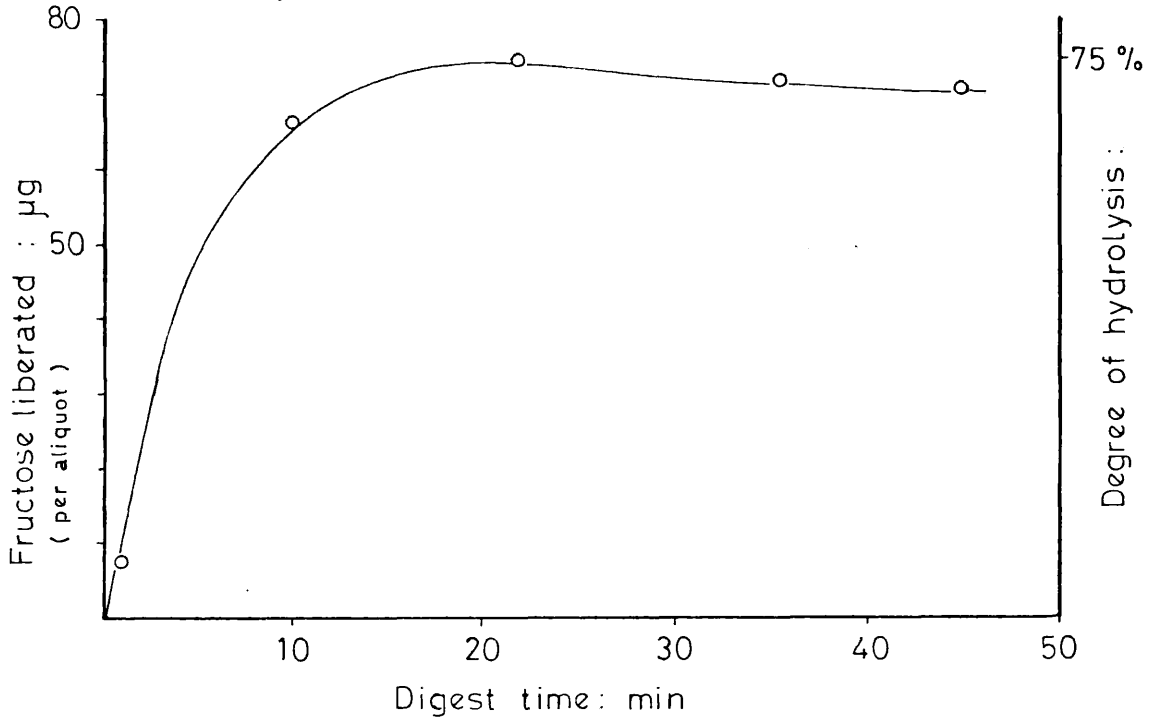
IV. D. iv. A comparative study of the levanase of S. salivarius strain 51 with the invertase of Candida utilis

It was noted by Leach[196] et al, in 1972, that a preparation of yeast invertase (E.C. 3.2.1.26), a non-specific β-D-fructofuranosidase, is capable of degrading fructans of oral origin. It was suggested that the mechanism of this hydrolysis might be the same as that occurring in dental plaque. As it seems likely that fructan metabolism in dental plaque is due to the action of levanases and as no significant studies have been performed on the fructan hydrolases elaborated by oral streptococci, it seemed pertinent to perform a comparative study of the levanase of S. salivarius with a yeast invertase. It was decided to compare the activity of the two enzymes towards methyl β-D-fructofuranoside (a model substrate for invertase), levan (the typical levanase substrate) and inulin. Inulin was chosen as a substrate because it is a linear or unbranched polysaccharide composed of β-D-fructofuranosyl residues linked through positions 2 and 1.[3-7] Because such β-2⟶1-linkages occur in levan at branching positions (see IV. B., p. 110) and, in this environment, are hydrolysed by levanase, it was thought worthwhile to discover whether a polymer composed of contiguous β-2⟶1-linked residues would serve as a substrate.

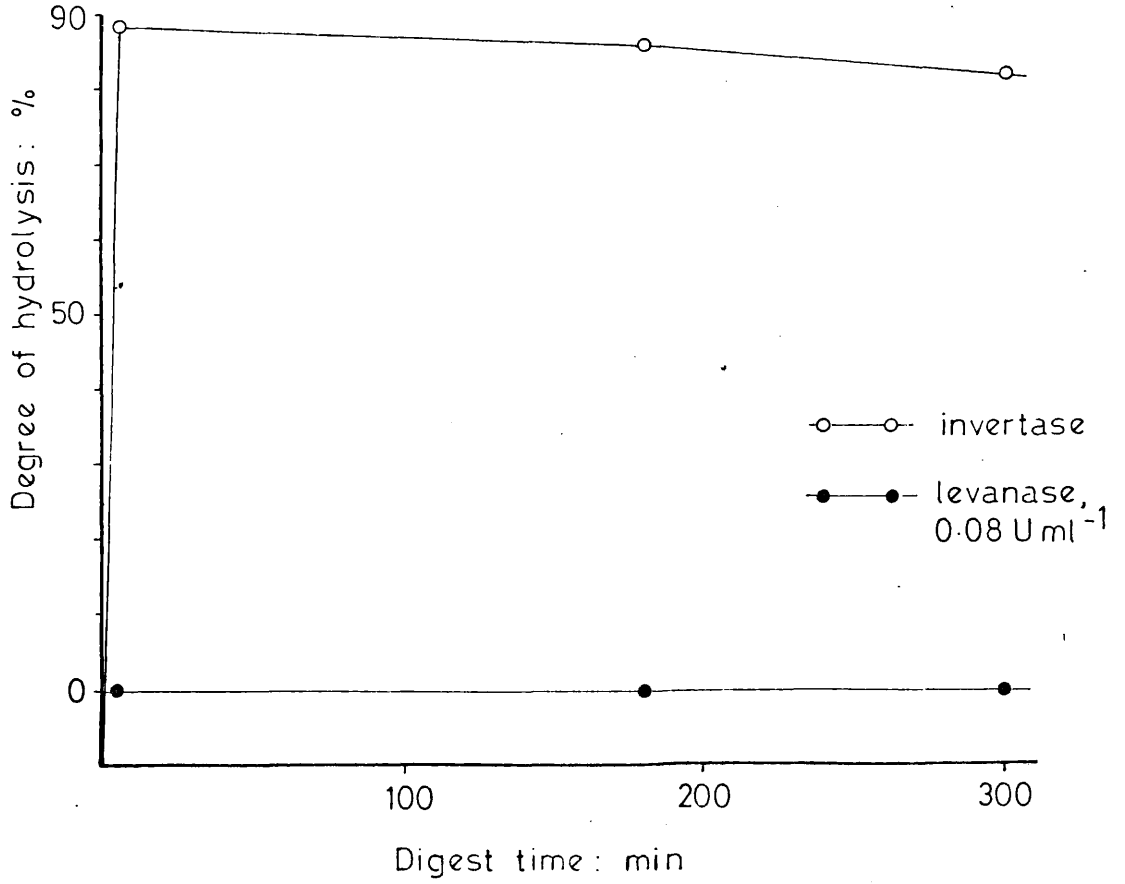
IV. D. iv. a. Action of levanase and invertase on methyl β-D-fructofuranoside. Methyl β-D-fructofuranoside, a model invertase substrate, prepared by the method of Horvath and Metzenberg[171] as described in III. P., had $[\alpha]_D^{20}$ - 36.2° (cf. Lit. value - 60° ± 3°). This substrate was digested with levanase and invertase as described in III. Q., the results, shown graphically in Fig. IV. 20. b., clearly demonstrating that the levanase preparation has negligible action on this substrate and differs in this respect from yeast invertase. It was assumed in this

Figure IV. 20.

a. Action of invertase on methyl β-D-fructofuranoside (III.Q., digest a.)



b. Action of levanase and invertase on methyl β-D-fructofuranoside (III.Q., digests b., c.)



experiment, that the methyl β -D-fructofuranoside, for the purposes of calculating percentage hydrolysis values, was completely dry and pure. Being a syrup, it had retained some moisture, and the rapid hydrolysis to 90%, obtained with yeast invertase, suggests that the moisture content plus any impurities amount to about 10% by weight of the syrup. Interestingly, the degree of hydrolysis, after attaining a maximum value, thereafter decreases slowly with time, evidence that the products of the hydrolysis serve as substrates for a synthetic reaction, a process known as 'reversion'.[197]

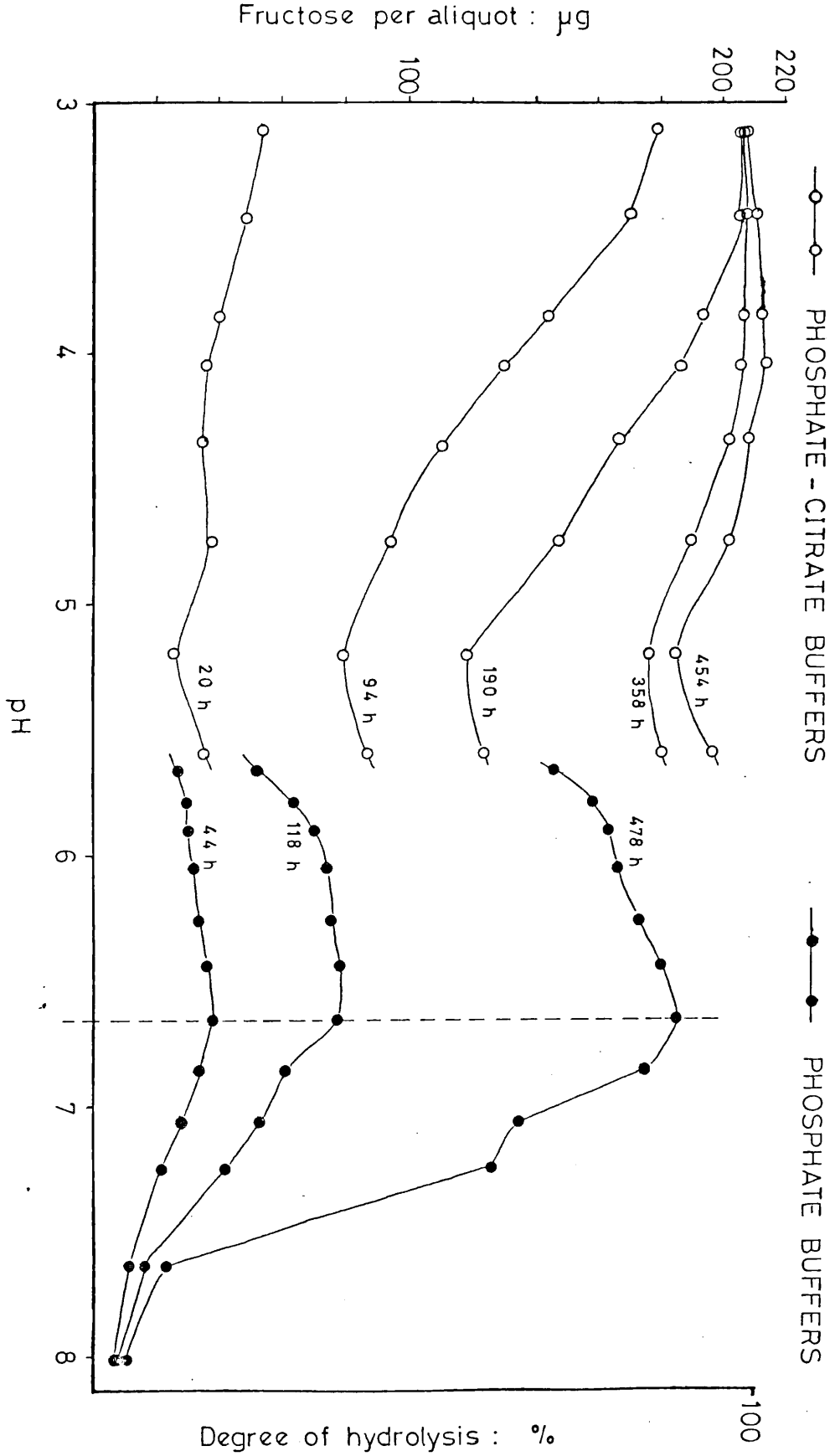
From Fig. IV. 20. a., the rate of D-fructose release, from methyl β -D-fructofuranoside by the invertase preparation, was estimated from the initial linear part of the curve and was found to be 24 μg min^{-1} or 0.13 μmole min^{-1}.

IV. D. iv. b. Action of invertase on levan

(The action of levanase on levan and the activity towards this substrate was discussed in IV. D. i. c.).

An unbuffered invertase-levan digest was prepared as described in III. R. i. and examination of aliquots, at time intervals, by paper chromatography indicated D-fructose to be the only product of the reaction. This suggests that C. utilis invertase, like levanase, has an exo action on levan. In order to make a comparison of the rates of levan hydrolysis displayed by these two enzymes the optimum pH of the invertase reaction was initially found as described in III. R. ii. The hydrolysis curves shown in Fig. IV. 21. indicate the optimum pH to be 6.65. The increasing rates of hydrolysis at pH values less than 5 were not taken as evidence of increasing enzymic activity since levans are highly labile under conditions of low pH.

Figure IV. 21. Optimum pH for the hydrolysis of levan by C. utilis invertase



A quantitative invertase-levan digest was then performed as described in III.R. iii. at pH 6.65. The results, shown graphically in Fig. IV. 22., demonstrate that invertase hydrolyses levan extremely slowly. The extent of hydrolysis reaches almost 100% before subsequently decreasing, evidence, again, that yeast invertases possess reversion activity. The invertase concentration in this experiment was 0.8 mg ml^{-1} and the stated activity (with respect to sucrose hydrolysis) was 535 U mg^{-1}. The rate of release of D-fructose from levan was calculated from the initial gradient of the hydrolysis curve shown in Fig. IV. 22., the resulting activity being 0.75×10^{-3} U mg^{-1}, lower by a factor of 7×10^{-5} than the stated activity towards sucrose and considerably lower than that of the levanase preparation (3.3 U mg $protein^{-1}$, Table IV. 8.).

IV. D. iv. c. Action of levanase and invertase on inulin

Inulin was incubated with the levanase preparation and invertase as described in III.S., the course of the reactions being monitored by measuring the reducing sugar content at time intervals. Assuming the inulin preparation to be dry and pure, the degrees of hydrolysis were calculated and plotted against digest time in Fig. IV. 23. It is evident that invertase smoothly hydrolyses this substrate to completion, whereas the levanase preparation has little or no action. From the initial gradient of the invertase curve the rate of D-fructose release was calculated as 2.19 μg min^{-1}, which is equivalent to an activity of 0.012 μmole min^{-1} (U) mg^{-1}.

The activities of the levanase and invertase preparations towards sucrose, methyl β-D-fructofuranoside, levan and inulin are summarised in Table IV. 9.

Figure IV. 22. Invertase – levan digest at pH optimum (6.65)

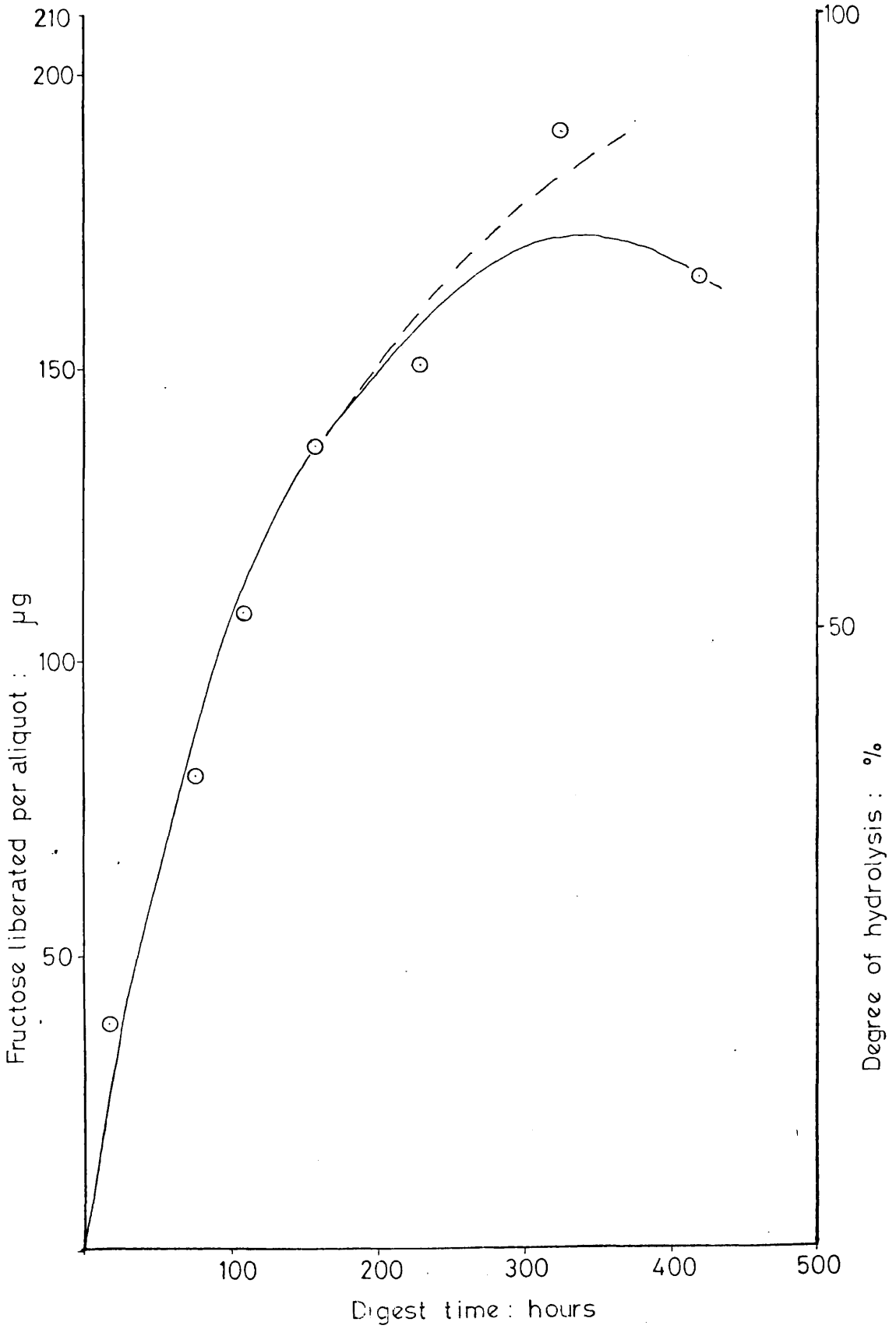


Figure IV. 23. Hydrolysis of inulin by levanase and invertase

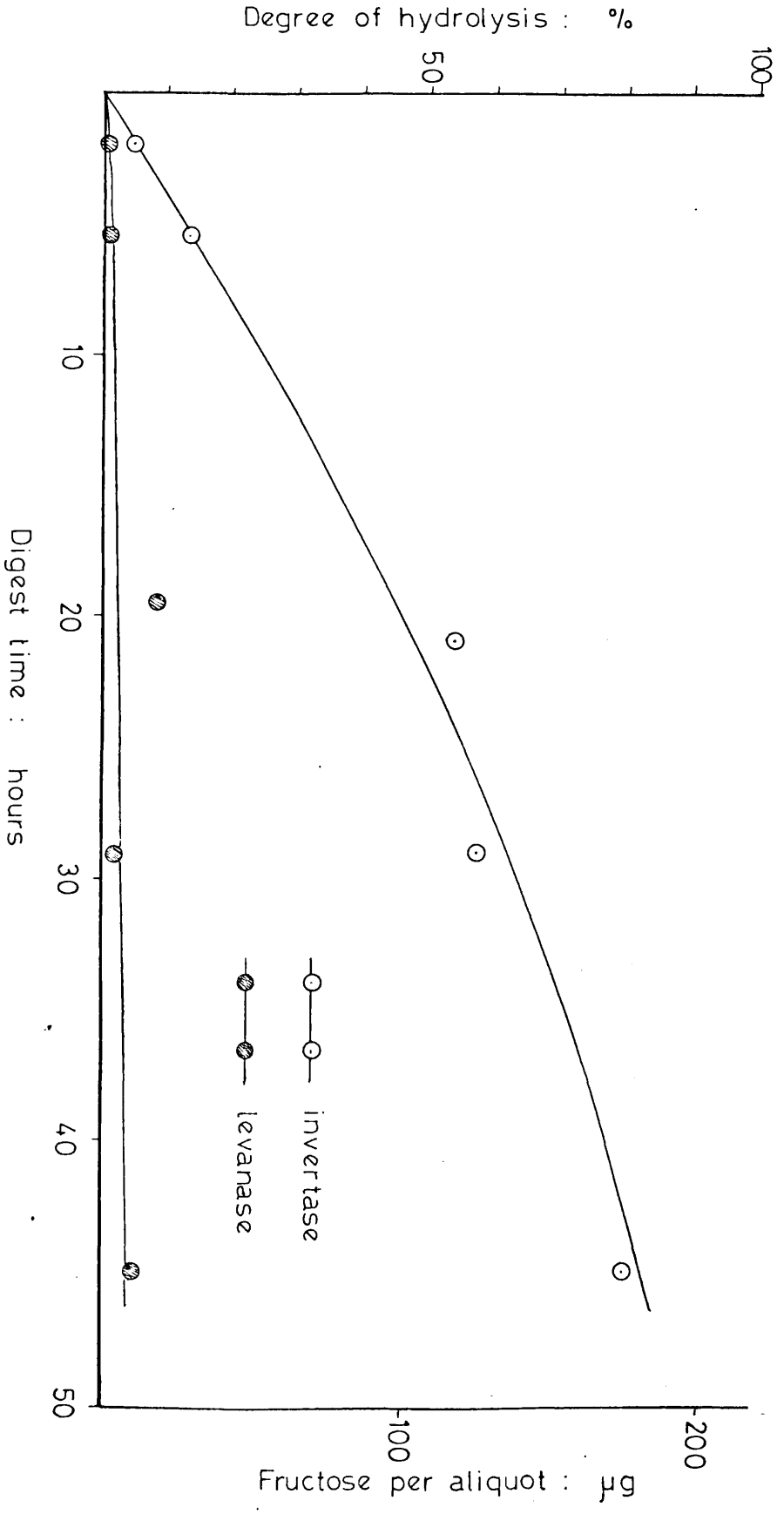


Table IV.9. Activities of levanase and invertase preparations towards various substrates

Substrate	Specific activity: μmoles fructose released min^{-1} mg^{-1}	
	levanase *	invertase **
sucrose	-	535 ***
methyl β-D-fructofuranoside	negligible	1.3×10^{-1}
levan	3.3	7.5×10^{-4}
inulin	negligible	1.2×10^{-2}

* U mg protein^{-1}

** U mg solid^{-1}

*** Manufacturers' stated activity

Clearly, the levanase of S. salivarius strain 51 possesses a far greater substrate specificity than C. utilis invertase. The most striking differences between the two hydrolases being in their affinities for levan and methyl β-D-fructofuranoside. The invertase preparation was found to hydrolyse all the substrates tested, the order of rates of hydrolysis being sucrose > methyl β-D-fructofuranoside inulin > levan.

The fact that the levanase preparation did not hydrolyse inulin to any significant extent suggests that it is able to hydrolyse the $\beta - 2 \longrightarrow 1$ linkages as they occur at branching residues in levan but not in the $\beta - 2 \longrightarrow 1$ linked polymer. An attempt is made, in the following sub-section, to rationalise this aspect of the specificity of the levanase by digesting oligosaccharides obtained from inulin.

IV.D.v. Action of S. salivarius strain 51 levanase on the oligosaccharides obtained from inulin

It was demonstrated in IV.D.iv.c. that the levanase of S. salivarius strain 51, unlike invertase, is unable to hydrolyse inulin, although it hydrolyses the $\beta - 2 \longrightarrow 1$ linkages in levan. It was thought that this specificity must be due to either the need to have the $\beta - 2 \longrightarrow 1$ linkage at a branching residue linked also through positions 6 and 2 or the simple fact that the levanase cannot degrade molecules, above a certain size, possessing contiguous $\beta - 2 \longrightarrow 1$-linked fructofuranosyl residues. In order to test the latter possibility a series of digests were performed, qualitatively and quantitatively in which homologues of inulobiose (XLII) from DP2 to DP7 served as substrate.

Oligosaccharides from a partial acid hydrolysate of inulin, up to DP7, were prepared as described in III.F.ii. The resulting preparations were homogeneous to paper chromatography and had DP values verified as described

in II.H.iv. Each oligosaccharide was digested with levanase as described in III.T. The products of the enzymic degradations were examined by paper chromatography, the results being given in Table IV.10. Relative increases in reducing sugar content with time, in each case, are shown graphically in Fig. IV.24. From these results, percentage hydrolysis values were calculated with the expression: -

$$\text{degree of hydrolysis, \%} = \frac{\text{\% relative increase in reducing sugar content}}{\text{DP} - 1}$$

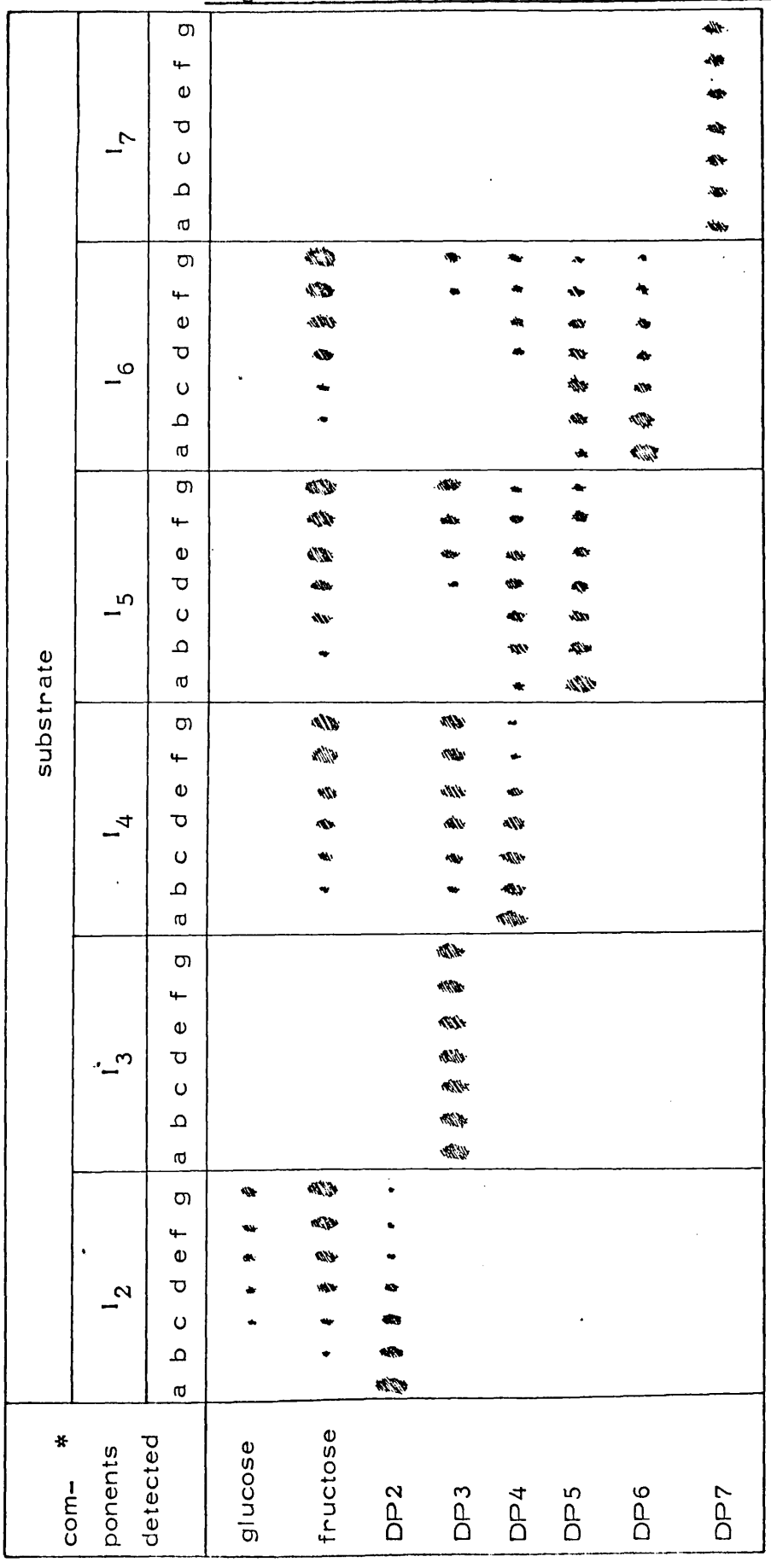
Curves showing degree of hydrolysis values against time are given in Fig. IV.25.

From the results several points appear to emerge concerning the specificity of the enzyme preparation towards these substrates. The apparent degree of hydrolysis of the disaccharide, in excess of 100% is no doubt due to the presence of a certain amount of sucrose in the disaccharide preparation. Sucrose, presumably originating from the "reducing" end of inulin during partial acid hydrolysis, is a commonly encountered impurity in inulobiose preparations [198] and its presence here is further evidenced by the presence of a component with a mobility similar to that of D-glucose upon subjecting the disaccharide hydrolysate to paper chromatography.

On consideration of the hydrolysis curves, it is evident that substrate activity decreases with increasing DP up to DP7 which is essentially undegraded. This is confirmed by the chromatography and serves as an explanation for why inulin, which has a DP value of ca. 34, [198] does not possess substrate activity.

An interesting and unexpected finding was that the trisaccharide preparation, which presumably consists mainly of inulotriose, I_3 (O-β-D-fructofuranosyl-(2$\longrightarrow$1)-O-β-D-fructofuranosyl-(2$\longrightarrow$1)-β-D-fructose, XL IV), does not appear to be hydrolysed by levanase very readily, if at all, on the evidence of its hydrolysis curve and paper chromatography. This finding

Table IV.10. Components detected upon paper chromatography of oligosaccharides obtained from inulin and digested with levanase



Digest times : a, zero ; b, 10 min ; c, 30 min ; d, 1 h ; e, 2 h ; f, 3.5 h ; g, 17.5 h

* size of spots approximately proportional to intensity of spots on chromatograms

Figure IV. 24. Time dependence of reducing sugar content in digests of oligosaccharides obtained from inulin with levanase

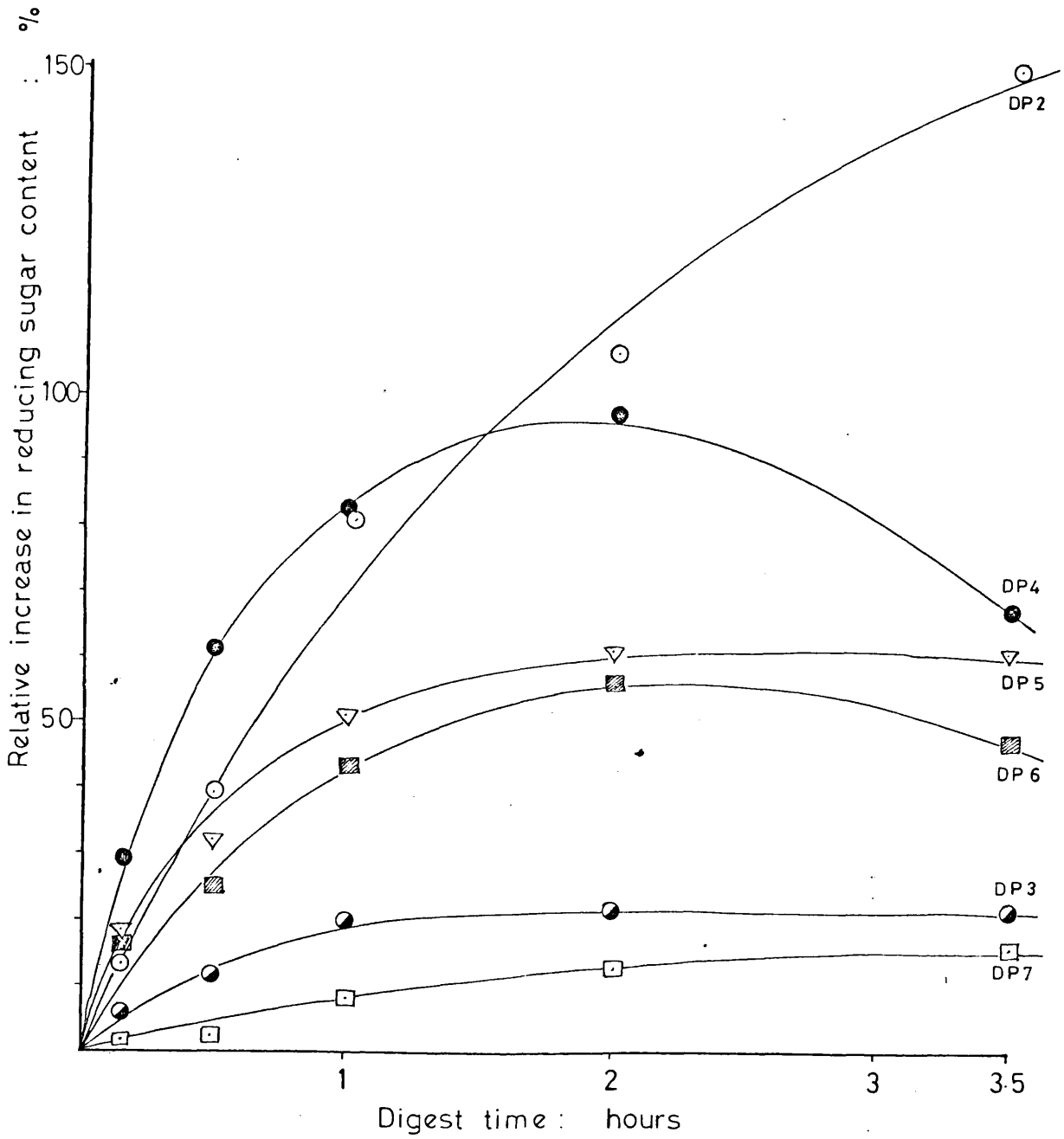
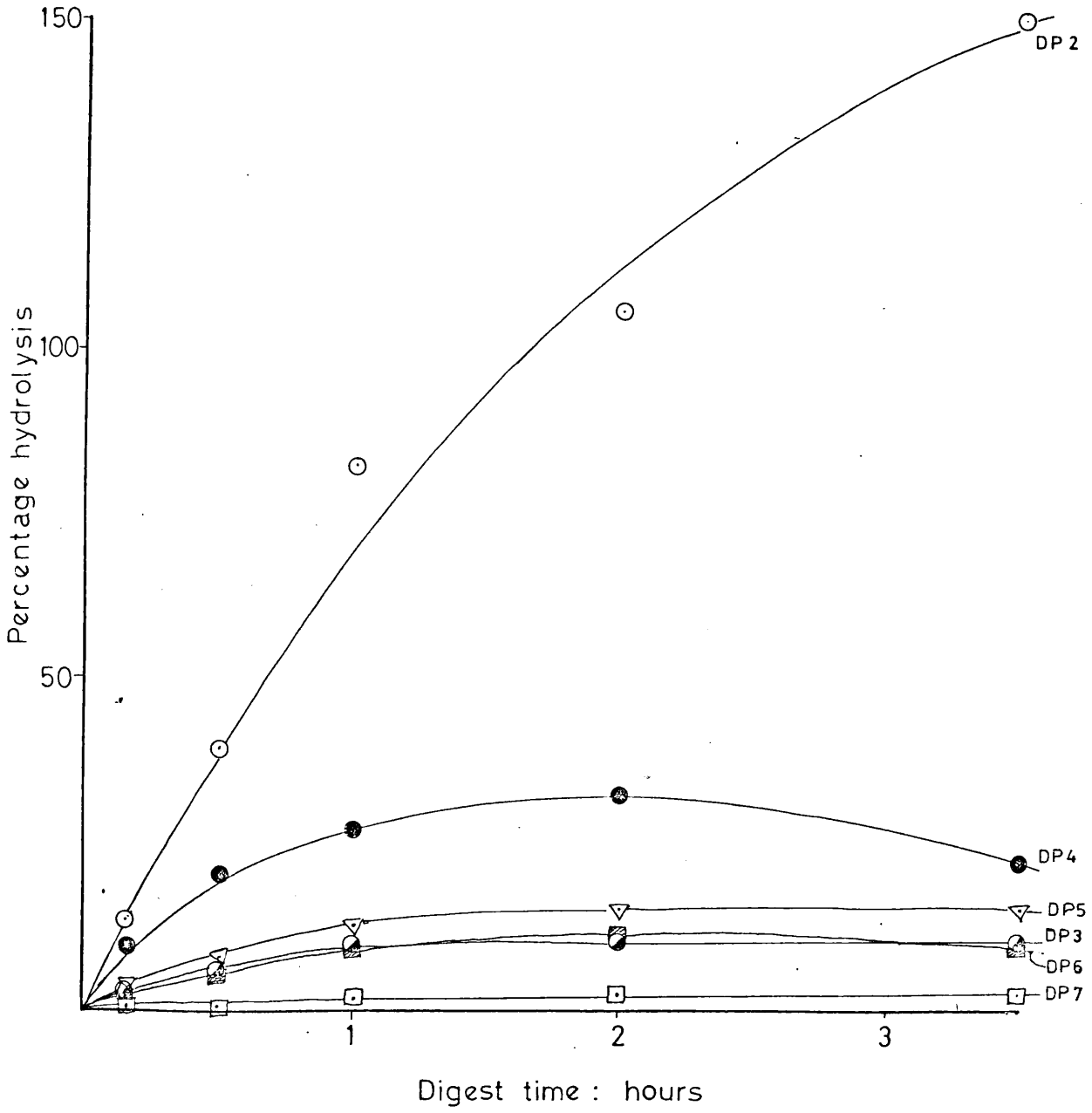
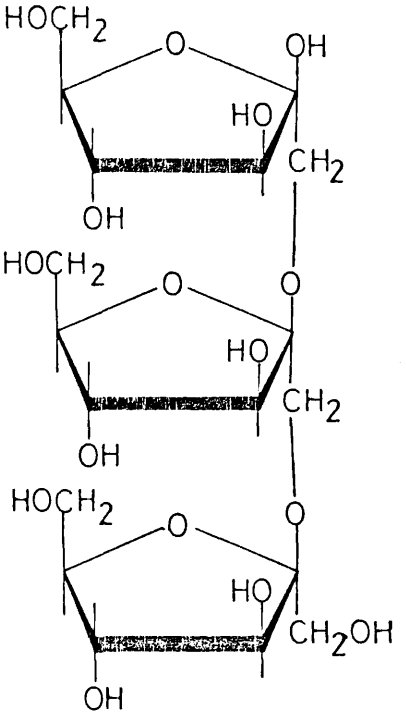


Figure IV. 25. Time dependence of degree of hydrolysis of oligosaccharides obtained from inulin digested with levanase





XLIV Inulotriose

is supported by the observation that the tetrasaccharide, I_4, upon levanase degradation apparently loses only one fructose residue, as evidenced by paper chromatography, leaving I_3, which remains undegraded. The fact that I_4 is only hydrolysed to an extent of ca. 30% is compatible with the loss of one D-fructose residue. Higher homologues, similarly, are hydrolysed by sequential loss of fructosyl residues until I_3 is reached. Chromatographic evidence (Table IV. 10) also indicates the absence of disaccharide in the reaction products from the degradation of oligomers of DP3 and above, further supporting the action pattern postulated above. The apparent lack of substrate activity displayed by inulotriose suggests that a strong steric specificity is in operation which inhibits its accommodation at the active sites on the enzyme at which hydrolysis can occur. Since the disaccharide and larger oligosaccharides are hydrolysed it is difficult to visualise only one site for the accommodation of these substrates. Clearly, this is

an area for further investigation that, unfortunately, falls outside the scope of this present work.

Upon re-examination of the hydrolysis curves shown in Fig. IV. 25., it can be seen that some pass through maxima and others do not reach the levels that would be expected on the basis of the action pattern postulated above. This could be due to the operation of a reversion mechanism as in the case of the invertase hydrolysis of levan discussed in IV. D. iv. b.

In conclusion, the levanase preparation of S. salivarius strain 51 possesses a fairly high substrate specificity. Many facets, however, of the properties of this hitherto unstudied enzyme remain to be investigated. For example, the possibility cannot be excluded that more than one hydrolase is present, specific for the β - 2$\longrightarrow$6 and β - 2 $\longrightarrow$ 1 interfructofuranosidic linkages in levan.

IV.E. Size of Branches through 2 ⟶ 1 - Linkages in the Levan Elaborated by Streptococcus salivarius strain 51

The gross structures of three levans elaborated by strains of S. salivarius were elucidated in section IV.B. They were found to be branched D-fructans composed of β-(2⟶6)-linked fructofuranosyl residues with branching residues linked through positions 1, 2 and 6, the average repeating unit of the levan elaborated by strain 51 being shown in Fig. IV.9. It was also estimated that the degrees of branching for the three polysaccharides lie in the range 9 - 12%.

It is desirable, in a structural study of a polysaccharide, to gain information not only about the types and proportions of constituent monosaccharides, the total number of them and the positions of the linkages joining them but also about such aspects as the lengths of branches, whether branches themselves are rebranched i.e. the type structure, and the general conformation.

To gain information concerning such finer aspects of polysaccharide structure it is invariably necessary to degrade or fragment the polymer in a controlled manner and investigate the structures of the resulting fragments. Of the methods that can be employed for effecting fragmentation, partial acid hydrolysis has proved useful in the study of sizes of branches of levans, a branch in this context meaning a continuation of the polysaccharide through a β-2⟶1-linkage, such linkages being in the minority and occurring only at branch points.

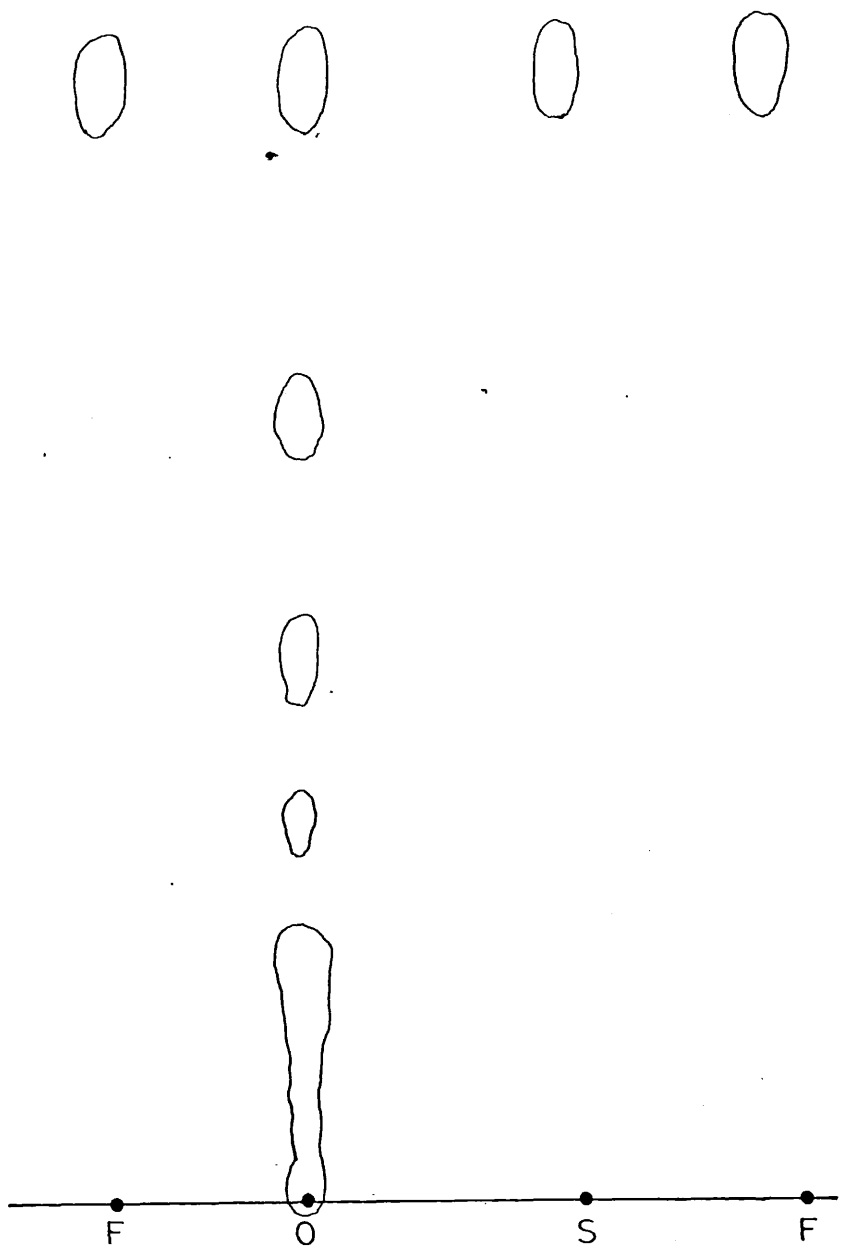
It was noted in IV.B.iii. that a disaccharide preparation from partially acid hydrolysed levan contained a significant (ca. 20%) amount of the β-2⟶1-linked disaccharide inulobiose (XLII) in addition to levanbiose (XLI), a finding that suggests that β-2⟶1-linkages at branching positions are relatively resistant to acid hydrolysis in comparison to

β-2⟶6-linkages. Feingold and Gehatia [52] noted that paper chromatography of a partial acid hydrolysate of the so-called levan of Aerobacter levanicum showed a series of components corresponding to D-fructose, a disaccharide, trisaccharide and higher saccharides with two spots at DP values of 4 and above. This was considered as being evidence that oligosaccharides were generated containing β-2⟶1-linkage(s) or terminated by β-2⟶1-linked reducing groups which would be expected if the β-2⟶1-linkage is, indeed, less susceptible to acid hydrolysis than the β-2⟶6-linkage. It was therefore considered probable that partial hydrolysis would yield oligosaccharide fragments from levan which, upon structural determination, might provide information concerning the structure of the parent polysaccharide. Feingold and Gehatia [52] unfortunately furnished no details of the conditions employed for the degradation of their polysaccharide and it was therefore necessary to ascertain suitable conditions for the partial acid hydrolysis of the levan of S. salivarius strain 51 (available in the greatest yield).

IV.E.i. Partial acid hydrolysis of the levan of S. salivarius strain 51 : Homologous series of the oligosaccharide products

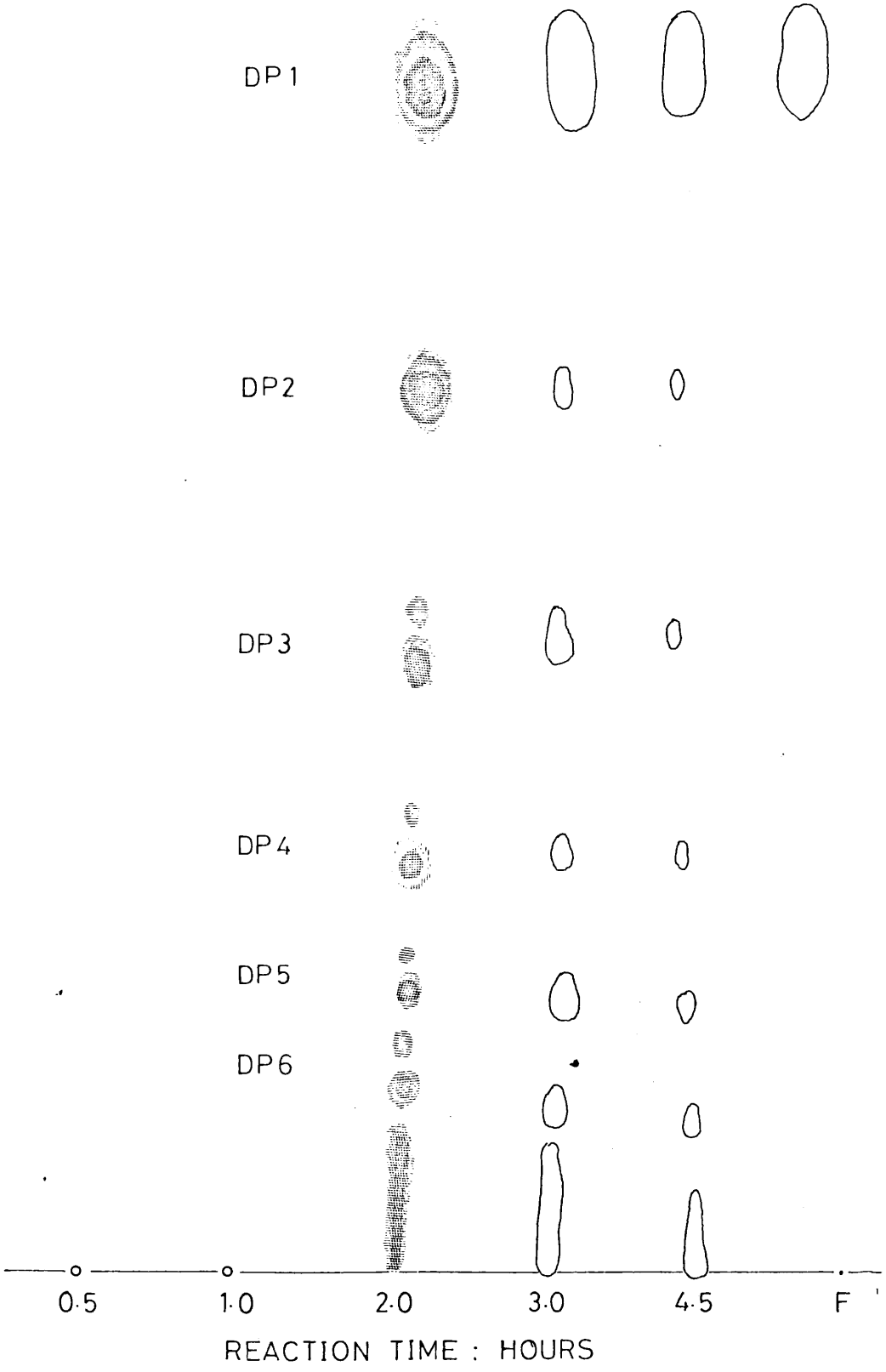
Initially, hydrolyses were performed with 0.005 M sulphuric acid and 0.0005 M oxalic acid as described in III.C.i. Examination of the chromatograms of the hydrolysates, shown in Fig. IV.26., indicates that the sulphuric acid treatment caused virtually complete hydrolysis to D-fructose whereas the oxalic acid treatment produced a series of components. A further experiment was then performed, as described in III.C.ii. to determine a suitable hydrolysis time for the generation of oligosaccharides by degradation with 0.005 M oxalic acid at 70°. Examination of the partial hydrolysates by paper chromatography (Fig. IV.27.) leads to the conclusion that the optimum time is ca. 2 h. It is evident from the 2 h

Figure IV. 26. Paper chromatograms of oxalic acid (0. 005 M) and sulphuric acid (0. 005 M) degraded levan



Key: F = D-fructose
O = oxalic acid hydrolysate
S = sulphuric acid hydrolysate

Figure IV. 27. Paper chromatography of oxalic acid (0.005 M) hydrolysates of S. salivarius 51 levan : Effect of reaction time



chromatogram that two series of components are present at DP values of 3 and higher, essentially in agreement with the finding of Feingold and Gehatia[52], except that these workers found component duplication to begin at DP4.

To ascertain whether the two series of components form members of homologous series, R_f values were found as described in III.C.iii., the results being given in Table IV.11. R_M values were calculated and plotted against assumed DP values as shown in Fig. IV.28. Two parallel straight lines result indicating that the two series of components do, indeed, form members of homologous series. The upper line, A, corresponds to the slower moving, more intense series of components which most probably comprises unbranched saccharides possessing only $\beta-2\longrightarrow6-$ linkages. The lower line, B, corresponding to the fainter, faster moving components, is probably due to saccharides possessing a $\beta-2\longrightarrow1-$ linkage or linkages in addition to $\beta-2\longrightarrow6-$ linkage(s). The component corresponding to the disaccharide, which has been shown to be predominantly the $\beta-2\longrightarrow6-$ linked levanbiose (XLI) plus some $\beta-2\longrightarrow1-$ linked inulobiose (XLII), also falls on the B - series line.

IV.E.ii. Preparation of oligosaccharides from the levan of S. salivarius strain 51 and determination of degree of polymerisation (DP) values.

Having established suitable conditions for the partial degradation by acid hydrolysis of the levan of S. salivarius strain 51 it was then necessary to implement these conditions in a large scale hydrolysis and separate the resulting oligosaccharides. Charcoal-Celite adsorption chromatography has found application in this field.[199] The technique relies upon the increasing eluting power of aqueous ethanolic solutions of increasing strength, the weakest solutions removing only the smallest sugars

Table IV.11. Oligosaccharides obtained from levan : R_f and R_M values

DP		R_F	R_{DF}	R_f	R_M
1		1.00	1.44	0.234	0.504
2		0.70	1.00	0.163	0.710
3	B	0.47	0.68	0.111	0.904
	A	0.42	0.61	0.099	0.959
4	B	0.31	0.45	0.073	1.104
	A	0.27	0.39	0.063	1.173
5	B	0.21	0.30	0.049	1.288
	A	0.18	0.26	0.042	1.358
6	B	0.145	0.21	0.034	1.453
	A	0.120	0.17	0.028	1.540
7	B	0.094	0.137	0.022	1.648
	A	0.081	0.115	0.019	1.713

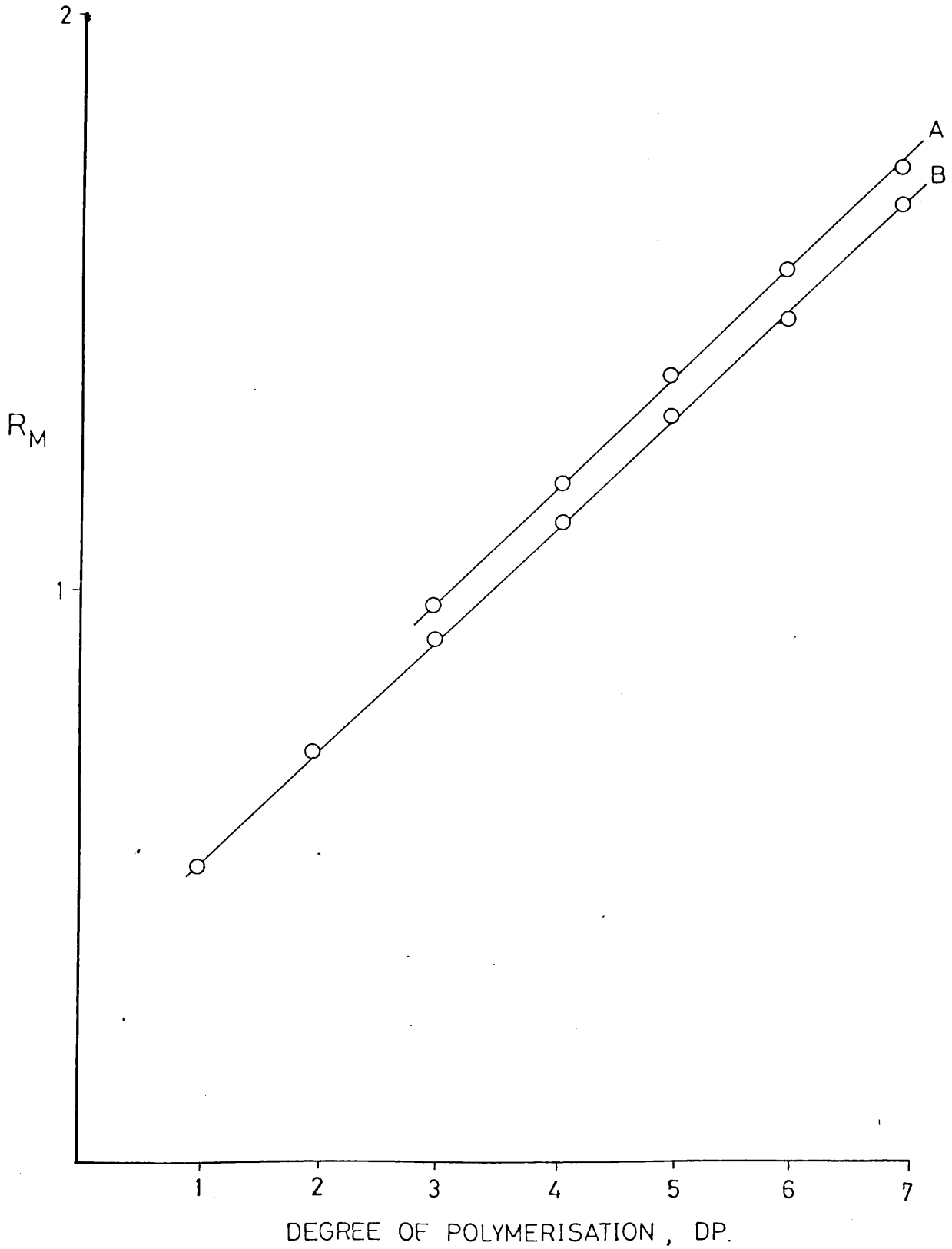
Key : R_F = distance moved by component / " " " D-fructose

R_{DF} = distance moved by component / " " " disaccharide

R_f = distance moved by component / " " " solvent front

$$R_M = \log_{10} \frac{1 - R_f}{R_f}$$

Figure IV.28. Oligosaccharides obtained from levan : Homologous series



from the adsorbent bed. This technique was employed during the preparation of levan oligosaccharides as described in III.F.i. Unfortunately removal, under reduced pressure, of the large volumes of eluent needed resulted in partial hydrolysis of the resolved components to smaller sugars, presumably due to the lability of oligosaccharide products which are composed of furanosyl residues. It was therefore necessary to re-chromatograph on paper the fractions obtained and lyophilise the resulting resolved components to prevent further degradation. Unfortunately, the preparative paper chromatographic method employed did not permit separation of members of the A and B series (Fig. IV.28 , IV.E.i.) and oligosaccharides of common DP were therefore co - lyophilised. Five preparations were obtained which proved to be homogeneous to paper chromatography. The yields of the preparations, designated A - E in Table IV.12., were very low considering that the amount of levan initially degraded was 15 g. The DP values of the preparations were estimated as described in II.H.iv., the results being given in Table IV.12 , together with R_f values.

IV.E.iii. Size of branches

An approach to the problem of lengths of branches in levans has previously been proposed by Zelikson and Hestrin.[200] By means of an endo hydrolase, obtained from a soil bacterium which possessed the characteristics of Arthrobacter tumescens , they obtained a partial hydrolysate of the levan of Aerobacter levanicum. Paper chromatography revealed the presence of two series of oligosaccharides up to DP8. Members of one series reduced triphenyltetrazolium chloride (TTC), a reagent specific for reducing sugars except when position(s) adjacent to the reducing group is/are substituted, as in saccharides terminated by a reducing D-fructose residue linked through position C-1 (e.g. inulobiose, XLII).

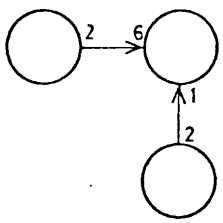
Table IV.12. Yields, chromatographic data and DP values of oligosaccharides obtained from the levan of S. salivarius strain 51

Preparation	Yield [a] mg	R_f [b]	DP determined	DP
A	62	0.171	2.2	2
B	213	0.105	3.3	3
C	146	0.071	3.6	4
D	100	0.045	4.9	5
E	18	0.024	5.5	6

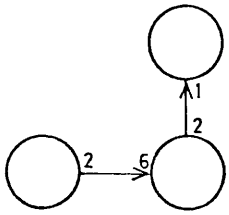
a. from 15 g levan, b. solvent system 1 (II.C.i.)

It was suggested that members of the series that did not reduce TTC were terminated by reducing D-fructose units linked through position 1, and concluded that up to at least 7 residues were attached to branching residues via 2 ⟶ 1-linkages.

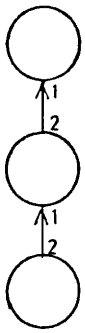
Such an approach to the problem is unsatisfactory since it relies upon the non-formation of a colour complex with a specific reagent. Furthermore, the possible presence of oligosaccharides terminated by branched reducing units linked through positions 6 and 1, as in the trisaccharide XLV, was not considered. Such oligosaccharides



XLV



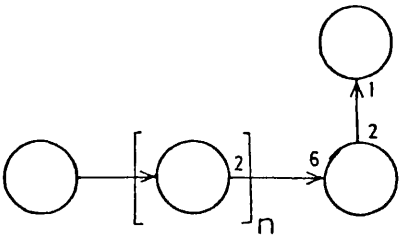
XLVI



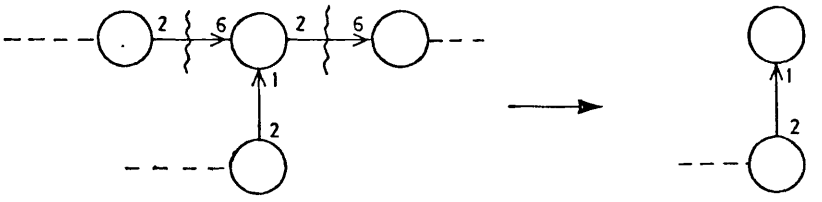
XLVII

would also be inactive towards TTC and, as a result, the TTC test has no relevance to lengths of branches through β-2 ⟶ 1-linkages in levans.

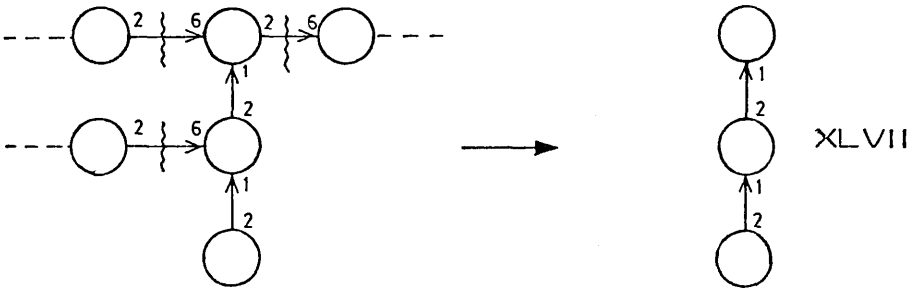
A chemical method was therefore devised for the investigation of branch sizes in levans. Having obtained oligosaccharide preparations from the levan of S. salivarius strain 51 and established that the β-2 ⟶ 1-linkages in the polymer are, relative to the β-2 ⟶ 6-linkages, resistant to acid hydrolysis, it was thought likely that the preparations contained saccharides possessing β-2 ⟶ 1-linkages in addition to β-2 ⟶ 6. Although it was not found to be practical to separate members of the A and B series (IV.E.i., Fig. IV.28.), a method was devised for the determination of the sizes of branches by establishing the presence in each oligosaccharide preparation of saccharides terminated by reducing D-fructose residues linked through position 1 only.



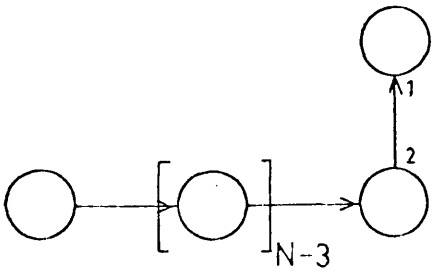
It was realised that such compounds could only be generated by hydrolysis of the β-2⟶6-linkages at a branch point.



Of the possible trisaccharides originating from levan, for example, two (XLVI and XLVII) are terminated by a reducing D-fructose residue linked only through position 1. If either or both were present then branches in the parent polysaccharide would be concluded to possess at least 2 residues. Indeed compound XLVII, if present, would have originated from a branch containing at least 3 residues since all 2 ⟶ 1-linkages occur at branching positions.



In general, the detection of reducing residues linked only through position 1 in a preparation with a DP value of N would be indicative of branches, linked through the C-1 position of branching residues, containing up to at least N-1 residues.



During the determination of ring size of non-terminal residues in the levan of S. salivarius strain 51 (IV.B.iii.), the sequence : -

disaccharide

1. reduction with $NaBD_4$
2. methylation
3. hydrolysis
4. reduction with $NaBD_4$
5. acetylation

gave a mixture of 2 - d - O - acetyl - O - methyl - hexitols (Table IV.4.), containing a component, R_{tmg} = 0.50, which proved to be a mixture of 2 - d - 6 - O - acetyl - and 2 - d - 1 - O - acetyl - penta - O - methyl - hexitols (XXXVIII, XL respectively), the latter compound indicating that the disaccharide preparation contained a component, the reducing end group of which being linked through position 1 only. It was therefore thought likely that a similar procedure could be used to detect higher saccharides the reducing end groups of which being similarly linked only through position 1, thereby yielding information concerning the sizes of branches in the parent polysaccharide.

Samples of the tri-, tetra-and pentasaccharide preparations were, therefore, subjected to the above sequence as described in III.H.ii. The resulting 2 - d - O - acetyl - O - methyl - hexitol mixtures were subjected to gc-ms as described in II.J.ii. Gas chromatograms of these mixtures are shown in Figs. IV.29., 30., 31. Partial E.I. mass spectra of the components obtained from the trisaccharide preparation are shown in Fig. IV.32. and partial E.I. mass spectra of the components with an R_{tmg} value of 0.50, obtained from the tetra- and pentasaccharide preparations, are shown in Fig. IV.33. In addition a partial E.I. mass spectrum of the component with R_{tmg} = 1.18, found in the mixture of hexitol derivatives from the tetrasaccharide preparation, is shown in Fig. IV.34. Gas chromatographic retention data and interpretations of the E.I. mass spectra are given in Table IV.13.

It is evident that the component with an R_{tmg} value of 0.50, derived from the tri-, tetra- and pentasaccharide preparations, is a

Figure IV. 29. Gas chromatogram of the 2-d-O-acetyl-O-methyl-hexitol mixture derived from the trisaccharide preparation obtained from the levan of S. salivarius strain 51

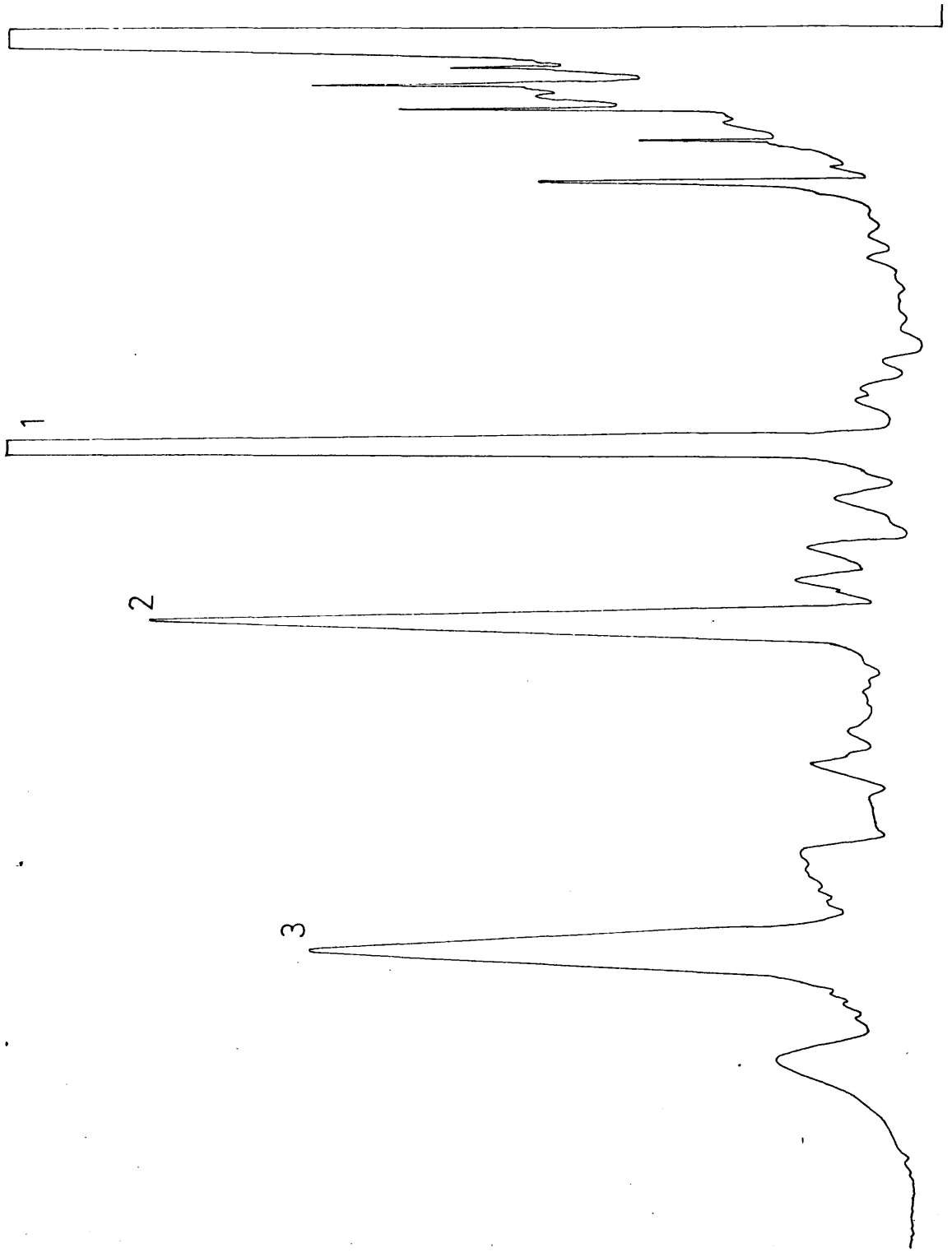


Figure IV. 30. Gas chromatogram of the 2 - d - O - acetyl - O - methyl - hexitol mixture derived from the tetrasaccharide preparation obtained from the levan of S. salivarius strain 51

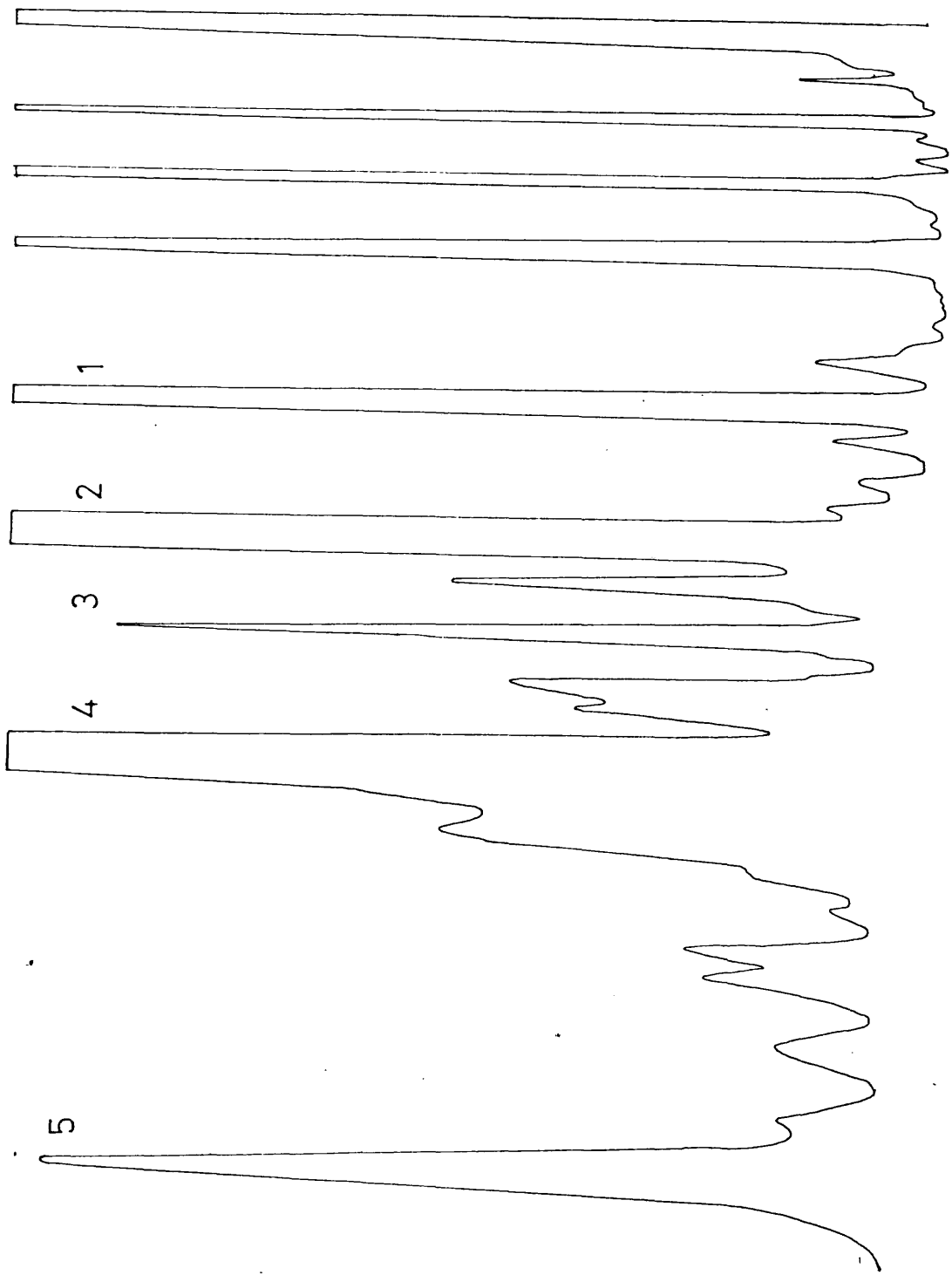


Figure IV. 31. Gas chromatogram of the 2-d-O-acetyl-O-methyl-hexitol mixture derived from the pentasaccharide preparation obtained from the levan of S. salivarius strain 51

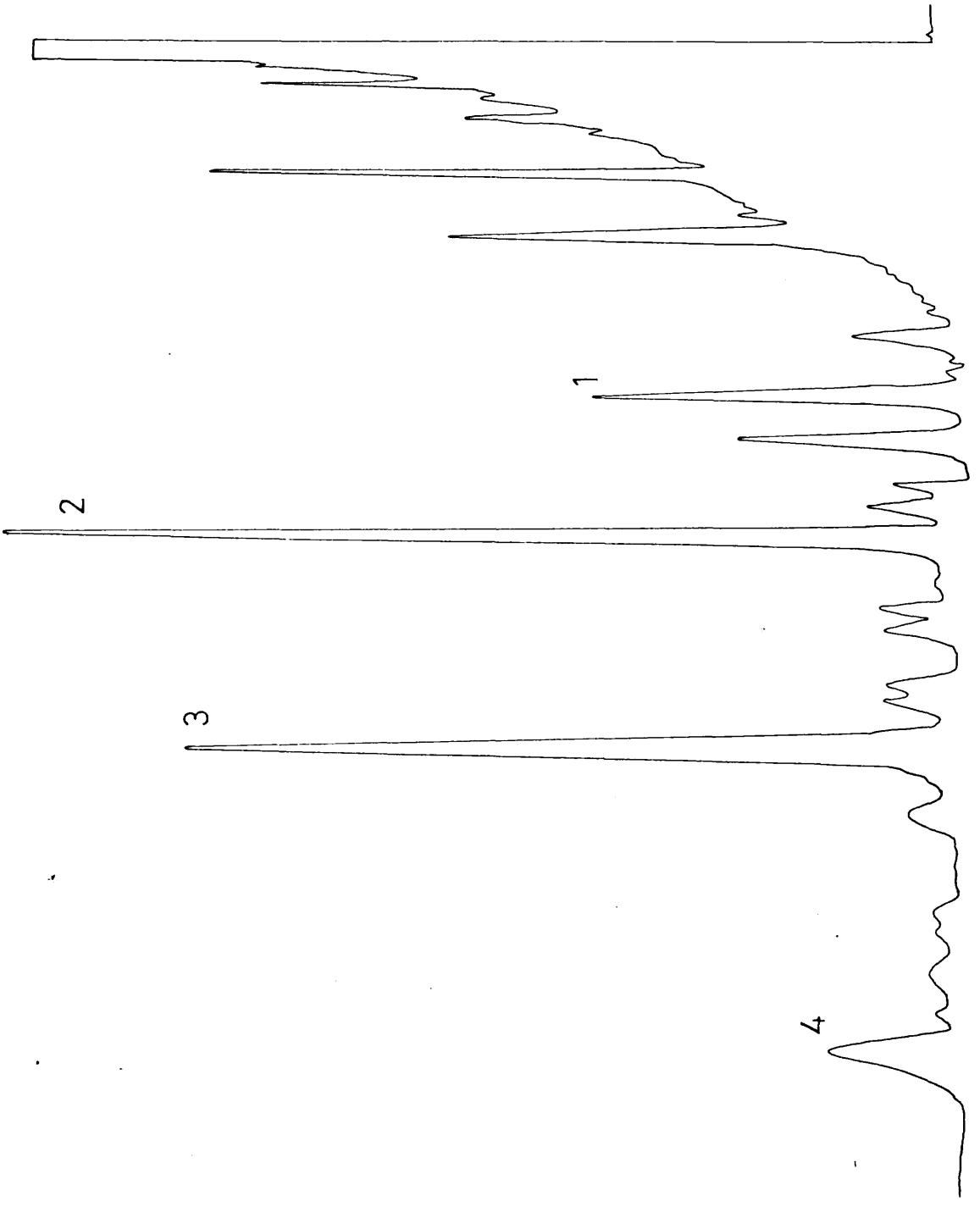


Figure IV. 32. Partial electron impact mass spectrum of 2-d-O-acetyl-O-methyl-hexitols derived from the trisaccharide preparation obtained from the levan of S. salivarius strain 51

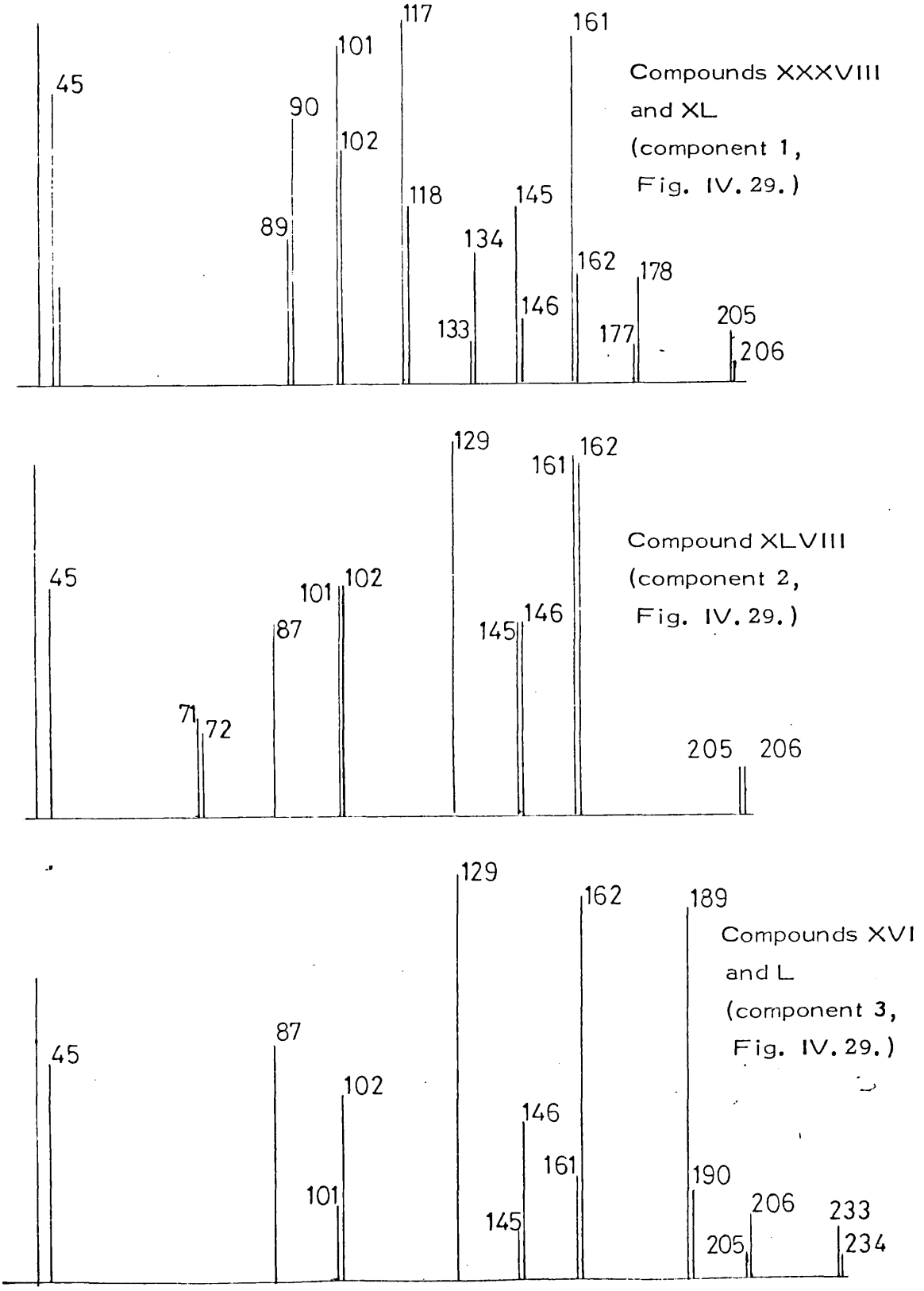
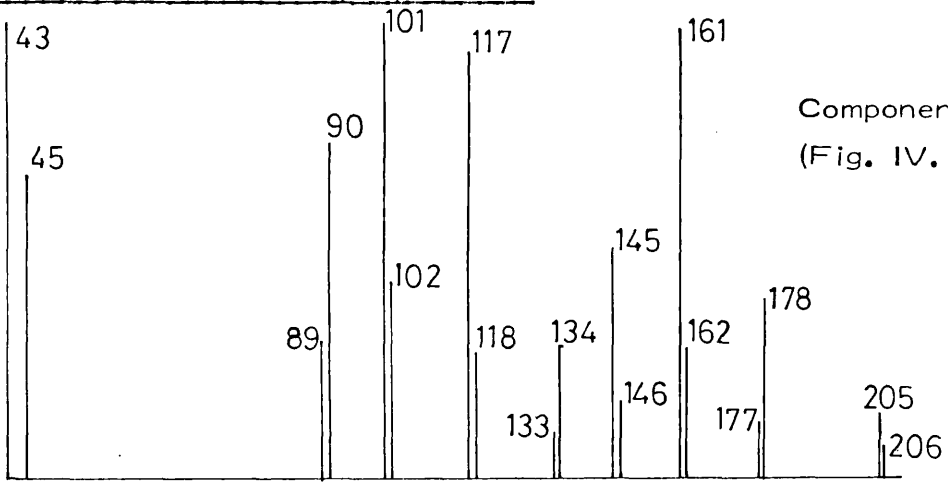


Figure IV.33. Partial electron impact mass spectra of mixtures of 2-d-6-O-acetyl-1,2,3,4,5-penta-O-methyl hexitol (XXXVIII) and 2-d-1-O-acetyl-2,3,4,5,6-penta-O-methyl-hexitol (XL)

a. from tetrasaccharide preparation



b. from pentasaccharide preparation

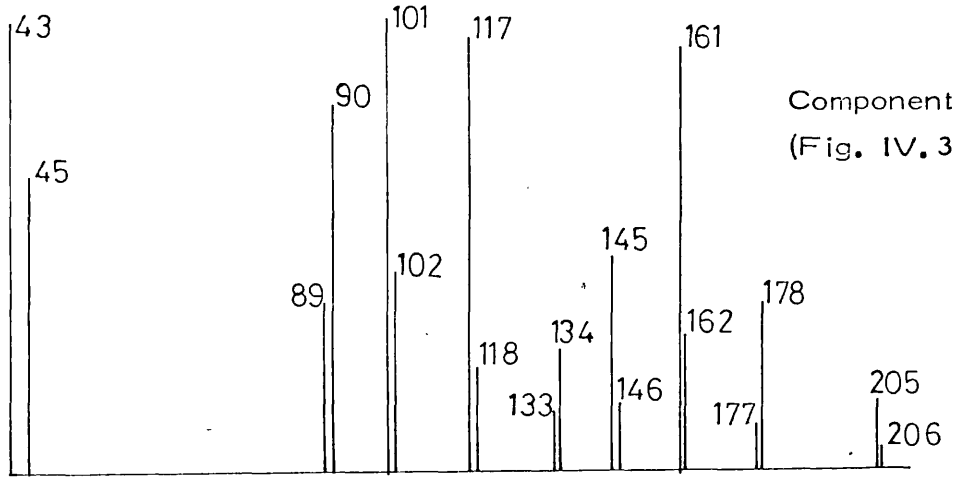


Figure IV. 34. Partial electron impact mass spectrum of 2-d-1,6-di-O-acetyl-2,3,4,5-tetra-O-methyl-hexitol (XLIX) obtained from the tetrasaccharide preparation

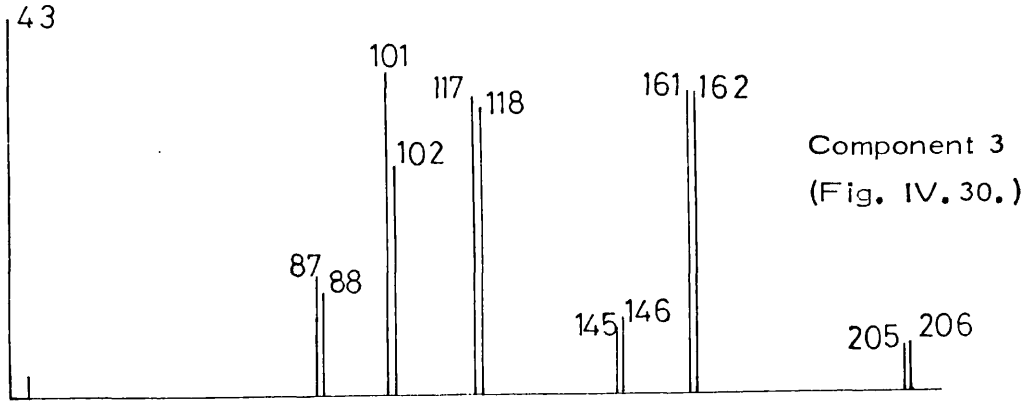
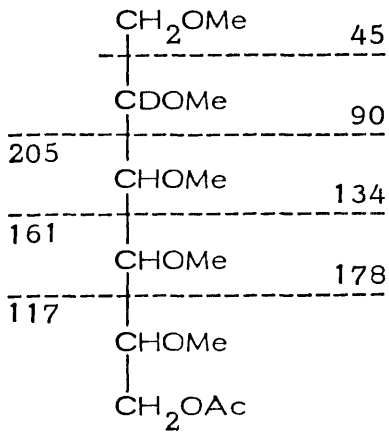


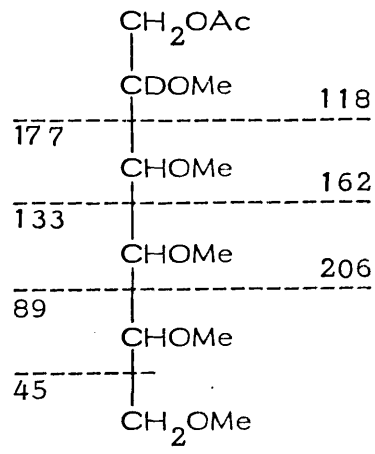
Table IV.13. Chromatographic properties and mass spectrometric analysis of 2-d-O-acetyl-O-methyl-hexitols derived from the tri-, tetra- and pentasaccharide preparations obtained from the levan of S. salivarius strain 51

Component	R_{tmg}	E.I. Mass spectrometry		Parent structural unit
		primary fragments m/e	positions of methoxyl groups in hexitol derivative	
XXXVIII[a,b,c]	0.50	45,90,117,134, 161,178,205	1,2,3,4,5	terminal reducing group linked through position 6 (L II)
XL[a,b,c]	0.50	45,89,118,133, 162,177,206	2,3,4,5,6	terminal reducing group linked through position 1 (L III)
XLVIII[a,b,c]	0.79	45,161,162, 205,206	1,3,4,6	non-reducing D-fructofuranosyl terminus (XXXIV)
XLIX[b]	1.18	117,118,161, 162,205,206	2,3,4,5	terminal reducing group linked through positions 1 and 6 (L IV)
XVI[a,b,c]	1.70	45,162,189, 206,233	1,3,4	D-fructofuranose residue linked through positions 2 and 6 (V)
L[a,b,c]	1.70	45,161,190, 205,234	3,4,6	D-fructofuranose residue linked through positions 1 and 2 (XXXV)
LI[b,c]	3.95	189,190,233, 234	3,4	branching D-fructofuranose residue linked through positions 1,2 and 6 (XXXVI)

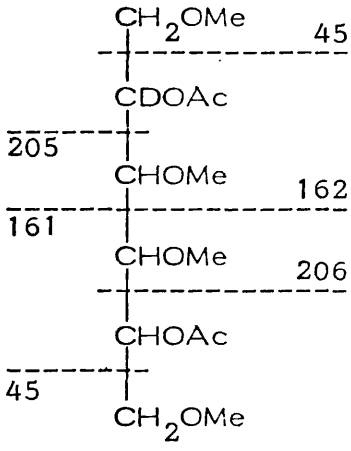
key: a obtained from trisaccharide preparation
b " " tetrasaccharide "
c " " pentasaccharide "



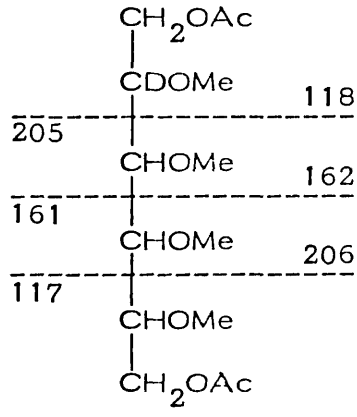
XXXVIII



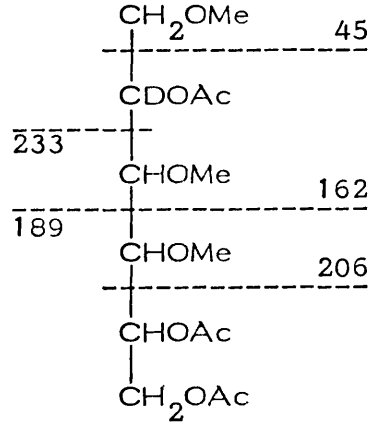
XL



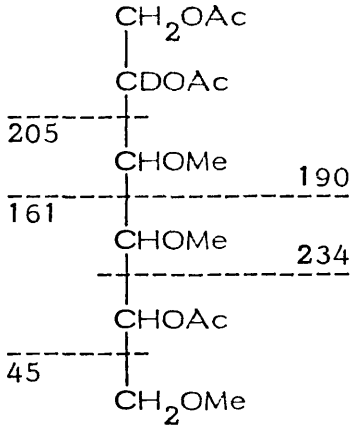
XLVIII



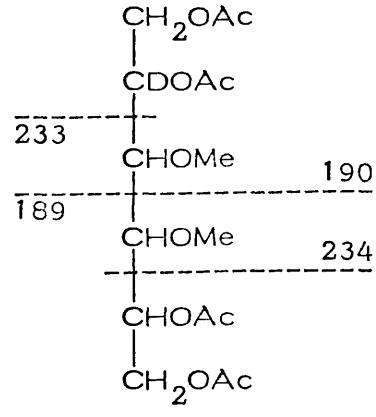
XLIX



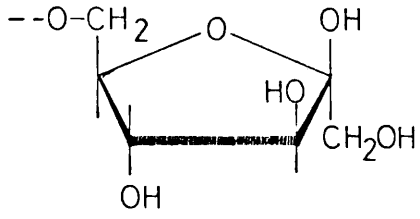
XVI



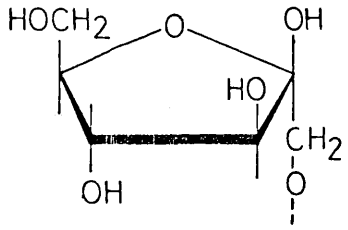
L



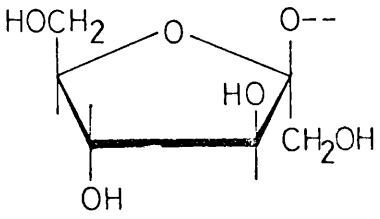
LI



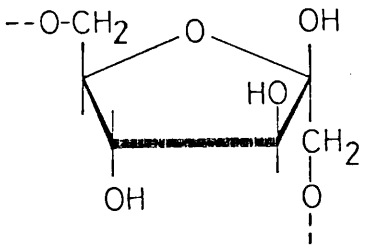
LII



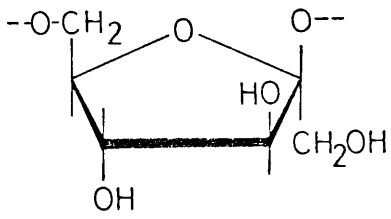
LIII



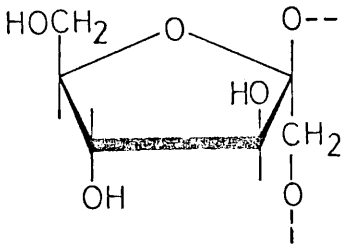
XXXIV



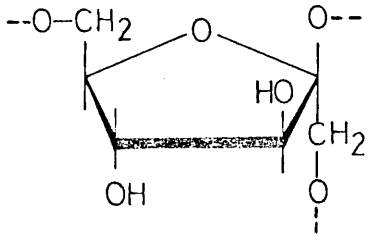
LIV



V

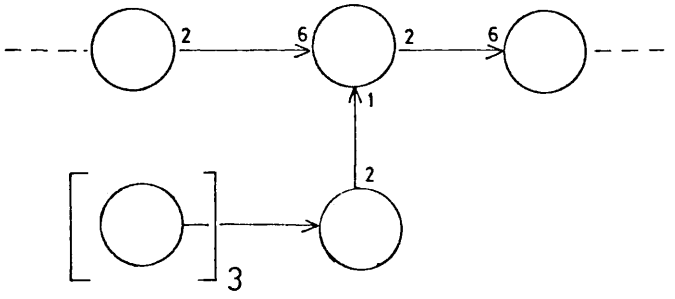


XXXV

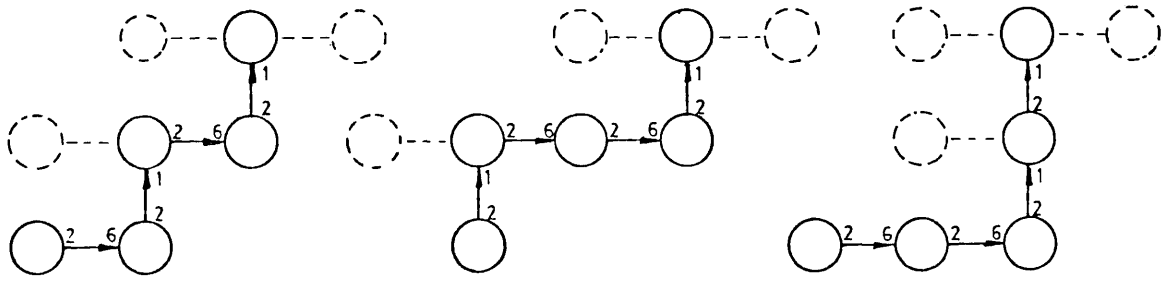


XXXVI

mixture of 2-d-6-O-acetyl-1,2,3,4,5-penta-O-methyl-hexitol (XXXVIII) and 2-d-1-O-acetyl-2,3,4,5,6-penta-O-methyl-hexitol (XL) identical to that obtained from the disaccharide preparation in IV.B.iii., the latter (XL) being evidence for the presence of compounds possessing reducing terminii linked only through C-1 in the three preparations. The fact that the pentasaccharide preparation contains components with reducing terminii thus linked can be considered to be conclusive evidence that branches in the levan elaborated by S. salivarius strain 51 contain up to at least 4 D-fructose residues.



Although the method yields information concerning the sizes of levan branches, it is unable to yield information concerning the arrangement of the residues contained in the branches. For example, if segments of polysaccharide terminated by a 2 ⟶ 1-linkage to a branching position and containing 4 residues are considered, some of the possible structures such as LV, LVI and LVII contain further β-2 ⟶ 1-linkages. It has been established (section IV.B.) that such linkages occur exclusively at branching positions. If structures such as LV, LVI and LVII are present then there must have been in excess of 4 residues contained in the branches concerned: hence the generalisation "the detection of saccharides with a DP value



LV LVI LVII

of N, terminated by reducing groups linked only through C-1, is evidence that branches in the parent polymer contain up to at least N-1 residues".

Clearly the approach employed in this section could equally well be applied to other levans and branched fructans in general. Indeed the branched "inulin-like" fructans elaborated by strains JC2[1] and Ingbrett[2] of Streptococcus mutans, which have been shown[1,2] to comprise D-fructofuranose residues linked principally through positions 2 and 1 with branching through C-6, would also be amenable to branch length analysis by a similar procedure. In these cases, however, it would be necessary to detect saccharides terminated by reducing groups linked only through C-6 in the presence of those linked through C-1.

The compound with R_{tmg} = 1.18 (Fig. IV.30., component 3), detected in the mixture of hexitol derivatives obtained from the tetrasaccharide preparation, proved to be, on the basis of E.I. mass spectrometry (Fig. IV.34.), 2-d-1,6-di-O-acetyl-2,3,4,5-tetra-O-methyl-hexitol (XLIX). This was considered to be derived from reducing D-fructose terminii linked through both positions 1 and 6 (LIV) by implementing the sequence of reactions described above. Presumably compound XLIX should also be

present in the mixtures of hexitol derivatives obtained from the tri- and penta-saccharide preparations. It is thought that the concentrations of XLIX in these mixtures were too low to be detectable by the methods employed.

IV.F. Chemical Ionisation Mass Spectrometry of 2-d-O-Acetyl-O-Methyl-Hexitols

When the work on the sizes of branches in the levan of S. salivarius strain 51 was completed it became possible to examine some of the 2-d-O-acetyl-O-methyl-hexitols mixtures by combined gas chromatography-chemical ionisation (C.I.) mass spectrometry.

In E.I. mass spectrometry, the major disadvantage usually encountered is that the energy of the electron beam required to ionise a sufficiently large proportion of the sample molecules is such that excess energy is available for the extensive fragmentation of the resulting molecular ion-radical ($M^{+\cdot}$). In consequence the $M^{+\cdot}$ ion is rarely discernible in the spectrum of a complex molecule such as an O-acetyl-O-methyl-alditol. In assigning structures to such derivatives it is necessary therefore to rely on chromatographic retention data and comparative data of reference compounds or literature values in order to assign values for the numbers of methoxy and acetate groups and the number of carbon atoms in the chain. Clearly if a mass spectrum of such a compound contained a readily discernible peak corresponding to the molecular ion, the molecular weight and hence carbon chain length and numbers of methoxy and acetate groups could be established.

Chemical ionisation mass spectrometry has recently emerged as an important technique which can provide information additional to that obtainable by electron impact mass spectrometry.

Essentially the C.I. process of sample ionisation occurs with a much lower transfer of energy to the sample molecules resulting in a greatly diminished fragmentation. The parent ion is, however, not usually abundant as a result. A quasi-molecular ion, formed by loss or gain of one hydrogen atom, is usually the most prominent feature of the spectrum. C.I. spectra are therefore able to provide information about molecular weights.

Chemical ionisation results from interactions between sample molecules at a low pressure and a reactant gas at a higher pressure. Because of the low abundance of sample molecules, the reactant gas molecules undergo primary ionisation when bombarded by electrons. The ionised reactant gas then undergoes ion-molecule reactions with itself and the resultant "steady state plasma" reacts chemically with the sample molecules. The process results in fragment and product ions characteristic of the unknown sample. Typically ion chamber pressures will be 0.3 - 3 torr for the reactant gas, which is most commonly methane or iso-butane, and 10^{-6} torr for the vaporised sample.

If the reactant gas iso-butane is considered, important reactions that occur are as follows:-

$$C_4H_{10} + e \longrightarrow C_4H_{10}^{\cdot +} + 2e \quad \text{(primary ionisation)}$$

$$C_4H_{10}^{\cdot +} \longrightarrow C_3H_7^{+} + {}^{\cdot}CH_3$$

$$C_3H_7^{+} + C_4H_{10} \longrightarrow C_4H_9^{+} + C_3H_8$$

In iso-butane mediated mass spectra the ions $C_3H_7^+$ and $C_4H_9^+$ give rise to prominent peaks at $^m/e$ 43 and 57 respectively and are, therefore, abundant species in the steady state plasma.

In the presence of proton acceptors, ions such as $C_3H_7^+$ and $C_4H_9^+$ can act as Brönsted acids and protonate the sample molecule.

$$C_3H_7^+ + AH \longrightarrow AH_2^+ + C_3H_6$$

$$C_4H_9^+ + AH \longrightarrow AH_2^+ + C_4H_8$$

Sample molecules such as alcohols, aldehydes and esters will react as above and a dominant feature of a C.I. mass spectrum of such a compound is generally the high abundance of ions at $^m/_e$ M+1 , where M is the molecular weight.

The mixtures of hexitol derivatives from oligosaccharide preparations studied in IV.E.iii. were subjected to gc-C.I. ms as described in Appendix I. The resulting mass spectra contained very strong peaks at $^m/_e$ M+1 for the various components, the data being given in Table IV.14.

From the results, it proved very easy to assign values for the numbers of methoxy and acetate groups in each hexitol derivative. The results obtained confirm the values already assigned in IV.E.iii. (Table IV.13.).

Since all fully substituted partially methylated alditol acetates possess characteristic molecular weights, the technique of C.I. ms enables the assignment of length of carbon chain and numbers of methoxy- and acetate groups to be made, without the need to resort to the use of chromatographic retention data, and therefore represents an advance.

Table IV. 14. Chemical ionisation mass spectrometry of 2-d-O-acetyl-O-methyl-alditols derived from the tri-, tetra- and pentasaccharide preparations obtained from the levan of S. salivarius strain 51: Molecular weights, lengths of alditol chain and numbers of methoxy and acetate groups

Component	C.I. mass spectrometry		E.I. mass spectrometry: Identity of alditol derivative
	M + 1 m/e	Alditol-2-d-derivative	
1[a,b,c]	296	O-acetyl-penta-O-methyl-hexitol	XXXVIII + XL
2[a,b,c]	324	Di-O-acetyl-tetra-O-methyl-hexitol	XLVIII
3[b]	324	Di-O-acetyl-tetra-O-methyl-hexitol	XLIX
4[a,b,c]	352	Tri-O-acetyl-tri-O-methyl-hexitol	XVI + L
5[b,c]	380	Tetra-O-acetyl-di-O-methyl-hexitol	LI

a. obtained from the trisaccharide preparation
b. " " " tetrasaccharide "
c. " " " pentasaccharide "

IV. G. An Attempted Study of the Type-Structure of the Levan Elaborated by Streptococcus salivarius strain 51

IV. G. i. Introduction

It has been established that the levans elaborated by strains of S. salivarius are branched molecules possessing β-2 ⟶ 6-linked D-fructofuranose residues with branching occurring at C-1. A method for investigating the sizes of branches in levans has been devised. Using this method the levan elaborated by strain 51 has been shown to contain branches, linked via 2 ⟶ 1-linkages to branching fructofuranose residues, that contain up to at least 4 residues. To conclude this study it was thought desirable to devise and apply an approach for the investigation of some finer aspects of the structure of levans in pursuance of the ultimate aim of having a complete knowledge of levan structures.

Structural studies on branched homoglycans usually, at best, provide information on Average Repeating Structures because of the difficulty in establishing such polymers to be structurally homogeneous. The most extensively studied of branched homoglycans, viz. amylopectin, the branched component of starches, possesses α-D-glucopyranose residues linked through positions 1 and 4 with branching occurring at C-6 (Fig. IV. 35. a.). For the purpose of establishing a nomenclature for possible levan type-structures, that developed for amylopectin will be briefly considered here.

The arrangement of residues within the amylopectin molecule has been the subject of much controversy and is a facet of starch chemistry that is still not entirely resolved. Peat, Whelan and Thomas[201] distinguished 3 types of chain, viz. A-, B- and C- chains (Fig. IV. 35. a.). An A-chain is linked to the rest of the molecule through only its reducing end group; a B-chain, in addition to being linked as an A-chain, is also substituted through C-6 of one or more of its constituent glucose residues; a C-chain, of which

there is only 1 per molecule, carries the reducing end group.

As a result of early structural studies, three models or 'type' structures were proposed for amylopectin (and glycogen). The laminated structure proposed by Haworth, Hirst and Isherwood in 1937[202] (Fig. IV. 35. b.) contains only one A - chain per molecule, whereas that suggested by Staudinger and Husemann[203], the herring-bone or comb-like structure (Fig. IV. 35. c.), contains A - chains, but no B - chains. An intermediate structure (Fig. IV. 35. d.), proposed by Meyer and Bernfeld[204], contains both A and B chains and is often referred to as a tree-like (randomly branched, ramified) structure.

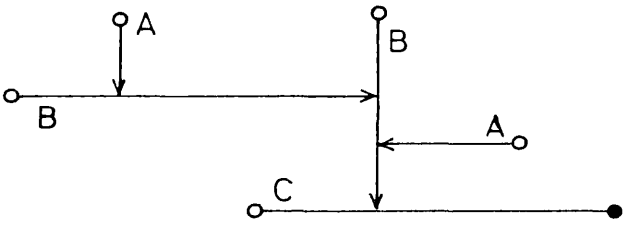
By way of an analogy, a levan molecule may be considered to possess one of the above 3 type-structures. In these instances, an A - chain would consist of β - 2 ⟶ 6 - linked D - fructofuranose residues linked to the rest of the molecule via a 2 ⟶ 1 - linkage, a B - chain would be linked as an A - chain and, in addition, substituted through C - 1 of one or more constituent residues and the C - chain would be terminated by a reducing group or initiator molecule. Possible type-structures for the levan molecule are depicted in Fig. IV. 36.

A variety of amylopectin degrading enzymes have been used as probes for the elucidation of type structure of this polysaccharide[205]. Peat, Whelan and Thomas[206], for example, were the first investigators to submit evidence that waxy maize starch (amylopectin) possesses a randomly branched or tree-like structure. This was achieved by examining the products obtained upon debranching the β - limit dextrin from amylopectin (produced by degradation with crystalline β - amylase) with the so-called R - enzyme[207].

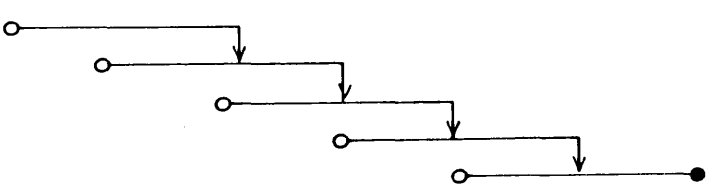
In recognising that specific enzyme degradations must be employed, in addition to chemical methods, in order that progress can be made towards a knowledge of the finer aspects of the structure of a branched homoglycan, it was considered that the exo - levanase preparation, isolated and partially characterised in this present work, might be used to provide information concerning the type-

Figure IV.35. Proposed type-structures for amylopectin

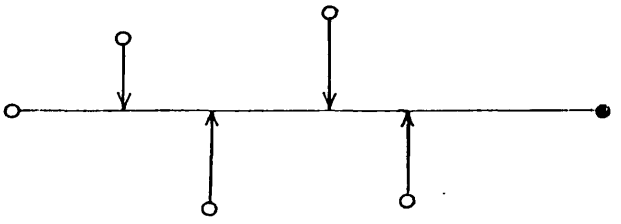
a. chain types[201]



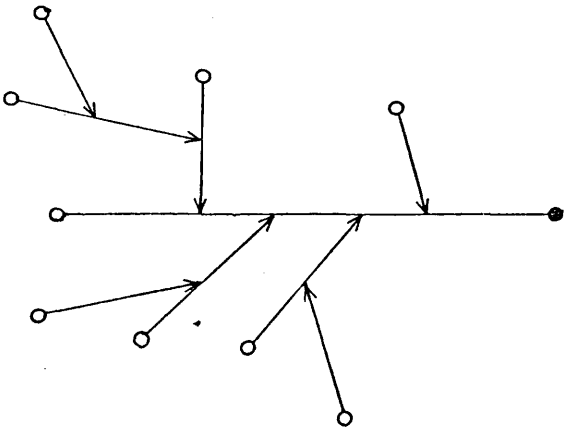
b. laminated [202]



c. comb-like [203]



d. tree-like [204]

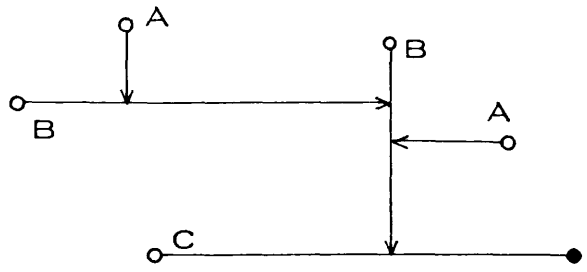


Key :

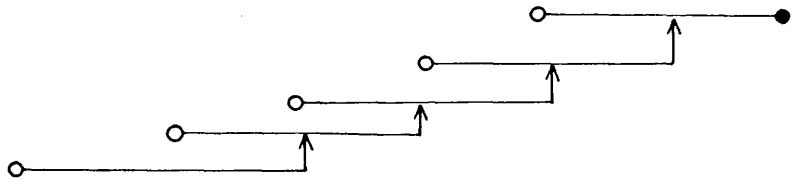
- ● reducing end group
- ○ non reducing end group
- → α-1 ⟶ 6-linkage to branch point
- — chain of α-1 ⟶ 4-linked residues

Figure IV. 36. Possible levan type structures

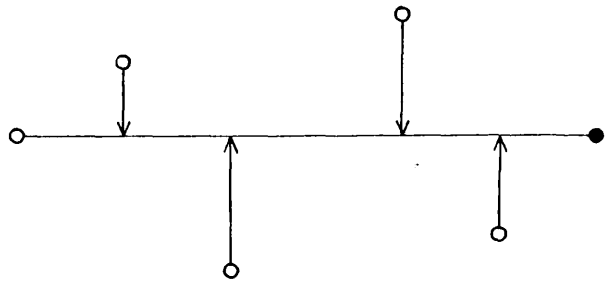
a. chain types



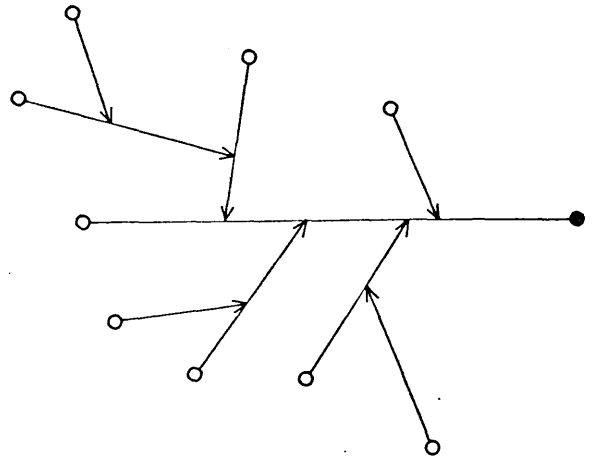
b. laminated



c. comb-like



d. tree-like



Key : ● reducing end group (initiator molecule)

o non reducing end group

→ β - 2 ⟶ 1 - linkage to branch point

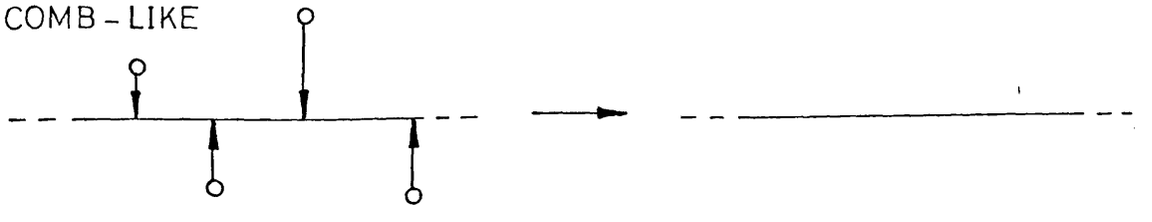
— chain of β - 2 ⟶ 6 - linked fructofuranose residues

structure of levans, although it was realised that the amount of information obtainable, as a result of using a single enzymic structural probe, would be limited.

It was established in section IV.D. that the partially purified levanase preparation elaborated by S. salivarius strain 51 completely degrades levan in an exo fashion, D-fructose being the sole product. The enzyme preparation must therefore hydrolyse both the $\beta-2\longrightarrow 6-$ interfructofuranosidic linkages and the $\beta-2\longrightarrow 1-$ linkages that occur at branching positions. Because the hydrolysis occurs in a strictly exo manner, the enzyme must work by removing residues from the non-reducing ends of the levan chains. It was realised that, during the course of exo-levanase degradation, the types and proportions of linkages remaining in the residual polysaccharide could be dependant on the type-structure. If segments of the extreme possible structures (viz. the laminated (Fig. IV.36.b.), which contains only 1 A-chain per molecule, and the comb-like (Fig. IV. 36.c.) which contains only A-chains in addition to the C-chain) are considered, possible intermediate structures, as a result of partial exo-levanase degradation would be as shown below, it being assumed, in the absence of any evidence to the contrary, that the enzyme is able to remove residues linked via $2\longrightarrow 6-$ linkages or $2\longrightarrow 1-$ linkages to branching positions.

LAMINATED

COMB-LIKE

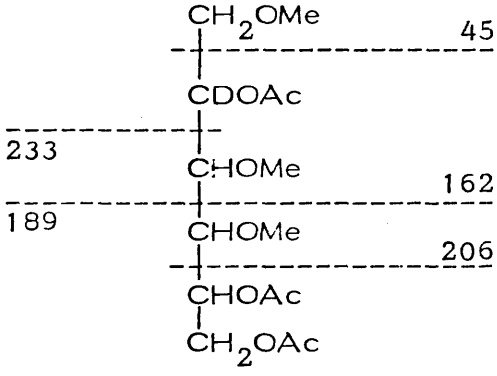


Clearly, there would be some difference in the linkages remaining between the two partially degraded polymers. A comb-like structure could give rise to a partially degraded intermediate consisting of a chain of β-2 ⟶ 6-linked residues whereas that from a laminated structure could contain some 2 ⟶ 1-linked residues in the chain. In either case, the degree of branching of the polymer could be drastically reduced as a result of exo-levanase degradation.

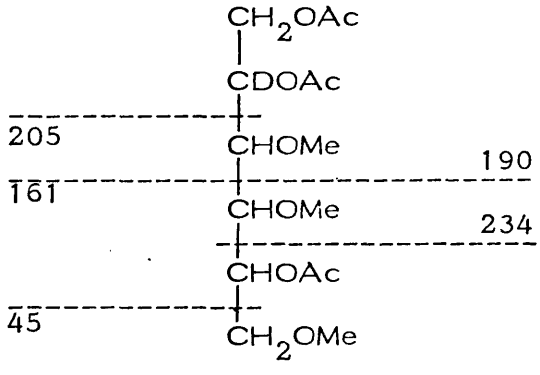
A tree-like structure (Fig. IV. 36. d.), being intermediate between the laminated and comb-like extremes, in that it possesses many A- and B- chains, would , during the course of exo-levanase degradation, be expected to acquire some 2 ⟶ 1-linked residues in its chains, as a result of the removal of chains linked via 2 ⟶ 6-linkages to branching positions. Such a structure would, in this respect be similar to a laminated structure. It is difficult, however, to envisage how the degree of branching of a randomly branched levan could be greatly affected during the course of exo- degradation.

As a result of these arguments it was decided to make an attempted study of the type-structure of the levan elaborated by S. salivarius strain 51 by monitoring the types and proportions of the variously linked residues and the degree of branching during the course of exo-levanase degradation. To do so on a continuous basis would be a virtually impossible task and so it was decided that the linkage analyses would be performed on a series of samples of the degraded polysaccharide, the extent of degradation being up to 60%. This was considered to be a reasonable practical upper limit since it is highly unlikely that a branched polysaccharide with a degree of branching of 11.5% (i.e. with 9 residues in its Average Repeating Unit, see IV. B. ii, p. 113) would still possess an intact set of branching residues after removal of 60% of the constituent residues. It was thought, therefore, that a series of levans degraded by 10, 20, 40 and 60%, in addition to the undegraded material, would constitute an adequate range for this study.

It was considered that the methods for the linkage analysis of levans employed earlier in these studies (see Section IV.B., p.100) would be suitable for the linkage analysis of the partially degraded levans, remembering that an essential requirement is the need to be able to detect and differentiate between two types of linkage viz. 2 ⟶ 6- and 2 ⟶ 1-linkages occurring at non-branching positions. Residues thus linked would, as discussed in Section IV.B, p. 110 give rise respectively to a 2,5,6-tri-O-acetyl-1,3,4-tri-O-methyl-hexitol (XIV) and a 1,2,5-tri-O-acetyl-3,4,6-tri-O-methyl-hexitol (XV). These compounds would only be differentiable by E.I. mass spectrometry if a deuterium label be introduced at C-2 during the reduction step of the reaction sequence by using sodium borodeuteride as the reducing agent. The corresponding 2-d-hexitol derivatives (XVI) and (XVII) would then give rise to different mass spectra.



XVI



XVII

Because these derivatives carry the same numbers of methoxy and acetate groups and possess similar patterns of substitution, it is unlikely that they would be separated by gas chromatography and consequently would be simultaneously present in the mass spectrometer during analysis by g.c.-m.s. If the latter compound (XVII) was indeed present, in additon to the expected XVI, in hexitol derivative mixtures from one or more of the degraded levan preparations, its presence would be apparent by a change in the mass spectrum of

the tri-O-acetyl-tri-O-methyl-hexitol component. The expected result would be that the ratios of the abundances of the pairs of fragments $^{m}/_{e}$ $^{162}/_{161}$, $^{189}/_{190}$ and $^{206}/_{205}$ would decrease relative to the ratios of the same fragment pairs in the mass spectrum of compound XVI (which would be obtained from the undegraded levan). Determinations of the degrees of branching would be possible by the procedure used for degree of branching determinations in undegraded levans as described in IV. B. ii., p. 113.

IV. G. ii. Preparation of levans partially degraded by the exo-levanase of S. salivarius strain 51

Initially the levan of S. salivarius strain 51 was digested with the exo-levanase preparation as described in III. U. i. From the hydrolysis curve, shown in Fig. IV. 37., the digest times required for 10, 20, 40 and 60% hydrolysis were arrived at. These were 20 min, 45 min, 2 h and 3. 5 h. A large scale digest was then prepared as described in III. U. ii. At the digest times arrived at above, progressively larger aliquots (12, 16, 20 and 32 ml respectively) were removed and further treated as described in III. U. ii. In addition small aliquots of 50 µl were removed at zero time, at the times stated above and at further intervals up to 22 h for reducing sugar content and determination of degree of hydrolysis as described in III. U. ii. The hydrolysis curve, shown in Fig. IV. 38., is similar to that of the small scale digest (Fig. IV. 37.). The degree of hydrolysis and yield of each degraded levan product are given in Table IV. 15.

Figure IV.37. Preparation of levans partially degraded by levanase: Small scale digest

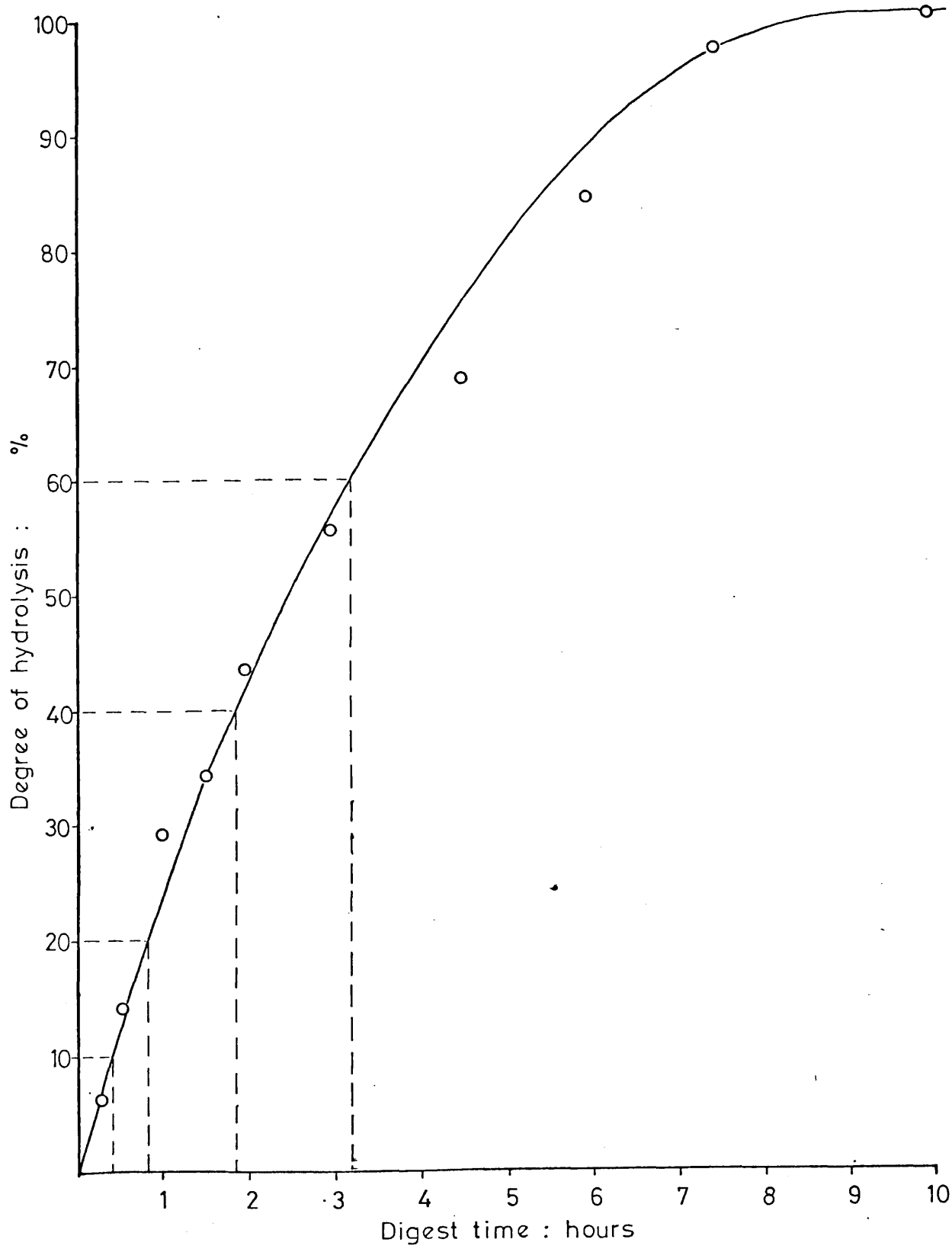


Figure IV. 38. Preparation of levans partially degraded by levanase: Large scale digest

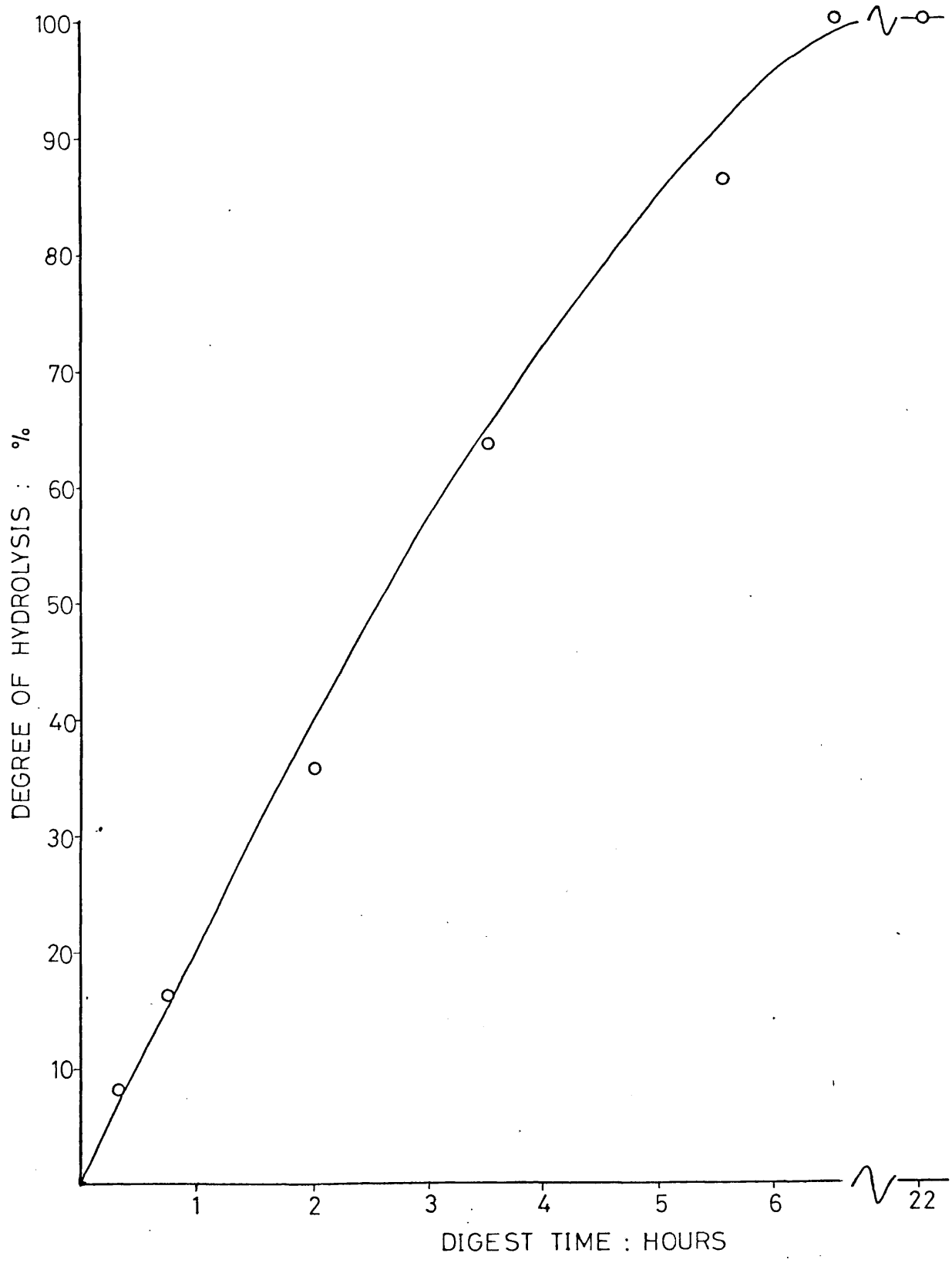


Table IV. 15. Partially levanase degraded levans

Digest time	$A_{520\ nm}$	Degree of hydrolysis, %	Yield [a] mg
20 min	0.021	7.9	15
45 min	0.042	15.8	26
2 h	0.094	35.3	30
3.5 h	0.168	63.2	33
5.5 h	0.229	86.1	-
6.5 h	0.267	100.0	-
22 h	0.266	100.0	-

a. From 165 mg levan

IV. G. iii. Linkage analysis and degree of branching of levans of S. salivarius strain 51 partially degraded by exo-levanase

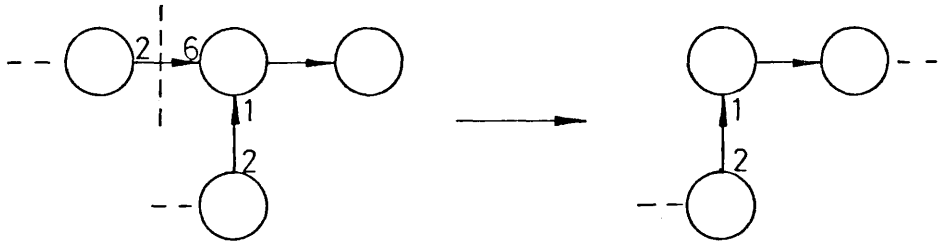
The partially degraded products, obtained as described in IV. G. ii, together with the undegraded polymer were converted to mixtures of 2 - d - O - acetyl - O - methyl - hexitol derivatives as described in III. E., with the exception that 10 mg of each material was subjected to the reaction sequence described. The resulting mixtures of hexitol derivatives were analysed by g. c. - E. I. m. s. as described in III. J. ii. and the areas of the peaks in the chromatograms corresponding to the hexitol derivatives were measured by electronic integration as described in III. J. iii.

From an examination of the mass spectra of the various 2-d-O-acetyl-O-methyl-hexitols, it was evident that components with R_{tmg} values of 0.79 and 3.95, in each case, are the expected 2-d-2,5-di-O-acetyl-1,3,4,6-tetra-O-methyl-hexitol (XLVIII) and 2-d-1,2,5,6-tetra-O-acetyl-3,4-di-O-methyl-hexitol (LI) derived respectively from non-reducing D-fructofuranosyl terminii (XXXIV) and branching D-fructofuranose residues linked through positions 1, 2 and 6 (XXXVI)(see Table IV.13., p 179). The undegraded polysaccharide gave the expected 2-d-2,5,6-tri-O-acetyl-1,3,4-tri-O-methyl-hexitol (R_{tmg} = 1.70, XVI) derived from D-fructofuranose residues linked through positions 2 and 6 (V). The mass spectra of compounds with R_{tmg} = 1.70 derived from all the partially exo-levanase degraded levans contained increased abundances of fragments $^{m}/e$ 161, 190 and 205 (characteristic of 2-d-1,2,5-tri-O-acetyl-3,4,6-tri-O-methyl-hexitol, L) relative to those of $^{m}/e$ 162, 189 and 206 (characteristic of XVI). These findings, given in Table IV.16., were obtained by direct measurement of the peak heights in the mass spectra and accordingly must be treated with some caution (see later, p. 203).

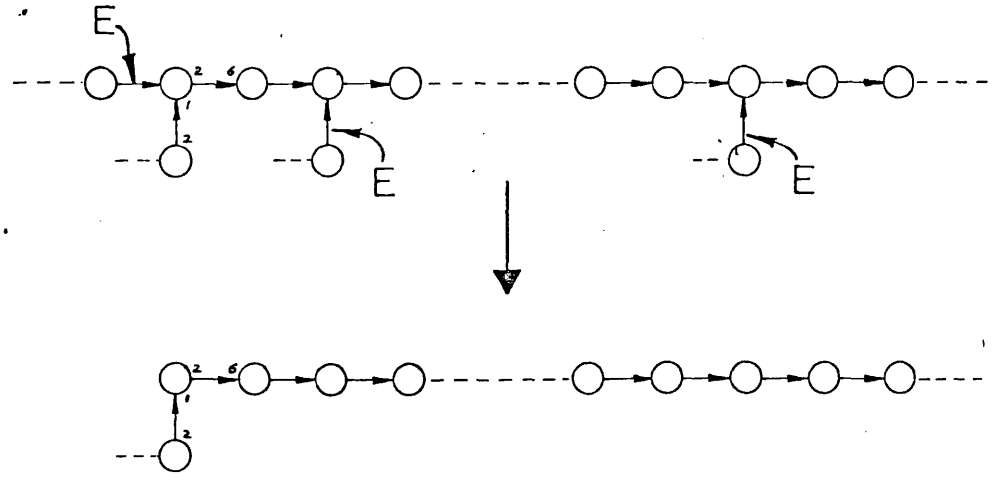
Table IV.16. Ratios of abundances of pairs of fragments occurring in electron impact mass spectra of 2-d-tri-O-acetyl-tri-O-methyl-hexitol (R_{tmg} = 1.70) components derived from the undegraded and partially exo-levanase degraded levans of S. salivarius strain 51

Degree of hydrolysis	Approximate fragment-pair ratios			Interpretation
	$^{162}/161$	$^{189}/190$	$^{206}/205$	
zero	7.3	10.4	5.4	Compound XVI only (9.6% fragment $^{m}/e$ 190 (e.g.) due to natural abundance ^{13}C)
7.9	5.4	7.1	4.2	Compound XVI + some Compound L
15.8	(7.6)	8.3	3.9	
35.3	5.2	8.9	4.2	
63.2	6.1	9.0	4.4	

If the assumption is made that these results, although unconfirmed, are valid, then they constitute evidence for the presence of 2 ⟶ 1 - linked D - fructofuranose residues in the partially degraded polysaccharides. Such a feature could only arise as a result of hydrolysis of 2 ⟶ 6 - linkages to branching residues in advance of the 2 ⟶ 1 - linkages to these positions.



Furthermore, a detectable amount of residues, thus linked, would essentially preclude the possibility of the levan of this investigation possessing a comb-like structure (Fig. IV. 36. c.) since each molecule with such a structure during exo - levanase degradation (D - fructose being the sole product, see IV. D. iii., p. 143) could at any instant in time possess, at most, only 1 such residue by removal of the residue linked via position 6 to the branching residue nearest to the non-reducing end of the basal C - chain. Removal of side chains (A - chains) attached via 2 ⟶ 1 - linkages to the basal chain would simply generate new 2 ⟶ 6 - linked residues in lieu of branching residues: -

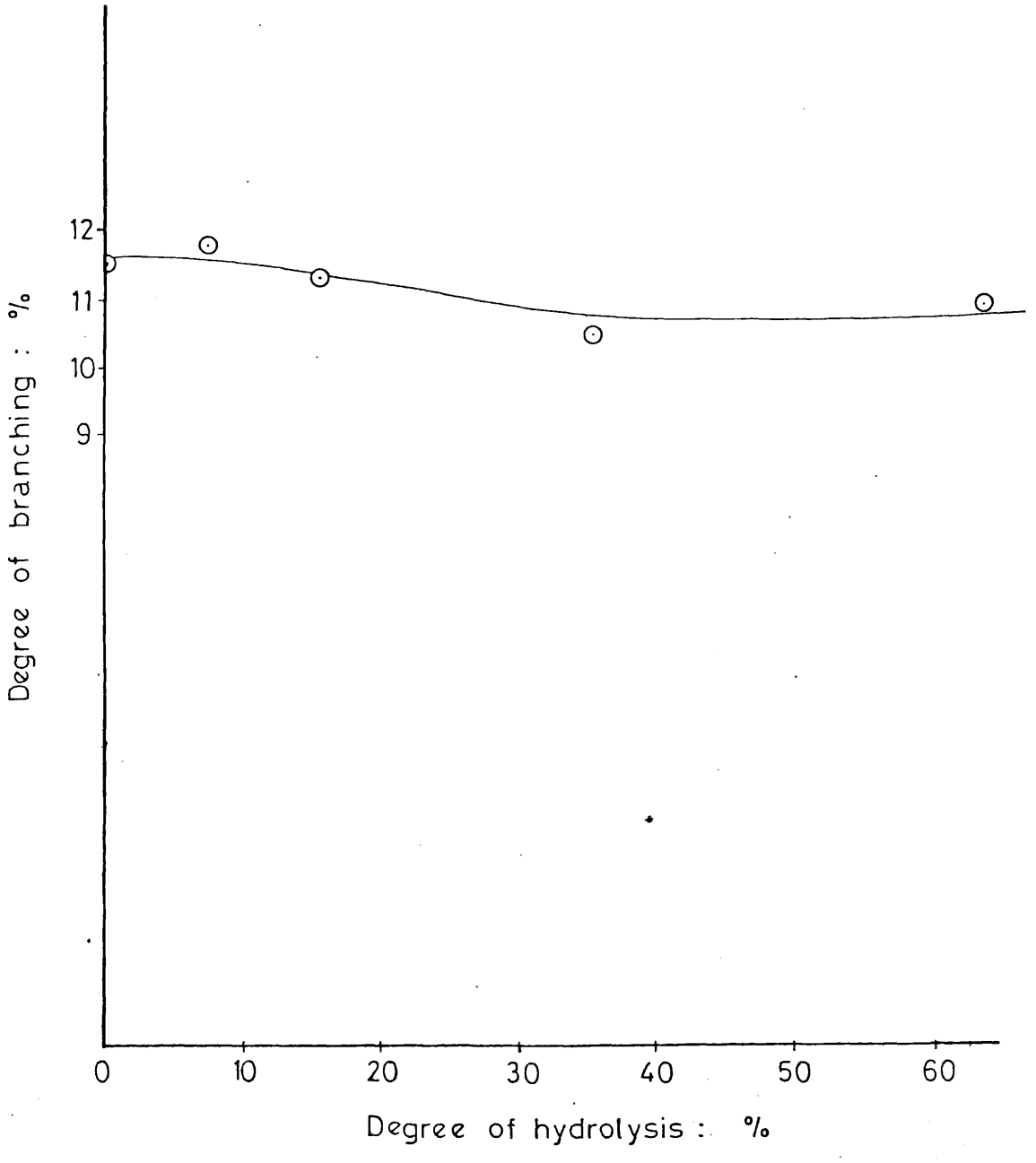


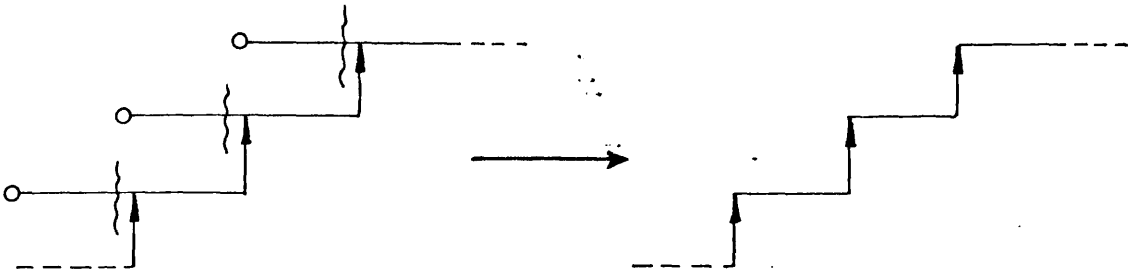
If the above results are valid then the levan elaborated by S. salivarius strain 51 must possess, therefore, either a laminated type-structure (Fig. IV. 36. b.) or a tree-like type-structure (Fig. IV. 36. d.).

The degree of branching for each preparation was determined as described in IV. B. ii., the calculations being based, as before, upon the areas of peaks corresponding to the tetra-O-acetyl-di-O-methyl-and tri-O-acetyl-tri-O-methyl-hexitols. Remethylation of portions of the methylated levan preparations resulted in no change in their degrees of branching. It was therefore concluded that the methylations had been complete. The results, shown graphically in Fig. IV. 39 as a function of degree of hydrolysis, demonstrate that the degree of branching remains fairly constant during the course of exo-levanase degradation up to an extent of hydrolysis of 60%.

This finding, in itself, does not enable the assignment of type-structure to be made. As the degree of branching of the original polysaccharide is ca. 11%, the evidence suggests that during the course of the degradation 8 D-fructofuranose residues are removed, on average, for the disappearance of each branching residue. Since it can be readily envisaged that such a situation could occur with either a laminated or randomly branched (tree-like) structure, it is not possible to differentiate between them on the basis of this evidence. A tree-like structure (Fig. IV. 36. d.) can be considered to consist of (commencing at non-reducing terminii and proceeding "inwards" towards the basal C-chain) outer chains terminating at "outermost" branch points followed alternately by interior chains and branch points. The degree of branching of such a structure, upon exo-degradation, would remain essentially constant, whereas this need not be the case with a laminated structure. If, for example, the enzyme was capable of splitting off residues from the B-chains in a laminated structure right up to the branching positions (thereby generating a significant number of 2 ⟶ 1-linked residues in the remaining basal chain), a drastic reduction in degree of branching would be the expected result.

Figure IV. 39. Variation of degree of branching with extent of exo - levanase degradation for the levan of S. salivarius strain 51





Conversely, it is difficult to reconcile a laminated structure with the appearance of a significant number of 2 ⟶ 1 - chain - linkages and a little changed degree of branching during exo - levanase degradation.

On the basis of the above reasoning it is thought that the levan elaborated by S. salivarius strain 51 may well possess a randomly branched, tree-like structure. Circumstantial evidence, viz. the fact that the levan of S. salivarius ATCC 13419 possesses a spherical or ellipsoidal molecular shape, as observed by electron microscopy (unpublished data of Newbrun and Jahn, see ref.[159]), would be consistent with such a structural type. Furthermore, on the basis of comparative biochemistry, such a structure is likely since other examples of reserve or storage polysaccharides (notably glycogen and amylopectin) possess a randomly branched structure. Finally a tree-like structure would be consistent with the mechanisms proposed for the enzymic synthesis of levans[113,117] and the origins of branches[119], as discussed in Section I.C.

In order to confirm the somewhat meagre evidence presented above for the occurrence of 2 ⟶ 1 - linked residues in partially exo - levanase degraded levan, it is recognised that the linkage analysis should be repeated and incorporate accurate (instrumental) measurement of the peak heights (and hence fragment abundances) in the resulting mass spectra, thereby enabling the ratios of

abundances of fragments $^{m}/e$ 161, 190 and 205 to those of $^{m}/e$ 162, 189 and 206 to be accurately found. In the case of the fragment pair $^{m}/e$ 189 and 190, it would be necessary to correct for the contribution to the latter from the former due to naturally occurring ^{13}C. Similar contributions to fragments $^{m}/e$ 162 and 206 from those with $^{m}/e$ 161 and 205 would be of far less importance since the latter pair would be present in a relatively far lower abundance.

By carrying out this work (which unfortunately could not be completed as a result of difficulties experienced with methylations and a resultant exhaustion of available degraded polysaccharides) it is felt that not only could the presence of 2 ⟶ 1 - linked residues in exo - levanase degraded levans be confirmed but also the proportion of the total residues that are thus linked could be established and monitored during the course of the degradation. Such data, in addition to confirming the structure not to be of the comb-like type would be of value in the positive assignment of the type-structure prevailing.

IV.H. General Conclusions

In these studies it has been established that the fructans elaborated by strains of Streptococcus salivarius are, according to the definition given in Section I.A., members of the levan class of polysaccharide. That these polymers are of high molecular weight, there can be little doubt, although the methods employed, strictly speaking, yield information leading to particle weights. Whether or not these represent true molecular weights remains to be answered and future work in this field might at least be partly directed at finding a reliable solution to this problem.

Levans elaborated by oral strains of streptococci undoubtedly play a role in the balance of ecological factors in the oral cavity. The fact that they are readily degraded by the action

of extracellular levanases is strong evidence that their biological function is one of carbohydrate storage. The levanase of one of the strains studied has been found to possess an exo-hydrolytic action towards the levan elaborated by Streptococcus salivarius strain 51. The sole product of the enzymic degradation has been shown to be D-fructose, which would most certainly be metabolised by the indigenous microbial flora in the in vivo situation. In the context of the process leading to dental caries, the levan synthesis-hydrolysis system may indeed play an important role since it undoubtedly contributes to the growth and activity of cariogenic streptococcal strains. The possibility of caries prevention by the inhibition of levansucrase or levanase activity has not been investigated, however, and it is suggested that here might be a worthwhile area for future research.

The exo-levanase elaborated by S. salivarius strain 51, which has been partially characterised in the present work has been hitherto neglected and it is felt that a detailed investigation of this enzyme system is warranted. For example, it is unknown whether the preparation, as obtained, contains a single hydrolytic enzyme or enzymes specific for the cleavage of the β-2 $\longrightarrow$ 6- and β-2 $\longrightarrow$ 1-interfructosidic linkages in the levan molecule. The possibility of a debranching enzyme being present, analogous to the amylopectin debranching enzymes R-enzyme, pullulanase and iso-amylase, is ruled out because such an enzyme would be incompatible with the strictly exo-action found for the degradation of levan by the levanase preparation. The possible existence, however, of a separate enzyme which is capable of removing β-2 $\longrightarrow$ 1-linked fructose residues, when exposed as stubs at branching positions by the previous action of a specific β-2 $\longrightarrow$ 6-fructofuranosidase, cannot be ruled out.

The use of the exo-levanase preparation as a probe into the type-structure of levans has been attempted and the results obtained suggest that the levan of S. salivarius strain 51 does not possess a comb-like structure. It is thought likely that the structure prevailing is, in fact, of the randomly branched tree-like type.

This hypothesis remains, however, to be experimentally substantiated and in order to do so it would be necessary to isolate and employ additional structural enzymic probes as in the analogous cases of amylopectin and glycogen which were structurally investigated using R-enzyme[206], iso-amylase[208], phosphorylase[209] and rabbit muscle amylo-1,6-glucosidase[209].

An advance has been made in the development and application of a method for reducing end linkage analysis in mixtures of oligosaccharides, obtained from levan, of common degree of polymerisation and resultant elucidation of sizes of branches in levans. It is believed that the procedure could find application in structural studies on levans in general and other branched fructans.

Finally, a further advance has been made in the use of Chemical Ionisation mass spectrometry in the assignment of structures to O-acetyl-O-methyl-alditol derivatives without the need to resort to chromatographic retention data.

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APPENDIX I

Gas chromatography - Chemical ionisation mass spectrometry

Instruments:

Pye gas chromatograph, model 104
VG Micromass mass spectrometer, model 16F

Experimental conditions:

column : glass (3.0 m x 5.0 mm), packed with OV225, 3%, on Chromasorb Q (80-100 mesh)

oven temperature : programmed from 170^{o} to 225^{o} with 5^{o} min^{-1} rate of increase

C.I. reactant gas : iso-butane, 0.5 torr

APPENDIX II

PUBLICATIONS

Reprinted from

Carbohydrate Research, 34 (1974) 279–288

STUDIES ON DEXTRANS AND DEXTRANASES

PART XI[1]. THE STRUCTURE OF A DEXTRAN ELABORATED BY *Leuconostoc mesenteroides* NRRL B-1299

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ABSTRACT

In dextran *S* elaborated by *Leuconostoc mesenteroides* NRRL B-1299, the polymeric chain consists, principally, of segments of isomaltose homologues which are mutually linked through positions 1 and 2 of their terminal D-glucose residues. The average repeating-unit, containing a total of fifteen D-glucose residues, possesses five branches which occur at each position 6 of such segments and at positions 2. Branches consist mainly of α-D-glucopyranosyl groups and some appear to be terminated by α-nigerosyl groups.

INTRODUCTION

Analysis of the complex mixtures of oligosaccharides produced by the action of dextranases on native dextrans possessing secondary linkages has afforded an insight into both the patterns of the action of the enzymes[2] and the structures of these dextrans[3,4]. We now report the results of studies on the action of the dextranase of *Penicillium lilacinum* (CMI 79197; NRRL 896) (dextranase A of Part II[5]) on the native, water-soluble dextran (dextran *S*) of *Leuconostoc mesenteroides* NRRL B-1299 and on the acid-degraded dextran *S* (dextran *S-AD*). Of the reports concerning the structure of the dextrans of *L. mesenteroides* NRRL B-1299, the following are pertinent to the present investigation. Jeanes and associates[6] concluded, on the grounds of statistical analysis of products of low molecular weight obtained by partial hydrolysis with acid, that most of the external branches in dextran *S* consist of single α-D-glucopyranosyl residues. Immunochemical studies by Kabat and co-workers[7–11] have indicated that terminal glucosidic linkages on non-reducing chain ends in dextran *S* and dextran *L* (the less-soluble fraction produced by *L. mesenteroides* NRRL B-1299) are most frequently of the α-(1→2) type and that others are of the α-(1→3) and α-(1→6) types. We have recently reported[1] that the approximate number of terminal, non-reducing D-glucose residues and those linked through positions 1 and 6, 1 and 3, as well as 1, 2, and 6 in the average repeating-unit of dextran *S* are 5, 4, 1, and 5, respectively. The corresponding figures for dextran *L* are 5, 4, 3, and 5.

RESULTS AND DISCUSSION

Dextran *S* was only slightly degraded by dextranase A. This result was expected in view[5] of the high content (41%) of α-D-glucopyranosyl residues linked through secondary linkages; the same result was obtained with the dextranase of *P.funiculosum* (C.M.I. 79195; NRRL 1132) (dextranase B of Part II[5]). However, partial hydrolysis with acid produced a polymeric material (dextran *S-AD*) which retained a proportion of secondary linkages, and yet was readily degraded by dextranase A. Conditions for obtaining such a non-dialysable material in reasonable yield were obtained from a series of trial experiments. The results of periodate oxidation suggested that all (1→3)-linkages in dextran *S* were hydrolysed during its acidic degradation, and that 9% of α-D-glucopyranosyl residues in dextran *S-AD* were linked through secondary linkages of the (1→2) type (the method[1,2] does not distinguish between residues linked through positions 1 and 2, and positions 1, 2, and 6), the remainder being terminal, non-reducing residues and residues linked through primary linkages only.

Paper chromatography of a dextranase–dextran *S-AD* digest revealed, in addition to products expected[2] from sections of dextran molecules possessing only primary linkages (*i.e.*, D-glucose, isomaltose, and isomaltotriose), several higher saccharides, three of which were isolated by methods similar to those described earlier[3,4]. The assignment of their molecular size was made on the basis of reducing power (Table I). The molecular size of the heptasaccharide *A* was deduced from its rate of chromatographic migration. Tetrasaccharide *A* and pentasaccharide *A* were selected for further studies.

TABLE I

YIELDS AND PROPERTIES OF PRODUCTS FROM DEXTRAN *S-AD* PRODUCED BY DEXTRANASE A

Product	*Yield (g from 50 g of dextran* S-AD)	*D.p.*[a]	$[\alpha]_D^{20}$ [b] (*degrees*)	R_G	M_G(B)	M_S(Mo) [c]
D-Glucose	0.60			1.00	1.00	1.00
Isomaltose	10.20	1.9	+120	0.74	0.70	0.70
Isomaltotriose[d]	7.10	2.9		0.56	0.60	0.58
Tetrasaccharide *A*	1.10	4.0	+157	0.42	0.50	0.46
Pentasaccharide *A*	1.60	5.0	+169	0.26	0.45	0.41
Hexasaccharide *A*	0.60	5.9	+171	0.16	0.41	0.38
Heptasaccharide *A*	Not measured	—	—	0.11	—	—

[a]Determined by the method of Timell[23]. [b]In water (*c* 0.6–0.7). [c]After reduction with $NaBH_4$. [d]This fraction contained a trace component, possibly a trisaccharide, with R_G 0.60.

Electrophoresis of a saccharide and its reduction product in borate and molybdate solutions, respectively, can be used for determining the position of the glycosidic linkage to the reducing unit of the original saccharide[13]. Accordingly, the

rates of electrophoretic migration of the tetra- and penta-saccharides *A* in borate solution and of their reduction products in molybdate solution (Table I) show that the reducing D-glucose residues in the oligosaccharides are linked only through position 6.

The results of oxidation with periodate of the tetra- and penta-saccharides *A* (Table II) are those expected from oligosaccharides possessing one (1→2)-linkage to units other than the reducing D-glucose residue, the remainder being (1→6)-linkages; methylation analysis of dextran *S* and the oligosaccharides (see below) rule out the possibility of a (1→4)-linkage.

TABLE II

PERIODATE OXIDATION OF OLIGOSACCHARIDES

Oligosaccharide	*Periodate reduced (mol.)*		*Formic acid produced (mol.)*	
	Found	*Calc.*[a]	*Found*	*Calc.*[a]
Tetrasaccharide *A*	9.0	9.0	5.9	6.0
Pentasaccharide *A*	10.8	11.0	6.9	7.0

[a]Calculated for oligosaccharides possessing one (1→2)-linkage, but not to the reducing D-glucose residue, the remainder being (1→6)-linkages.

G.l.c. of the methanolysates of the methylated tetra- and penta-saccharides *A* revealed components having retention volumes identical with those of the methyl α- and β-pyranosides of 2,3,4,6-tetra-, 2,3,4-tri-, and 3,4,6-tri-*O*-methyl-D-glucoses. On the evidence presented so far, the possible structures for tetrasaccharide *A* are **1** and **2**, and those for pentasaccharide *A* are **3**, **4**, and **5** (Fig. 1). The structures were

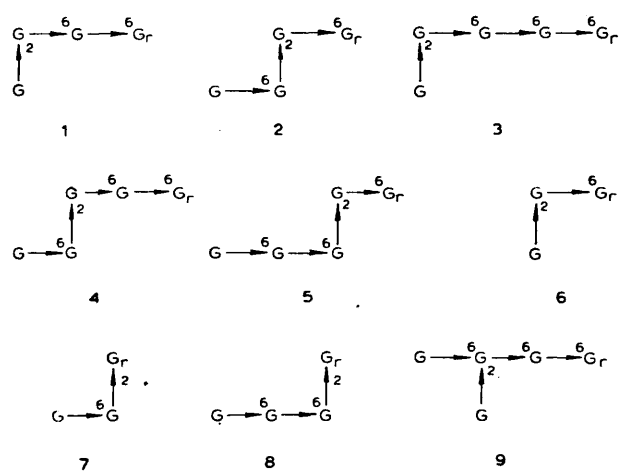

Fig. 1. Structure of oligosaccharides. G, α-D-glucopyranosyl unit; G_r, reducing D-glucose unit.

further elucidated by fragmentation by acetolysis and acid hydrolysis, and examination of products by paper electrophoresis and chromatography (Table III).

TABLE III

PRODUCTS OF ACETOLYSIS OF TETRASACCHARIDE *A* AND PENTASACCHARIDE *A*

Fraction	R_G	$M_G(B)$	$M_S(Mo)$ [a]	*Identity*
1	1.00	1.00	1.00	D-Glucose
2	0.83	0.30	0.70	Kojibiose
3	0.74	0.70	0.70	Isomaltose
4	0.56	(i) 0.60	0.58	Isomaltotriose and **6**
		(ii) 0.27		**7**
5[b]	0.42	0.50	0.46	Isomaltotetraose, **1**, and **2**

[a]After reduction with $NaBH_4$. [b]Only from pentasaccharide *A*.

Tetrasaccharide A. — The characteristic fragments of the tetrasaccharides **1** and **2** are isomaltotriose and *O*-α-D-glucopyranosyl-(1→6)-*O*-α-D-glucopyranosyl-(1→2)-D-glucose (**7**), respectively. The trisaccharide fraction obtained by acetolysis, followed by deacetylation, contained two components 4(i) and 4(ii), which had $M_G(B)$ 0.60 and 0.27, respectively, expected of trisaccharides in which the reducing D-glucose residue is linked through position 6 [isomaltotriose and *O*-α-D-glucopyranosyl-(1→2)-*O*-α-D-glucopyranosyl-(1→6)-D-glucose (**6**)] and position 2 (**7**), respectively*. Since the component with $M_G(B)$ 0.60 stained far more intensely than that with $M_G(B)$ 0.27, and as the trisaccharide **6** can arise from both tetrasaccharides **1** and **2**, it is likely that the faster-migrating component contained isomaltotriose and trisaccharide **6**. It is thus concluded that tetrasaccharide *A* is a mixture of *O*-α-D-glucopyranosyl-(1→2)-*O*-α-D-glucopyranosyl-(1→6)-*O*-α-D-glucopyranosyl-(1→6)-D-glucose (**1**) and *O*-α-D-glucopyranosyl-(1→6)-*O*-α-D-glucopyranosyl-(1→2)-*O*-α-D-glucopyranosyl-(1→6)-D-glucose (**2**).

Pentasaccharide A. — The tetrasaccharide fraction (5), obtained from pentasaccharide *A* by acetolysis followed by deacetylation, migrated during eletrophoresis in borate solution as a single component with $M_G(B)$ 0.50, expected of tetrasaccharides in which the reducing D-glucose residue is linked through position 6. As a component with an $M_G(B)$ value <0.27, expected of *O*-α-D-glucopyranosyl-(1→6)-*O*-α-D-glucopyranosyl-(1→6)-*O*-α-D-glucopyranosyl-(1→2)-D-glucose (**8**), could not be detected, it is likely that the pentasaccharide *A* contained only insignificant quantities, if any at all, of pentasaccharide **5**. This conclusion is in agreement with the action

*Since this work was completed[14], Sakakibara *et al.*[15] have obtained *O*-α-D-glucopyranosyl-(1→2)-*O*-α-D-glucopyranosyl-(1→6)-D-glucose (**6**) and *O*-α-D-glucopyranosyl-(1→6)-*O*-α-D-glucopyranosyl-(1→2)-D-glucose (**7**) by acetolysis of the dextran of *L. mesenteroides* NRRL B-1397, and have reported rates of electrophoretic migration that are virtually identical with those recorded in this paper.

pattern[2] of dextranase *A*. If liberated from dextran *S-AD*, pentasaccharide **5** would be hydrolysed by dextranase A to give isomaltose and trisaccharide **6**.

The trisaccharide fraction obtained by the above fragmentation could be resolved to give the same components as were obtained from tetrasaccharide *A*, *i.e.*, 4(i) and 4(ii). As structure **5** has been eliminated, the component with $M_G(B)$ 0.27 (**7**) undoubtedly arises from pentasaccharide **4**.

Acid hydrolysis of pentasaccharide *A* gave, *inter alia*, a tetrasaccharide fraction which was isolated by paper chromatography. Treatment of this material with dextranase A resulted in rapid and extensive degradation, giving isomaltose as the main product. The acid hydrolysate thus contained appreciable quantities of isomaltotetraose which, as indicated by the other evidence, could arise only from pentasaccharide **3**. Pentasaccharide *A* was thus a mixture of *O*-α-D-glucopyranosyl-

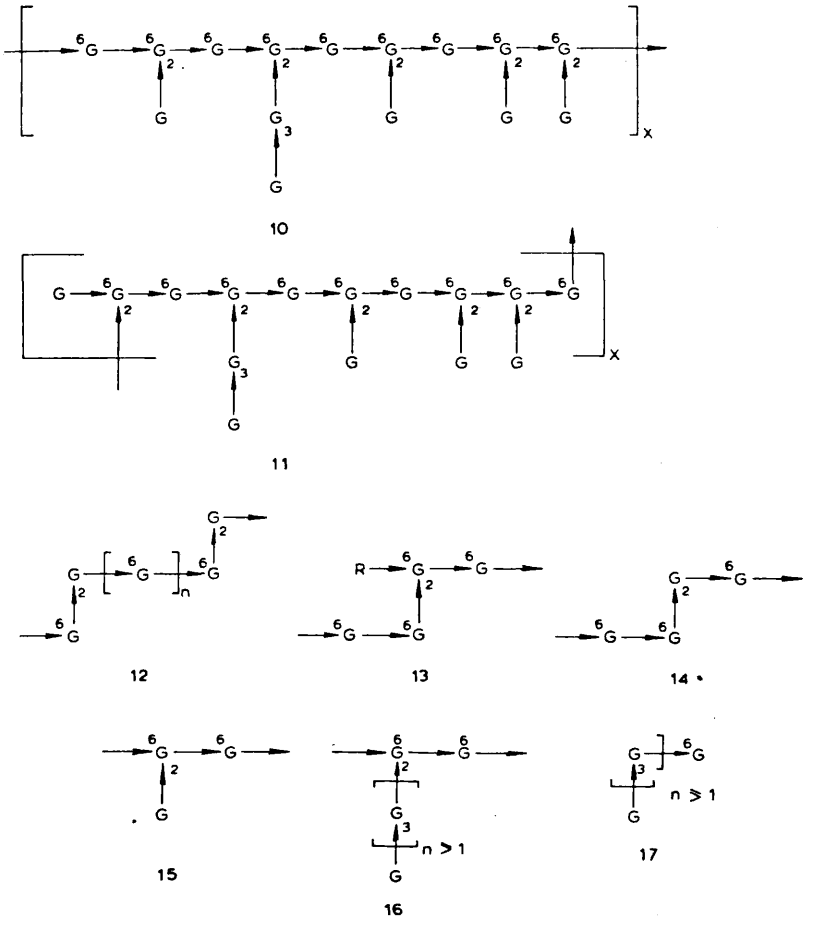

Fig. 2. Segments of dextran molecules. G, α-D-glucopyranosyl unit; R, α-D-glucopyranosyl unit or glycosyl units of isomaltose and its homologues.

(1→2)-*O*-α-D-glucopyranosyl-(1→6)-*O*-α-D-glucopyranosyl-(1→6)-*O*-α-D-glucopyranosyl-(1→6)-D-glucose (**3**) and *O*-α-D-glucopyranosyl-(1→6)-*O*-α-D-glucopyranosyl-(1→2)-*O*-α-D-glucopyranosyl-(1→6)-*O*-α-D-glucopyranosyl-(1→6)-D-glucose (**4**).

Structure of the dextrans. — Dextran *S* may possess two general types of structural features, consistent with the earlier reported[1] percentages of the variously linked D-glucose residues. The polymeric chain may continue either through α-(1→6)- or α-(1→2)-linkages, as exemplified by the average repeating-units **10** and **11** (Fig. 2), respectively. Some α-(1→3)-linkages in dextran *S* may[1,9,11] represent terminal linkages on non-reducing chain ends, as, for example, in **10** and **11**, although others may intersect a continuous chain of α-(1→6)-linked D-glucose residues. It is appreciated that some branches in both types of structure (**10** and **11**) may consist of more D-glucose residues than indicated. However, in view[1] of the high content (34%) of branching D-glucose residues in dextran *S* (*i.e.*, those linked through positions 1, 2, and 6), many must consist of only a few or, indeed, single D-glucose residues.

The results of the periodate oxidation of dextran *S-AD* show that many α-(1→2)- and all the α-(1→3)-linkages in dextran *S* had been hydrolysed when it was treated with acid. This is in agreement with the relative rates of hydrolysis of *O*-α-D-glucopyranosyl-D-glucoses[16–18]. The fact that the tetra- and penta-saccharides *A*, obtained from dextran *S-AD* by enzymic lysis, did not contain components possessing α-(1→3)-linkages is further evidence that dextran *S-AD* did not possess α-(1→3)-linkages.

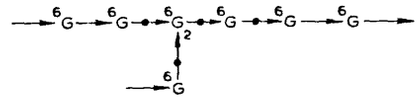

Fig. 3. Segment of dextran molecule showing glucosidic linkages which, if present, are resistant to hydrolysis by dextranase A. G, α-D-glucopyranosyl unit; —●—→, glucosidic linkage resistant to hydrolysis.

Fig. 3 shows a segment of a dextran molecule in the vicinity of a D-glucose residue linked through a secondary linkage, and indicates the glucosidic linkages which, if present at all, are resistant[2,4,14] to hydrolysis by dextranase A. According to this action pattern, the unbranched oligosaccharides **1**–**4** must have been produced from unbranched sections of dextran *S-AD*. In addition, the reducing D-glucose residue in tetrasaccharide **2** must also represent the reducing D-glucose residue of dextran *S-AD*. The fact that the branched pentasaccharide *O*-α-D-glucopyranosyl-(1→6)-*O*-[α-D-glucopyranosyl-(1→2)]-α-D-glucopyranosyl-(1→6)-*O*-α-D-glucopyranosyl-(1→6)-D-glucose (**9**) could not be detected when dextranase A had acted on dextran *S-AD* shows that such segments as that shown in Fig. 3 do not contribute significantly, if at all, to the structure of dextran *S-AD*. We thus conclude that the structure of dextran *S-AD*, in sections which can be degraded by dextranase A, is

essentially as represented by segment **12***. The structure of the oligosaccharides **1**, **3**, and **4**, together with the action pattern of dextranase A, further establish that the isomaltose homologues in segment **12**, which are mutually joined through positions 1 and 2 of their terminal D-glucose residues, have d.p. $\geqslant 4$, *i.e.*, in segment **12** $n \geqslant 2$.

In dextran *S*, all D-glucose residues linked through position 2 are also linked through position 6 and, hence, constitute branching residues. Therefore, the partial degradation of dextran *S* with acid to give dextran *S-AD* entailed debranching also by hydrolysis of the α-(1→6)-linkage on the non-reducing side of a branching D-glucose residue, *i.e.*, **13**→**14**.

It has been reported that acidic hydrolysis of dextran[6] (and of other glucans[19]) proceeds primarily by removal of small fragments (*i.e.*, D-glucose and small oligosaccharides) from non-reducing chain ends. The pronounced susceptibility to acidic hydrolysis of the α-(1→6)-linkage on the non-reducing side of some branching D-glucose residues in dextran *S* suggests, therefore, that these branching residues occur close to non-reducing ends of chains of α-(1→6)-linked D-glucose residues. As the majority of the "external" branches in dextran *S* appear to be single D-glucopyranosyl groups[6], we conclude that R in structure **13** also is most frequently an α-D-glucopyranosyl group (*cf.* the example **11** of an average repeating unit).

Kabat and co-workers[8,9,11] concluded, from immunochemical studies, that many terminal linkages in dextran *S* are of the α-(1→2) type. Sequences of α-(1→2)-linked D-glucose residues seem not to occur in dextran *S*, as the corresponding oligosaccharides [*e.g.*, *O*-α-D-glucopyranosyl-(1→2)-*O*-α-D-glucopyranosyl-(1→2)-D-glucose] could not be detected as products of acetolysis[14]. These observations, together with the fact that all D-glucose residues in dextran *S* which are linked through position 2 are branching residues[1], indicate that a high proportion of branches in dextran *S* are (1→2)-linked α-D-glucopyranosyl groups (as in segment **15**).

Present evidence (*cf.* Ref. 1) does not exclude the possibility that some of the α-(1→3)-linkages in dextran *S* intersect chains of α-(1→6)-linked D-glucose residues. However, the results of immunochemical studies[9–11] suggest that many of the α-(1→3)-linkages occur as terminal linkages on non-reducing chain ends. They are thus likely to occur at such chain ends as represented by segments **16** and/or **17**.

We now conclude that the polymeric chain in dextran *S* consists, principally, of segments of isomaltose homologues which are mutually linked through positions 1 and 2 of their terminal D-glucose residues. The average repeating-unit, containing a total of fifteen D-glucose residues, and of which structure **11** is one example, possesses five branches. These occur at each position 6 of such segments and at positions 2.

*[With Keith Marshall.] When we reached this conclusion, no more dextran *S-AD* was available. However, methylation analyses (*i.e.*, methylation, hydrolysis, reduction, acetylation, and g.l.c.–m.s.) of a sample of acid-degraded (hydrolysis for only 1.25 h) dextran *S* revealed, in addition to those D-glucose residues shown to be present in the native dextran *S*, D-glucose residues linked through positions 1 and 2 only. (The mass spectrum of the appropriate fraction showed a peak corresponding to *m/e* 189.) The results could not be evaluated quantitatively as, under the conditions used, 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol and 1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methyl-D-glucitol could not be resolved by g.l.c.

Branches consist mainly of α-D-glucopyranosyl groups and some appear to be terminated by α-nigerosyl groups.

EXPERIMENTAL

Paper chromatography. — The solvent used with Whatman No. 1 paper was ethyl acetate–pyridine–water (2:1:2, organic phase). Migration rates are expressed relative to the movement of D-glucose (R_G).

Paper electrophoresis. — The electrolytes used were those described[20] in Part VI.

Spray reagents. — The spray reagents used were (*a*) and (*b*), described[21] in Part IX.

Gas–liquid chromatography. — This was carried out as described by Aspinall[22], using columns containing (*a*) butane-1,4-diol succinate polyester or (*b*) polyphenyl ether [*m*-bis-(*m*-phenoxyphenoxy)benzene]. Retention times [*T* (*column*)] are expressed relative to that of methyl 2,3,4,6-tetra-*O*-methyl-β-D-glucopyranoside.

Dextran. — The dextran fraction (dextran *S*) used was elaborated by *L. mesenteroides* NRRL B-1299 and has been described[1] in Part X.

Dextranases. — The dextranase preparations of *P. lilacinum* (C.M.I. 79197; NRRL 896) (dextranase A) and *P. funiculosum* (C.M.I. 79195; NRRL 1132) (dextranase B) used in the present work were prepared as described previously[5].

Preparation of dextran S-AD. — Dextran *S* (200 g) was hydrolysed with 0.5M sulphuric acid (5 l) at 90° for 1.75 h. The hydrolysate was neutralised with barium carbonate and filtered. The filtrate was concentrated *in vacuo* at 40° to *ca.* 1 litre, filtered, and dialysed for 3 days against running tap water using Visking Cellulose Tubing. The dialysed solution was concentrated further, as described above, and freeze-dried to give dextran *S-AD* (55 g, zero ash content).

Periodate oxidation. — The method used was as described before[12]. Dextran *S-AD* reduced 1.91 mol. of periodate with concomitant formation of 0.91 mol. of formic acid per "anhydroglucose" unit. The results of periodate oxidation of oligosaccharides (see below) are shown in Table II. Under the conditions used, isomaltose reduced 6.1 mol. of periodate with concomitant formation of 5.0 mol. of formic acid.

Degradation of dextran S-AD *by dextranase A to oligosaccharides.* — Dextran *S-AD* (50 g) in 0.2M sodium citrate (pH 5; 500 ml) was incubated with dextranase A (60 units contained in 500 ml of 5mM sodium citrate, pH 6) at 37° for 6 days. Paper chromatography of the deionised solution revealed the components shown in Table I. These, except the heptasaccharide, were isolated by methods similar to those described earlier[3,4]. Properties of the saccharides are shown in Tables I and II.

Methylation analysis of tetra- and pentasaccharide A. — Dry silver oxide (1 g) and redistilled methyl iodide (3 ml) were added to separate solutions of each oligosaccharide (~12 mg) in *N*,*N*-dimethylformamide (1.5 ml, redistilled from calcium oxide). Each mixture was shaken at room temperature in a darkened, sealed flask for 24 h. Solid materials were filtered off and washed with chloroform, and the filtrates

and washings were concentrated under diminished pressure at 40°. The residues were again treated by the above procedure. Traces of *N*,*N*-dimethylformamide were removed under diminished pressure at 30°. Each residue was dissolved in methyl iodide (5 ml), dry silver oxide was added, and the mixture was refluxed for 6 h. Solid materials were removed as before. The residue obtained from each combined filtrate and washings was dissolved in 4% methanolic hydrogen chloride (4 ml), the solution was refluxed for 5 h, neutralised with silver carbonate, filtered, and concentrated. G.l.c. of the syrupy residue obtained from each oligosaccharide revealed components having retention times identical with those of methyl α- and β-pyranosides of 2,3,4,6-tetra-*O*-[*T* (*a*) 1.00 and 1.43, *T* (*b*) 1.00 and 1.33], 2,3,4-tri-*O* [*T* (*a*) 2.50 and 3.50, *T* (*b*) 1.33 and 1.83], and 3,4,6-tri-*O*-methyl-D-glucose [*T* (*a*) 2.93 and 3.50, *T* (*b*) 1.70 and 2.30].

Acetolysis of tetra- and penta-saccharide A. — Each oligosaccharide (~90 mg) was dissolved (by warming) in a mixture (3 ml) of acetic anhydride and sulphuric acid (100:9; v/v). Each solution was kept at 35° for 2 h and then poured onto crushed ice (10 g). Each mixture was adjusted to pH 6 by addition of sodium hydrogen carbonate, and the precipitated material was extracted with chloroform (4 × 10 ml). The extracts were washed with dilute, aqueous sodium hydrogen carbonate and water, dried (Na_2SO_4), and evaporated *in vacuo* at 40°. Each residue was dissolved in dry methanol (1 ml), and deacetylation was effected by addition of a small piece of sodium. After 24 h, each solution was deionised and concentrated, and the residue was fractionated by paper chromatography. Each fraction was subjected to electrophoresis in borate solution and, after treatment with sodium borohydride followed by deionisation, in molybdate solution. The results are shown in Table III.

Acidic and enzymic degradation of pentasaccharide A. — The oligosaccharide (~50 mg) was heated in 0.5M sulphuric acid (4 ml), in a capped tube, at 70° for 2.5 h. The hydrolysate was neutralised with barium carbonate, deionised, and fractionated by paper chromatography. The component (~5 mg) having R_G identical with that of isomaltotetraose was dissolved in 0.2M sodium citrate (pH 5, 2 ml) and incubated with dextranase A (0.24 unit contained in 2 ml of 5mM sodium citrate, pH 6) at 37° for 2 days. Paper chromatography of the deionised solution revealed that approximately half of the material had been degraded to the same compounds as was isomaltotetraose in a control experiment, *i.e.*, D-glucose, isomaltose (main product), and isomaltotriose.

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STRUCTURE OF THE LEVAN ELABORATED BY *Streptococcus salivarius* STRAIN 51: AN APPLICATION OF CHEMICAL-IONISATION MASS-SPECTROMETRY*

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ABSTRACT

The polysaccharide elaborated by *Streptococcus salivarius* strain 51 contains β-D-fructofuranose residues linked through positions 2 and 6, as well as 1, 2, and 6. The approximate numbers of terminal, non-reducing D-fructofuranose residues and those linked through positions 2 and 6, and through 1, 2, and 6 in the average repeating-unit are 1, 7, and 1, respectively. The branches through the β-(2→1)-linkage contain up to at least four D-fructofuranose residues. Chemical-ionisation mass-spectrometry aids the assignment of structures to *O*-acetyl-*O*-methylalditols obtained in methylation analysis.

INTRODUCTION

Strains of *Streptococcus salivarius* colonise in the human mouth and produce extracellular polysaccharides when grown on sucrose-containing media. These polysaccharides have previously been classed as levans, *i.e.*, D-fructans in which the principal glycosidic linkages are of the β-(2→6) type, the conclusions being based mainly on serological tests[1,2] and the infrared spectra[3] of the polysaccharides. As far as we are aware, the D-fructans have not been characterised chemically. Continuing our work on polysaccharides elaborated by organisms isolated from the human mouth[4], it seemed to us desirable to study in greater detail the chemical structure of these D-fructans. As a preliminary to detailed assignments of structures, we now report on the types and percentages of the glycosidic linkages, and the size of branches in the polysaccharide elaborated by *S. salivarius* strain 51.

RESULTS AND DISCUSSION

The extracellular polysaccharide was shown to be a D-fructan. Its negative rotatory power and the fact that invertase degraded the D-fructan almost completely to D-fructose indicated that it contained β-D-fructofuranose residues.

*Dedicated to the memory of Professor Edward J. Bourne.

TABLE I

PROPERTIES AND ANALYSIS OF HEXITOL-*2-d* DERIVATIVES OBTAINED FROM LEVAN

Component	*G.l.c.*		*C.i.–m.s.*		*E.i.–m.s.*		*Parent structural unit*
	T[a]	*Mole fraction*	$[M+1]^+$ (m/e)	*Hexitol*-2-d *derivative*	*Primary fragments* (m/e)	*Identity of hexitol derivative*	
A	0.79	0.14	324	Di-*O*-acetyl-tetra-*O*-methyl	45, 161, 162, 205, 206	**3**	**11**
B	1.70	0.75	352	Tri-*O*-acetyl-tri-*O*-methyl	45, 162, 189, 206, 233	**5**	**12**
C	3.95	0.11	380	Tetra-*O*-acetyl-di-*O*-methyl	189, 190, 233, 234	**7**	**14**

[a]Retention time relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol.

Since Lindberg and co-workers reported[5] that *O*-acetyl-*O*-methylalditols can be characterised by electron-impact (e.i.) mass-spectrometry, the procedures for the linkage analysis of polysaccharides have been greatly simplified[6]. The method involves methylation of the polysaccharide, followed by hydrolysis, reduction, acetylation, and g.l.c. of the alditol derivatives thus obtained. The results obtained with the levan of *S. salivarius* strain 51 when sodium borodeuteride[7] was used as the reducing agent are shown in Table I. It was expected that, on reduction, each *O*-methyl-D-fructose would give the corresponding D-glucitol and D-mannitol derivatives. However, g.l.c. of a sample of authentic 1,3,4,6-tetra-*O*-methyl-D-fructose, treated as described for the hydrolysate of the methylated levan, showed only a single peak, *i.e.*, 2,5-di-*O*-acetyl-1,3,4,6-tetra-*O*-methylhexitol. Thus, the conditions used do not allow the separation of the corresponding D-glucitol and D-mannitol derivatives.

The assignment of structure to *O*-acetyl-*O*-methylalditols is based on the g.l.c. retention times (*T*-values) and the pattern of electron-impact (e.i.) induced fragmentations. Although it is possible by e.i. mass-spectrometry to determine the pattern of substitution in an *O*-acetyl-*O*-methylalditol, the assignment of its structure is aided by determining first the number of methoxyl groups; this number is often estimated from the *T*-values. The ambiguities inherent in this method can be avoided by determining the molecular weight of the *O*-acetyl-*O*-methylalditols. Molecular ions, $[M]^+$, of *O*-acetyl-*O*-methylalditols produced by electron-impact ionisation are unfortunately rarely stable enough to be discernible in the e.i. mass-spectra. However, determinations of molecular weight can easily be carried out by chemical-ionisation

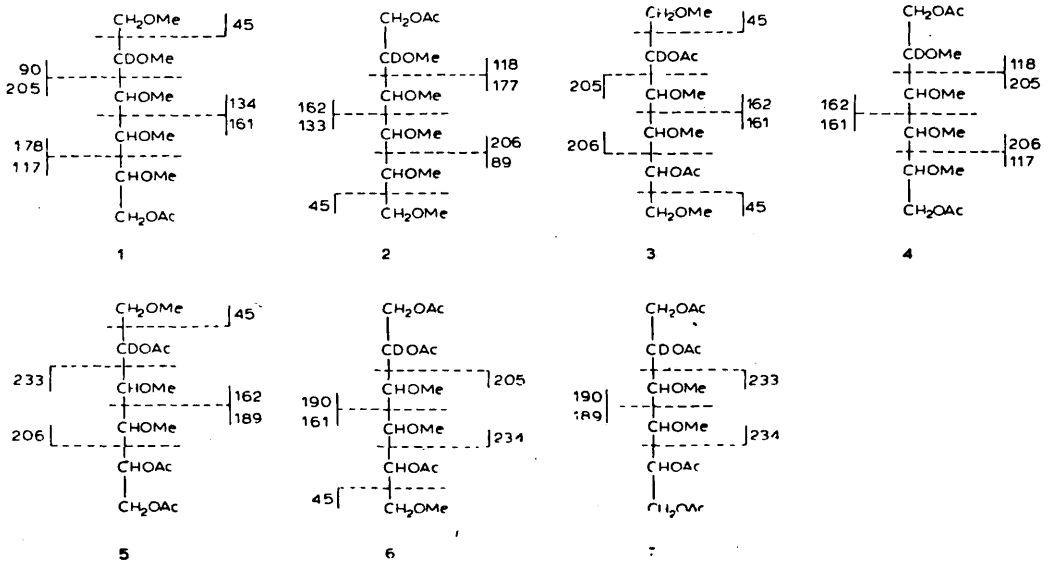

Fig. 1. E.i.-induced, primary fragmentation modes of *O*-acetyl-*O*-methylhexitol-*2-d* products (stereochemistry at asymmetric carbon atoms is not shown).

TABLE II

PROPERTIES AND ANALYSIS OF HEXITOL-*2-d* DERIVATIVES OBTAINED FROM DI- AND OLIGO-SACCHARIDES

Component	*G.l.c.* T[a]	*C.i.–m.s.*		*E.i.–m.s.*		*Parent structural unit*
		$[M+1]^+$ (*m/e*)	*Hexitol*-2-d *derivative*	*Primary fragments* (*m/e*)	*Identity of hexitol derivative*	
D[b–e]	0.50	296	*O*-Acetyl-penta-*O*-methyl	45, 89, 90, 117, 118, 133, 134, 161, 162, 177, 178, 205, 206.	**1** and **2**	**8** and **9**
E[b–e]	0.79	324	Di-*O*-acetyl-tetra-*O*-methyl	45, 161, 162, 205, 206.	**3**	**11**
F[d]	1.18	324	Di-*O*-acetyl-tetra-*O*-methyl	117, 118, 161, 162, 205, 206.	**4**	**10**
G[c–e]	1.70	352	Tri-*O*-acetyl-tri-*O*-methyl	45, 161, 162, 189, 190, 205, 206, 233, 234.	**5** and **6**	**12** and **13**
H[d,e]	3.95	380	Tetra-*O*-acetyl-di-*O*-methyl	189, 190, 233, 234	**7**	**14**

[a]Retention time relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol. [b]Obtained from disaccharide fraction. [c]Obtained from trisaccharide fraction. [d]Obtained from tetrasaccharide fraction. [e]Obtained from pentasaccharide fraction.

(c.i.) mass-spectrometry, as its commonly encountered feature is the abundance of $[M+1]^+$ ions. The molecular weights (M) of the alditol derivatives *A*, *B*, and *C* obtained from the levan (Table I), and determined from their $[M+1]^+$ ions in the c.i. mass-spectra, indicate them to be tetra-, tri-, and di-*O*-methylhexitol-*2-d* acetates, respectively. (C.i.-induced fragmentation modes of *O*-acetyl-*O*-methylalditols will be reported elsewhere). The e.i.-induced fragmentation patterns of the derivatives *A*, *B*, and *C* (Table I and Fig. 1) showed that they were, respectively, 2,5-di-*O*-acetyl-1,3,4,6-tetra-*O*-methyl- (**3**), 2,5,6-tri-*O*-acetyl-1,3,4-tri-*O*-methyl- (**5**), and 1,2,5,6-tetra-*O*-acetyl-3,4-di-*O*-methyl-hexitol-*2-d* (**7**).

The dubiety regarding the ring size of the D-fructose residues in the fructan was removed when the disaccharide fraction obtained by partial hydrolysis of the fructan (Table III) was reduced with sodium borodeuteride, followed by methylation, hydrolysis, reduction with sodium borodeuteride, and acetylation. The results (Table II and Fig. 1) of g.l.c.–m.s. (e.i. and c.i.) analysis show that the hexitol derivative *D* was a mixture of 6-*O*-acetyl-1,2,3,4,5-penta-*O*-methyl- (**1**) and 1-*O*-acetyl-2,3,4,5,6-penta-*O*-methyl-hexitol-*2-d* (**2**), whereas the hexitol derivative *E* was 2,5-di-*O*-acetyl-1,3,4,6-tetra-*O*-methylhexitol-*2-d* (**3**). Hence, the disaccharide fraction contained *O*-β-D-fructofuranosyl-(2→6)-D-fructose (levanbiose) and *O*-β-D-fructofuranosyl-(2→1)-D-fructose (inulobiose). We therefore conclude that the fructan of *S. salivarius* strain 51 is composed of β-D-fructofuranose residues linked through positions 2 and 6, as well as 1, 2, and 6.

The chemical evidence for the suggestion that levans contain β-D-fructofuranose residues is the observation[8] that the tri-*O*-methyl-D-fructose obtained by methylation of a fructan, followed by hydrolysis, could be converted into a methyl 1,3,4,6-tetra-*O*-methyl-D-fructofuranoside by methylanosis at room temperature

TABLE III

DI- AND OLIGO-SACCHARIDES OBTAINED BY PARTIAL HYDROLYSIS OF LEVAN WITH ACID

Fraction	R_{Fru}[a]	*Yield (mg from 15 g of levan)*	*D.p.*[b]
Disaccharide	0.70	62	2.2
Trisaccharide *X*	0.47	213	3.3
Trisaccharide *Y*	0.42		
Tetrasaccharide *X*	0.31	146	3.6
Tetrasaccharide *Y*	0.27		
Pentasaccharide *X*	0.21	100	4.9
Pentasaccharide *Y*	0.18		
Hexasaccharide *X*	0.15	18	5.5
Hexasaccharide *Y*	0.12		
Heptasaccharide *X*	0.09		
Heptasaccharide *Y*	0.08		

[a]Paper-chromatographic migration rate relative to that of D-fructose. [b]Estimated by the method of Timell[14].

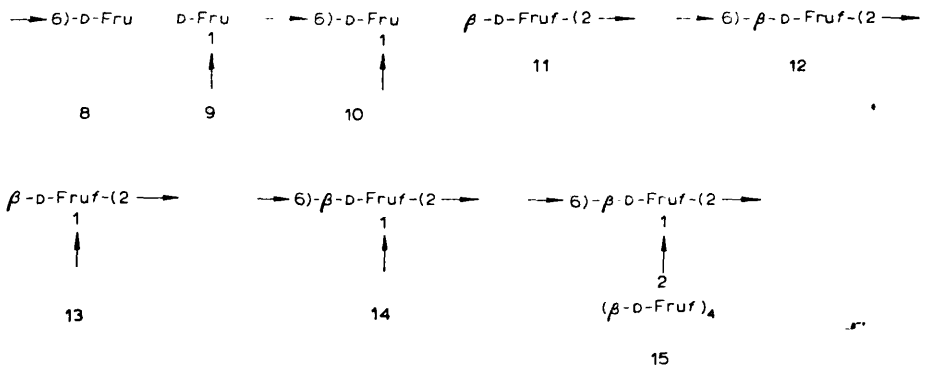

Fig. 2. Structural units of di- and oligo-saccharides, and levan.

and methylation. As 1,3,4-tri-*O*-methyl-D-fructose can give rise to furanosides and pyranosides, it is, however, likely that the intermediate, methyl 1,3,4-tri-*O*-methyl-D-fructofuranoside, was the kinetically controlled product of methanolysis. We believe that our results represent the first chemical evidence for levans to be composed of D-fructofuranose residues.

Complete methylation of polysaccharides is often assessed from the relative proportion of the terminal units and the branching residues. However, g.l.c. did not separate the tetra-*O*-methylhexitol acetate *A* (Table I) sufficiently from materials that arise from the reagents used. Nevertheless, when the methylated fructan was subjected to another methylation-analysis procedure, the molecular proportion of the tri-*O*-methyl- to di-*O*-methyl-hexitol derivatives remained constant. It was therefore assumed that the molecular ratio of the tetra-*O*-methyl- to di-*O*-methyl-hexitol acetates was unity. It could then be calculated that the approximate numbers of terminal, non-reducing D-fructofuranosyl units and those residues linked through positions 2 and 6, and through 1, 2, and 6 in the average repeating-unit of the levan are, respectively, 1, 7, and 1.

Partial, acid hydrolysis of the fructan yielded two series (*X* and *Y*) of oligosaccharides (Table III), for each of which the plot[9] R_M versus d.p. was a straight line. Although only 11% of all D-fructofuranose residues are branching residues, a considerable proportion of the disaccharide fraction was inulobiose. It is therefore likely that the susceptibilities to acid hydrolysis of the β-(2→1)- and β-(2→6)-D-fructosidic linkages are comparable, and that the oligosaccharides of one of the above series possessed also β-(2→1)-linkages.

Similar series of oligosaccharides, with d.p. up to 8, were obtained[10] by the action of an endo-hydrolase on the levan of *Aerobacter levanicum*. The members of one series reacted with triphenyltetrazolium chloride, and it was concluded that the reducing D-fructose residue in these was linked through position 1, and that the branches through the β-(2→1)-linkage in that levan contained up to seven D-fructose residues. Triphenyltetrazolium chloride can be used to distinguish between reducing

fructose residues linked through position 1 or 6. It fails, however, to distinguish between the former and those linked through positions 1 and 6.

It was conceived that the sequence of reduction with sodium borodeuteride, methylation, hydrolysis, reduction with sodium borodeuteride, acetylation, and analysis by g.l.c.–m.s. could also be used to ascertain the position of linkage to the reducing fructose residues in the oligosaccharides obtained by partial hydrolysis with acid. The results obtained with the tri-, tetra-, and penta-saccharide fractions (it was not practicable to separate the oligosaccharides of series *X* from those of series *Y*) are shown in Table II. The tri- and penta-saccharide fractions did not give all the hexitol derivatives which might be expected. It is significant, however, that each oligosaccharide fraction gave, *inter alia*, by the above sequence of reactions, 1-*O*-acetyl-2,3,4,5,6-penta-*O*-methylhexitol-2-*d* (**2**). Therefore, each oligosaccharide fraction contained a component in which the reducing D-fructose residue was linked only through position 1 (**9**). Methylation analysis of the fructan had already shown that those D-fructofuranose residues in the fructan that are linked through position 1 are also linked through positions 2 and 6, and hence constitute branching points (**14**). The di- and oligosaccharides (with d.p. $n+1$) which are terminated by D-fructose residues linked only through position 1 (**9**) thus contain n fructose residues of a branch of the original fructan. We now conclude that the branches through the β-(2→1)-linkage in the levan of *S. salivarius* strain 51 contain up to at least four D-fructofuranose residues (**15**).

The distribution of branching residues in the levan and the type of its branched structure will be reported elsewhere.

EXPERIMENTAL

Paper chromatography. — The solvent used was ethanol–butan-1-ol–water (40:11:19). Compounds were detected with silver nitrate in acetone–ethanolic sodium hydroxide[11]. Whatman No. 1 paper was used for the analysis of mixtures, and Whatman No. 17 paper for the fractionation of larger quantities of materials.

T.l.c. — Plates coated with silica gel (Polygram Sil G) were developed with butan-1-ol–acetic acid–water (4:1:5, upper layer). Compounds were detected by spraying with sulphuric acid (5% in ethanol) and heating at 120°.

G.l.c.–mass spectrometry. — (*a*) *C.i.–m.s.* A VG Micromass 16F mass spectrometer was used with 2-methylpropane, at a pressure of 0.5 torr, as the reactant gas. The gas chromatograph (Pye 104) contained a column (3.0 m × 5.0 mm) packed with 3% of OV 225 on Chromosorb Q (80–100 mesh), and was operated with temperature programming (170→225°) at 5°.min^{-1}.

(*b*) *E.i.–m.s.* A Perkin–Elmer F11 gas chromatograph, operating at 190° and containing a glass column (4.0 m × 1.6 mm) packed with 3% of OV 225 on Chromosorb Q (80–100 mesh), was used. The carrier gas, helium, was removed from the effluent by passage through a Biemann separator. The effluent was then passed into a Hitachi RMS-4 mass spectrometer operating at 80 eV and 50-μamp target current.

Preparation of levan. — The lyophilysed *Streptococcus salivarius* strain 51 micro-organism was reactivated at 37° for 24 h in a medium containing D-glucose (0.5%), Brain Heart Infusion concentrate (Oxoid, 5 tablets/100 ml), Thioglucolate (Difco, 2.4%), and with a bottom layer of calcium carbonate (2 g/100 ml). A suspension (~5 ml) was then used to inoculate media (1.25 l) containing sucrose (8%), dipotassium hydrogen phosphate (0.2%), and Tryptone (Difco, 1%). After incubation at 37° for 40 h, the culture fluid was centrifuged at 1000 *g* for 20 min. The levan was precipitated from the clear, supernatant liquid by the addition of 2.3 volumes of ethanol, and sedimented by centrifugation at 1000 *g* for 20 min. The levan was dissolved in water and precipitated by the addition of ethanol as before. The procedure of redissolution and reprecipitation was repeated three times. The levan was freeze-dried to a white powder, and finally dried *in vacuo* over phosphoric oxide at 60°. The levan had $[\alpha]_D^{20}$ −59.3° (*c* 1.0, water), and the average yield (based on sucrose used) was 37% [Found: fructose (determined by the method of Wise *et al.*[12]), 93.8; ash, 4.7; N, 0.5%].

Degradation of levan by invertase. — Digests contained levan (0.5%, w/v) and *Candida utilis* invertase (0.4%, w/v; Sigma, grade X) in phosphate buffer (0.1M, pH 6.6), and were incubated under toluene at 37°. The reducing sugar content, as D-fructose, was determined by the method of Nelson[13], and indicated, after 350 h, that 90% of the levan had been degraded. Chromatographic analysis of the digest revealed the presence of only D-fructose.

Methylation of levan. — Levan (20 mg) was dissolved in methyl sulphoxide (4 ml) in a MacCartney flask containing a Teflon-covered magnetic follower, and fitted with a drilled cap with a rubber seal. Sodium methylsulphinylmethanide (~2M in dimethyl sulphoxide, 1 ml) was added with the aid of a syringe, and the mixture stirred for 6 h, with warming at 40° for the first hour. Methyl iodide (0.1 ml) was added and the mixture stirred for a further 18 h. Further portions of sodium methylsulphinylmethanide (as above, 1 ml) and methyl iodide (1.5 ml) were added in the manner described. The reaction mixture was then poured into water (20 ml), and the suspension was dialysed against running tap-water and then against deionised water. The methylated levan was extracted with chloroform (2 × 10 ml) and the extract evaporated to dryness. A portion of the methylated levan was subjected to another methylation procedure.

Characterisation and determination of O-*acetyl*-O-*methylhexitols obtained from methylated levan.* — A solution of the methylated levan (~10 mg) in a mixture of methanol (10 ml) and 2.5M oxalic acid (3 ml) was boiled under reflux for 18 h. The methanol was removed by distillation under reduced pressure, water (10 ml) was added, and the solution was boiled under reflux for 4 h. After neutralisation with calcium carbonate, sodium borodeuteride (10 mg; 98% D, Merck) was added, and the solution kept for 4 h. Deionisation was effected by treatment with Dowex 50W-X8 (H^+) resin, evaporation to dryness, and repeated distillation of methanol from the residue. The residue was acetylated by using acetic anhydride (1 ml) and pyridine (1 ml). After 20 min at 90°, the solution was evaporated and the residue analysed by

combined g.l.c.–m.s. Retention times (T) and peak areas were determined separately with a Pye 104 gas chromatograph, operating at 175°, containing a glass column (3.0 m × 5.0 mm) packed with 3% of OV 225 on Chromosorb Q (80–100 mesh), using nitrogen as carrier gas, and linked to a Hewlett Packard 3370B integrator. The results are listed in Table I.

Acid hydrolysis of levan. — (*a*) The levan (~10 mg) was hydrolysed with 5mM sulphuric acid (1 ml) at room temperature for 15 h. After neutralisation (with barium carbonate), paper chromatography revealed D-fructose as the sole reducing-sugar present.

(*b*) The levan (~10 mg) was hydrolysed with 5mM oxalic acid (1 ml) at 70° for 2 h. After neutralisation (with calcium carbonate), paper chromatography of the hydrolysate revealed the components shown in Table III. Chromatography of a deionised hydrolysate of a larger sample of levan (15 g) on a charcoal–Celite column (7 × 44 cm; elution with 1, 5, 10, and 20% ethanol), with purification of the fractions thus obtained by paper chromatography, gave samples of di-, tri-, tetra-, penta-, and hexa-saccharide fractions. The yields are shown in Table III.

Characterisation of oligosaccharides obtained from the levan. — The di-, tri-, tetra- and penta-saccharide fractions (see Table III, ~10 mg), separately dissolved in water (10 ml), were reduced with sodium borodeuteride (5 mg). After deionisation (as described above), each product was desiccated and then shaken with methyl iodide (0.2 ml), *N*,*N*-dimethylformamide (0.2 ml), and silver oxide (0.2 g) at room temperature for 18 h in the dark. Each mixture was filtered through glass-fibre paper, and the filtrate evaporated to dryness. Each methylated product was dissolved in a mixture of methanol (2 ml) and 0.5M oxalic acid (0.5 ml), and the solution was boiled under reflux for 18 h. Methanol was removed by evaporation under reduced pressure, water (2 ml) was added, and heating was continued for a further 3 h. Each reaction mixture was neutralised with calcium carbonate, and the products were reduced with sodium borodeuteride and acetylated as described above. The results of analysis by g.l.c.–m.s. of the *O*-acetyl-*O*-methylhexitol-*2-d* products are shown in Table II.

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