STRUCTURAL STUDIES ON SOME BRANCHED POLYSACCHARIDES

A Thesis submitted by

RAMON L. SIDEBOTHAM

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Royal Holloway College,
University of London,
Englefield Green,
Surrey.

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ABSTRACT

Water soluble and water insoluble polysaccharides were isolated from sucrose-broth cultures of L. mesenteroides strain NRRL B-1299.

The polysaccharides were identified as α-D-glucopyrans and assigned to the dextran class of polysaccharides from a consideration of their properties.

Methylation and periodate oxidations of the polysaccharides established that they have highly branched structures. The branching occurs through 1→2,6 linked glucose units.

The majority of the glucose units in each case are, however, 1→6 linked although the polysaccharides do also contain smaller percentages of 1→3 linked glucose units in intra chain positions.

From the available data a repeating unit (see page 57) is proposed for each polysaccharide.

Isolation of a series of isomaltose homologues from partial acid hydrolysis indicated that the polysaccharides contain chains of α-(1→6) linked glucose units; which probably form the molecular skeletons.

Examination of oligosaccharides from a partial acetolysis indicated that branches of the water soluble polysaccharide are more than one glucose unit long.

Enzymic degradation of the water soluble polysaccharide gave oligosaccharides whose structures indicated that some branches are at least two glucose units long and probably consist of four glucosyl units.

I. INTRODUCTION

The term dextran has been applied to members of a class of bacterial polysaccharides, composed predominantly of α-D-glucopyranose units. These units are joined principally by the α-(1→6) primary glucosidic linkage, but may in addition contain varying percentages of α-(1→4), α-(1→3) and α-(1→2) secondary glucosidic links; usually situated at branching points.

The dextrans investigated in this work were produced by the action, on sucrose, of Leuconostoc mesenteroides NRRL B-1299, a strain of chain forming coccus belonging to the bacterial family Lactobacteriaceae, tribe Streptococcae, genus Leuconostoc, species Leuconostoc mesenteroides (Betacoccus Arabinosaceous).

Historical

The species of bacteria Leuconostoc mesenteroides was first noted almost a century ago.[1]

Cienkowski (1878) investigating an outbreak of slime in sugar factory vats, isolated and studied the organisms responsible. Among them he noted a chain forming coccus, producing about itself a large gelatinous capsule, which upon innoculation into sugar solutions produced slime. Cienkowski described the strain as Ascococcus mesenteroides, believing it to be related to a recently isolated, yet little known, Ascococcus.

Van Tiegham[2] (1878) studied the organism more fully and, deciding that it was morphologically and physiologically distinct from an Ascococcus, applied it its present generic name of Leuconostoc. The name Leuconostoc resulted directly from the microscopic appearance of the growing organism,

whose colourless, slimy, globular masses resembled the well known blue-green Nostoc algae.

Many different strains of L. mesenteroides have since been isolated from such widely separated sources as slimy sugar solutions, fermenting vegetable matter and milk products. In 1944 Sugg and Hehre[3] observed a new strain of the organism which they named Leuconostoc mesenteroides strain K. This organism was deposited with the U.S. Department of Agriculture collection of typed cultures (1951) as L. mesenteroides NRRL B-1299.

Beijerlinck (1912)[4] had first identified the slime produced by this species of organism as a dextran: the name dextran having been conceived by Scheibler[5] many years earlier (1869) to describe a polymer, related to starch and dextrin, which he had isolated from sugar vat mucilages.

Recent interest in dextrans was stimulated shortly after World War II, when it was realised that they could be used as blood plasma volume expander materials.[6] An intensive survey[7,13] of dextrans produced by many strains of micro organisms was initiated by the North Regional Research Laboratories of the United States Department of Agriculture. The research programme was concerned with typing dextran producing organisms and investigating the most suitable culturing conditions for dextran production.

Methods of isolation, fractionation and purification of the dextrans were examined. The purified dextrans were each subjected to a limited structural analysis and a selected few chosen for degradational studies: principally to assess commercial usefulness. (Since 1945 some 700 patents on dextrans and their derivatives have been issued in 26 countries.) 25

One of the most significant findings of this survey has been that each dextran was in itself unique: the strain of the parent micro organism not the culturing conditions determined both the chemical and serological properties of any dextran. As a consequence dextrans are usually named after the parent micro organisms. The dextrans produced by L. mesenteroides NRRL B-1299, after fractionation, are referred to as B-1299 S dextran and B-1299 L dextran. The suffix S denotes a culture medium Soluble dextran and L indicates a culture medium insoluble or Lesser soluble dextran.

Structural studies

The general chemistry of the dextran class of polysaccharides has been reviewed periodically: notably by Evans (1946),[8] Barker (1952),[9] Neely (1960)[10] and Manners (1966),[11] whilst selected bibliographies have been published by A. Jeanes[12] (U.S. Department of Agriculture) in 1952, 1960 and 1968.

Survey of this literature has indicated that the structures of the B-1299 dextrans have so far been investigated only by means of periodate oxidations,[13] partial degradations with acetolysis reagents[14-15] and through studies of their immunochemical reactions.[16-21]

A. The first structural information about the B-1299 dextrans was obtained as a result of oxidizing the molecules with sodium meta-periodate:[13a] when the amounts of periodate reduced and formic acid liberated in the reactions were used to calculate an approximate distribution of the glucosidic links in each polysaccharide fraction.

This analysis revealed little difference between the two dextrans:[13b]

in B-1299 S dextran 50% of the units were linked 1→6 and 50% could have been either 1→2 or 1→4 linked, whilst B-1299 L dextran contained 58% of 1→6 linked units, 38% of units which could have been either 1→2 or 1→4 linked and 6% of units which may have been 1→3 linked.

This method of analysis had been conceived as a means of obtaining structural data rapidly from dextrans produced by 96 strains of bacteria,[13b] and as such had limitations of accuracy. The method, together with the calculations involved, is discussed more fully on page 29–32.

Senti[22] et al. later modified these figures by studying the optical rotations of the dextrans in cuprammonium solution.

Reeves[23] had noted that the optical rotations (at 4358Å) of simple glucosides were greatly decreased if the molecules were complexed with cuprammonium at the 2,3 hydroxyl position. Complexing at the 3,4 hydroxyl position, however, caused an increase of rotation; if both these positions were complexed the rotational shift was negligible.

The rotational shift of a 1→6 linked glucose unit was calculated from experiments on cuprammonium complexes of the homologous series of α-(1→6) linked methyl glucosides of degree of polymerisation 1 to 11.[22] Using this value in conjunction with the earlier observations (of Reeves) an equation was derived which could distinguish between the 1→4 and 1→2 linkages in dextrans. Application of this analysis to the B-1299 dextrans indicated that each contained a high percentage of 1→2 links. The modified periodate figures were: B-1299 S dextran 50% 1→6 links, 38% 1→2 links, 12% 1→4 links; B-1299 L dextran 58% 1→6 links, 34% 1→2 links, 2% 1→4 links and 6% of links which could have been 1→3.

B. Chemical evidence to confirm the presence of the α-(1→2) linkage in the B-1299 dextrans came initially from Japanese workers.[14] They carried out a partial acetolysis on the unfractionated dextran and were able to isolate kojibiose (2-O-α-D glucopyranosyl-D-glucopyranose) in good yield. Smaller quantities of nigerose (3-O-α-D-glucopyranosyl-D-glucopyranose) were also found, but maltose was not observed.

Hehre[15] also applied this method of degradation to a series of dextrans, including B-1299 S dextran from which he identified, by paper chromatography, components with migrations identical to those of kojibiose and nigerose but not maltose.

This method of degradation has proved particularly valuable for identifying the secondary glucosidic links in dextran molecules. The attaching species is believed to be a solvated acetylium ion ($CH_3.CO^{\oplus}$) which, for possibly steric reasons, breaks primary glucosidic links faster than secondary ones. The method is discussed further on page 19.

C. The immunochemical studies on the B-1299 dextrans have been particularly extensive and stimulated much of the earlier interest in these dextrans. It was found that dextrans were able to participate in a type of precipitin reaction in which the dextran, acting as a soluble antigen, was able to react or cross react with soluble antibodies to produce a measurable precipitate.[24]

Neill and Hehre,[16] examining sucrose-broth supernatants, noted that Leuconostoc polysaccharides were serologically reactive not only with Leuconostoc antiserums but also by cross reaction with rabbit antiserums to types II and XX (and sometimes type XII) pneumococci. This led them

to divide the Leuconostoc bacteria into two serologically different groups, A and B. Serotype A Leuconostocs showed cross reaction in high dilution with types II, XX and XII antiserums, Serotype B Leuconostocs cross reacted only with types II and XX antiserums. Among the small number of Serotype A Leuconostocs was L. mesenteroides strain K (NRRL B-1299).

A structural basis for the unusual reactivity of the dextrans from NRRL B-1299 strain has been proposed by Kabat in a series of experiments with antidextran antiserums and cross reacting anti pneumococcal serums.[17-21]

Kabat[17] found that purified dextrans were antigenic in man: injection of small quantities of dextran stimulated production of antidextran antibodies (precipitins) and using refinements of the Landsteiner hapten inhibition reaction,[24] it became possible to elucidate the structural requirement of the combining site of the antidextran molecule.

Most dextrans tested produced antibodies with a specificity directed towards the α-(1$\rightarrow$6) glucosidic linkage;[17] as the immune reaction was strongly inhibited by the isomaltodextrins, with isomaltopentaose and isomaltohexaose (on a molar basis) being the best inhibitors. However, in one individual injected with B-1299 S dextran, a second antibody population was produced.[18] It was noted that with the isomaltose series of oligosaccharides inhibition was not great yet with oligosaccharides of the maltose series it was much improved; maltotriose and maltotetraose were notably better inhibitors than maltose itself.

These findings would establish the requirement for inhibition of the non 1$\rightarrow$6 specific antibody combining group as involving two α-glucosidic linkages and a minimum of two and a maximum of three pyranose rings. The

inference being that B-1299 S dextran could well contain short terminal chains of two or three α-(1→4) linked glucose units, and that all the α-(1→4) linkages do not occur at branching points on a primary chain of α-(1→6) linkages, as had already been noted for B-512 dextran.[51]

Later evidence[22] from cuprammonium rotational analysis, that many of the α-(1→4)-like linkages of the B-1299 dextrans were probably 1→2 linkages, and the availability of kojibiose and nigerose, allowed a reassessment of these findings.

Re-immunisation[19] of human subjects with B-1299 S dextran again provided antisera with non 1→6 specificity. The capacity of other dextrans to precipitate this type of antidextran could be correlated with the proportion of 1→2 linkages present, when the linkage values were calculated from cuprammonium rotational data.[22]

Among dextrans thus tested B-1299 L dextran showed one of the highest reactivities. Moreover, quantitative inhibition findings showed kojibiose to be the best inhibitor of precipitation in these reactions; maltose, nigerose and isomaltose showed significantly lower inhibitory capacity, suggesting an antibody specificity directed against glucosyl residues in α-(1→2) linkage. Earlier studies[17] had shown that sugars at non-reducing ends of chains contributed most to antibody reactivities, with each successive sugar contributing a smaller increment, and so it would seem probable that many chains in the B-1299 dextrans are terminated at non-reducing ends by kojibiosyl (2-O-α-D-glucopyranosyl-D-glucopyranosyl) units.

It has now been established that the cell wall polysaccharides of

the pneumococci, whose antiserums cross react with dextrans, contain α-D-glucose residues.[26] It is also certain that, for cross reactions to occur, the structural arrangements of the glucose units in both dextran and pneumococcus must be similar.

The cross reactions of types II, IX, XII and XX antipneumococcal sera with a number of dextrans, has been investigated.[20-21] Types II and XX antisera show the greatest reactivity with dextrans having a high percentage of 1→6 linkages (periodate data).[13] Inhibition of the cross reaction is greatest with isomaltodextrins.

The capacity of each dextran tested to precipitate these antiserums was found to be proportional to the content of 1→6 linkages.[20] B-1299 L dextran showed a weak cross reaction yet fell into the expected range. B-1299 S dextran was unusual: it had a greater capacity to cross react than could be expected from its content of 1→6 linkages. A possible explanation may be sought in the existence of short chains of non 1→6 linked units, an arrangement which, if present, would reduce the numbers of such units capable of interrupting sequences of 1→6 linkages and so give rise to longer sequences of terminal 1→6 linkages and result in the observed greater cross reactivity.

Antipneumococcal type XII cross reactivity was directed against the α-(1→2) linkage.[21] Dextrans with a high content of 1→2 linkages (as determined by cuprammonium rotational experiments)[22] showed the greatest capacity to precipitate antibody; whilst kojibiose was the best oligosaccharide inhibitor. Both the B-1299 dextrans had similar cross reactivities, and the highest of the dextrans tested, which were strongly inhibited by kojibiose. It would appear from these experiments that a

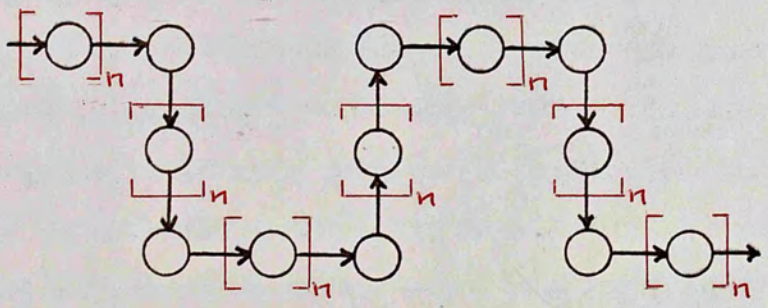
large number of chains in these dextrans are terminated by α-(1→2) linked glucose units.

Results obtained with type IX antipneumococcal serum proved more difficult to interpret.[21] The greatest cross reactivity occurred with a dextran having a high percentage of α-(1→3) linkages[42] and this cross reaction was greatly inhibited by nigerose. This would indicate a specificity directed towards the α-(1→3) linked glucose unit. However, the B-1299 dextrans had a far greater capacity to precipitate this antiserum than could be anticipated from the content of 1→3 linkages in each, and the reaction was inhibited equally well by nigerose, kojibiose and maltose. Also, a trisaccharide, 3-O-α-D-glucopyranosyl-4-Oα-D-glucopyranosyl-D-glucopyranose, on a molar basis, was three times as effective an inhibitor while the isomeric trisaccharide, 4-O-α-D-glucopyranosyl-3-Oα-D-glucopyranosyl-D-glucopyranose, proved to be even better.

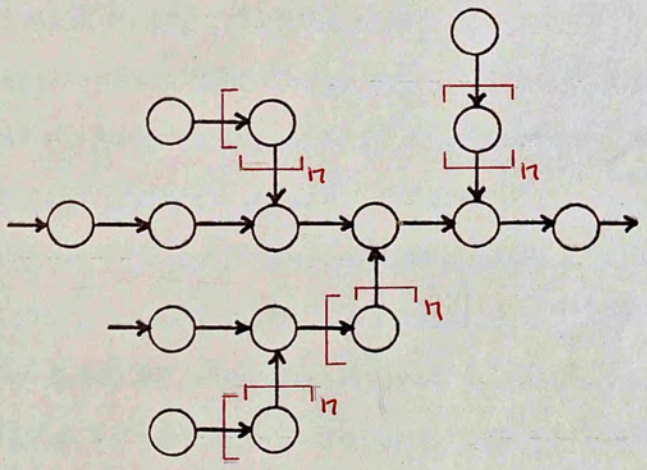
These experiments indicate the presence of two antibody populations: one directed against the 4-O-α-D-glucopyranosyl-3-O-α-D-glucopyranosyl arrangement of terminal units, and the other directed against terminal units linked α-(1→3) to the rest of the chain. If this is true then it could well indicate that certain chains in the B-1299 dextrans are terminated by α-(1→3) linked glucose units, whilst possibly others are terminated by an α-(1→4) linked glucose unit which is adjacent to a α-(1→3) linked unit.

This summarises the more important structural work so far reported for the B-1299 dextrans, from which it may be concluded that:

FIGURE I.1. —— POSSIBLE STRUCTURES FOR THE NRRL B-1299 POLYSACCHARIDES.



A



B

→ 1→6 LINK

⇅ NON 1→6 LINKS.

○ GLUCOSE UNIT.

n NUMBER OF UNITS, WHICH MAY BE 1, 2, 3 --- ETC.

1. the two polysaccharides contain α-(1→2), α-(1→3), α-(1→6) and possibly α-(1→4) linked glucose units.
2. the ratio of 1→6 linked units to secondary glucosidically linked units is almost unity.
3. sequences of secondary glucosidically linked units may be present at the non-reducing ends of the chains.
4. the polymers may or may not have a branching structure.

The possible structural arrangements encompassed by these observations are indicated in fig. 1. The typical branched dextran is represented by the structure shown in fig. 1B, which has a backbone of 1→6 linked glucose residues to which side chains are attached through the secondary, i.e. 1→2, 1→3 or 1→4, linked units. Many of these branches may consist of a single glucosyl unit.[27]

The unbranched (linear) structure shown in fig. 1A may be regarded as of a non dextran type, but can be compared with the alternating sequences of 1→3 and 1→4 linked α-D-glucose units seen in Nigeran.[28]

In the following sections of the thesis further chemical evidence will be presented with the intention of distinguishing more fully between these extremes of structure.

II. SOME ASPECTS OF THE STRUCTURES OF TWO DEXTRANS PRODUCED IN CULTURES OF Leuconostoc mesenteroides NRRL B-1299

A. Preparation and some physical properties of the B-1299 polysaccharides

The Leuconostoc mesenteroides NRRL B-1299 micro organism (donated by the U.S. Department of Agriculture) was preserved as described in expt. 1, and was inoculated into a sucrose-broth medium to synthesise the B-1299 polysaccharide when required (expt. 2).

The polysaccharide produced could be separated into culture medium soluble [S] and culture medium insoluble [L] fractions, which were purified by repeated precipitation from ethanolic solution, deproteinated and freeze dried to white powders containing less than 1% nitrogen (expt. 3).

Some physical characteristics of the two polysaccharide fractions are shown in Table II.1.

Table II.1. Some physical properties of B-1299 polysaccharides

Polysaccharide	Ash content (%)	$[\alpha]_D^{20}$, C=1.0 (N-NaOH)	Solubility	
			Water	N-NaOH
Fraction [S]	6.5	+ 211°	Soluble	Soluble
Fraction [L]	3.3	+ 220°	Insoluble	Soluble

Optical rotations were determined in N-NaOH (expt. 5) and ash contents by complete combustion (expt. 4).

Absorption peaks located in the infra red spectrum of each polysaccharide are shown in Table II.2.

The spectra resembled closely those reported for the primarily

α-(1→6) linked dextran polysaccharides.[29]

Table II.2. Absorption peaks located in the infra red spectra of B-1299 polysaccharides

Absorption peak (cm^{-1})	Origin[30]
764	α-(1→6) glucosidic linkage
847	α-D-glucopyranose ring
918	α-(1→6) linked glucose unit

The optical rotations shown in Table II.1. provided the first evidence - later substantiated by periodate oxidation, acid hydrolysis and acetolysis experiments - that the culture soluble [S] and insoluble [L] polysaccharides isolated in expt. 3 were identical to the B-1299S ($[\alpha]_D^{20}$ + 210°) and B-1299L ($[\alpha]_D^{20}$ + 216°) dextrans reported by Jeanes and co-workers.[13b]

In future discussions, therefore, the culture soluble [S] polysaccharide will be referred to as B-1299S dextran and the culture insoluble [L] polysaccharide as B-1299L dextran.

In certain respects this was a significant finding for it provided evidence that the B-1299S and B-1299L dextrans were (structurally) distinct polysaccharides synthesised by the same micro organism rather than fractions of the same polysaccharide: this point will, however, be discussed more fully later.

B. Complete acid hydrolysis of the B-1299 polysaccharides to determine their monosaccharide constituents

It has been reported that certain strains of L. mesenteroides, when cultured on a sucrose medium produce varying amounts of fructan in addition to dextran.[31,32,33]

Although the NRRL B-1299 micro organism appears to synthesise only dextrans (glucans) the possibility remained that the cultures used in the present work may have elaborated small amounts of fructan in addition to dextrans.

The monosaccharide constituents of each polysaccharide were, therefore, investigated after complete acid hydrolysis of the molecules. As the optimum conditions for cleavage of fructosidic[34] and glucosidic links[35] are not identical, two hydrolysis experiments were carried out.

In expt. 7(a), a small quantity of each dextran was mildly hydrolysed under conditions (0.2N sulphuric acid, 1 hour, 70°C) designed to completely cleave fructosidic links with the minimum degradation of any fructose liberated. After neutralisation, the hydrolysates were freed from non-hydrolysed polysaccharide, concentrated and spotted on paper chromatograms (with fructose and glucose as reference compounds) which were eluted with solvent (f).

The chromatograms were developed with spray reagents (a) and (d) (specific for ketohexoses)[34] which showed that fructose was absent, although a trace of a component migrating as glucose was present in each hydrolysate.

In expt. 7(b) a small quantity of each dextran was hydrolysed under conditions (2N sulphuric acid for 8 hours at 100°C) which would completely

cleave glucosidic links with the minimum degradation of any glucose liberated - although any fructose present would have been extensively degraded.

The hydrolysates were neutralised before being concentrated and spotted on paper chromatograms with glucose as the reference compound. The chromatograms were eluted with solvent (f), and then developed with spray reagent (a), which revealed that each dextran gave a single hydrolysis product whose migration rate was identical to that of glucose.

Each component from the hydrolyses had a mobility in borate electrophoresis identical to that of glucose and, after reduction, a mobility in molybdate electrophoresis consistent with that of glucitol.

The characterisation of each component was completed by converting it into the crystalline sugar (expt. 7(c)) and the acetate derivative (expt. 7(d)), which had physical constants comparable to those of D-glucose and D-glucose β-penta acetate respectively (see Table II.3.).

Table II.3. Properties of the monosaccharides liberated from B-1299 polysaccharides

Dextran	Crystalline sugar				Acetate	
	Mpt. °C	Mixed Mpt.	$[\alpha]_D$ (equilibrium)	Yield* (mg)	Mpt. °C	Mixed Mpt.
B-1299S	139-146	139-143	+52°	762	130-131	129-131
B-1299L	138-144	137-141	+51°	778	129-131	129-131

* From 1.5 gm. dextran.

Anhydrous α-D glucopyranose has a reported[36] melting point of 145-146°C, and an $[\alpha]_D^{20}$ + 112° → +52.5° (equilibrium) D-glucose β-penta acetate has a

reported[37] melting point of 133°C.

The glucose content of each polysaccharide was determined, after complete hydrolysis, with the enzyme reagent glucose oxidase (expt. 7(e)).

The results, uncorrected for ash content, were as follows:

B-1299 S dextran 95.3%

B-1299 L dextran 94.1%

It was, therefore, concluded that each of the B-1299 polysaccharides isolated in expt. 3 was a pure dextran (glucan).

C. Partial acid hydrolysis of the B-1299 polysaccharides: to establish the presence of 1→6 linked glucose residues

Whilst the basic monosaccharide units may be identified by complete acid hydrolysis of a polysaccharide; valuable information may be obtained by examining (oligosaccharide) products from the partially degraded molecule: characterisation of which will indicate how the monosaccharide units are assembled into the parent polysaccharide.

Extensive use has been made of acid hydrolysis as a means of partially degrading polysaccharides.[38]

Experiments with disaccharides have established the order of stability of α-glucosidic links to acid hydrolysis[39,40] - the α-(1→6) linkage being three or four times more resistant than a secondary α-linkage (see page105).

Hence, partial acid hydrolysis of an α-glucan would appear to provide one of the best means of obtaining fragments containing the α-(1→6) linkage, if present.

When this technique was applied to dextrans[41-45] the products consisted almost exclusively of α-(1→6) linked oligosaccharides

(isomaltose homologues) which suggested that this class of polysaccharide contained as a general feature a backbone of α-(1→6) linked glucose units.

To determine whether B-1299L and B-1299S polysaccharides - which had been provisionally classed as dextrans by earlier workers on the basis of serological reactivities,[16,21] and periodate oxidation data[13b] - had the typical dextran backbone, each was partially hydrolysed with dilute acid (expt. 8(a)).

After neutralisation, the hydrolysates were concentrated and spotted on paper chromatograms (with glucose and isomaltose homologues as reference compounds) which were then eluted with solvents (a) and (f) respectively.

The fractionated components 2 to 7 (shown in Table II.4.) had migration rates identical to the series of isomaltose homologues, and gave in addition a green stain with spray reagent (c), characteristic of glucose oligosaccharides with 6-o-substituted reducing units.[46]

When the R_M value (see page 57) of each component was plotted against a degree of polymerisation provisionally assigned on the basis of its chromatographic migration rate, a straight line (Fig.II.1.) characteristic of a homologous series[47] of oligosaccharides was obtained.

FIGURE II.1. —— OLIGOSACCHARIDES FROM THE PARTIALLY HYDROLYSED B-1299 DEXTRANS.

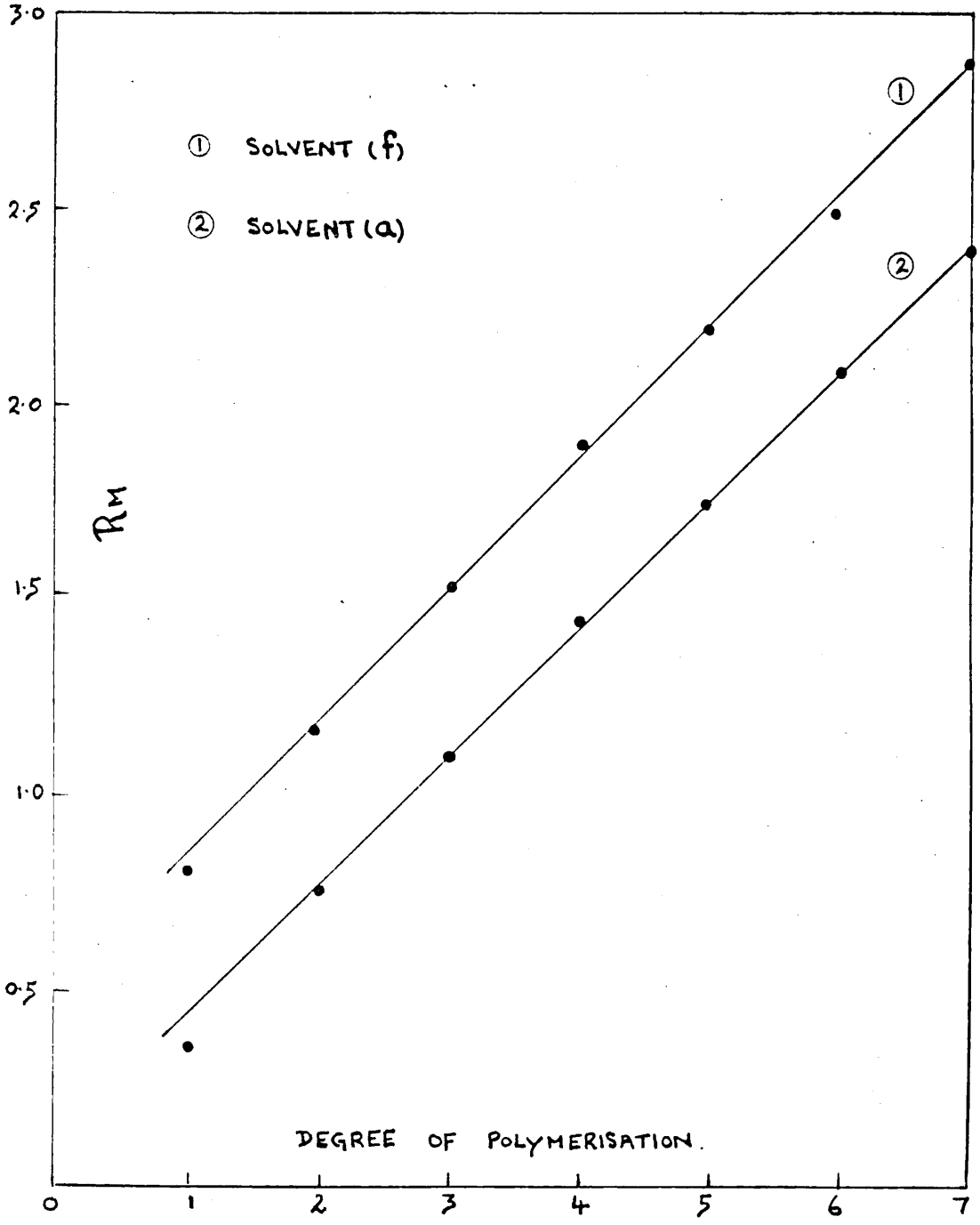


Table II.4. Chromatographic properties of oligosaccharides produced by hydrolysis of B-1299 polysaccharides

Component	Solvent (f)		Solvent (a)		Staining Intensity spray (a)	Possible identity
	R_G	R_M	R_G	R_M		
1	1.00	0.78	1.00	0.35	10+	Glucose
2	0.46	1.15	0.50	0.74	3+	Isomaltose
3	0.20	1.54	0.24	1.09	2+	Isomaltotriose
4	0.09	1.88	0.12	1.43	+	Isomaltotetraose
5	0.04	2.19	0.06	1.75	+	Isomaltopentaose
6	0.02	2.49	0.03	2.09	+	Isomaltohexaose
7	0.01	2.88	0.01	2.37	+	Isomaltoheptaose

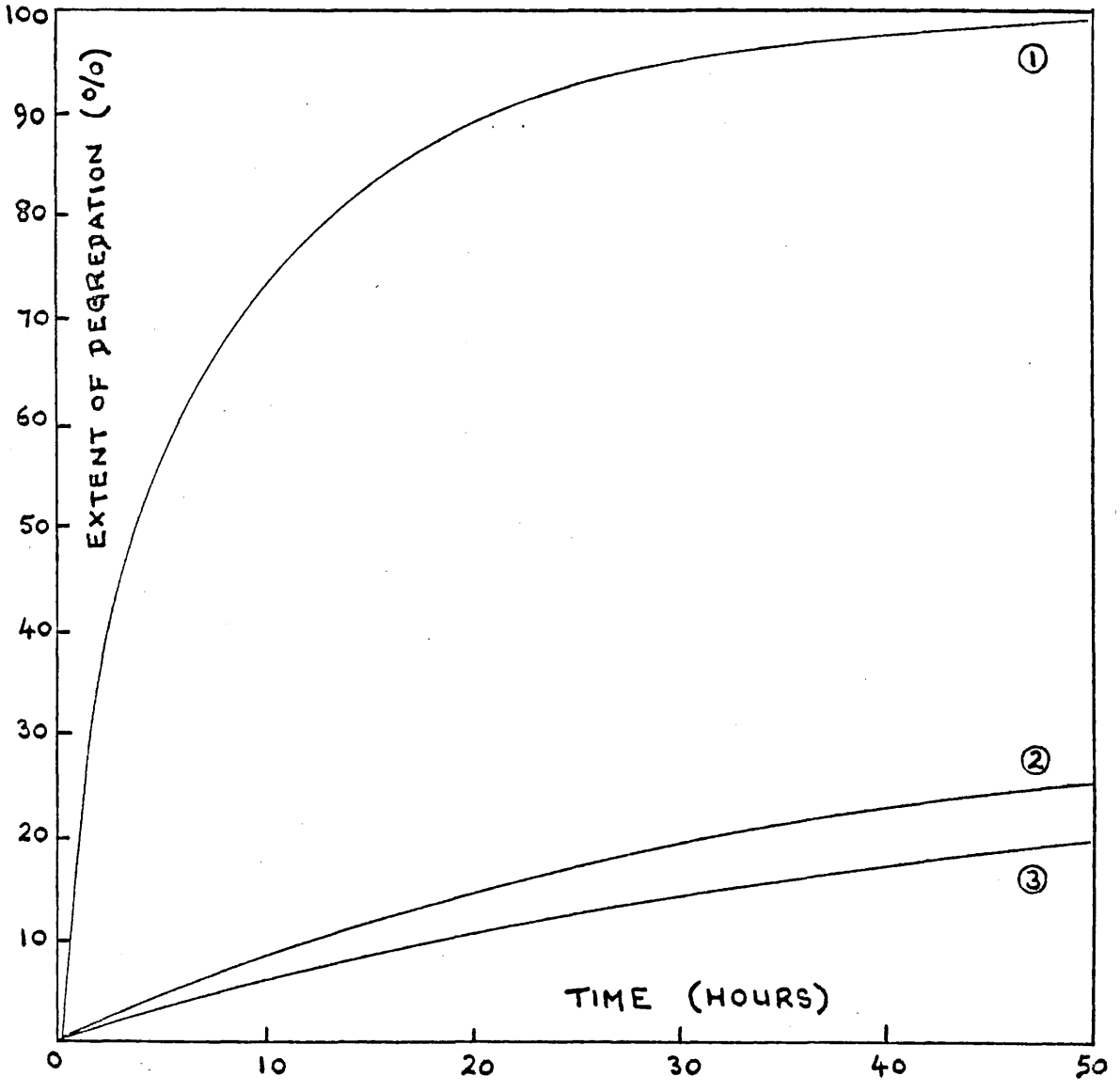
These preliminary results, therefore, indicate that both B-1299S and B-1299L polysaccharides have yielded a series of isomaltose homologues: this could point to the possibility that each has a backbone, typical of dextrans, consisting of α-(1→6) linked glucose units.

A more complete characterisation of the partial acid hydrolysis products from B-1299S dextran appears in section IIIB.

D. Acetolysis of B-1299 dextrans: to establish the presence of non-1→6 linked glucose residues

Matsuda and co-workers[40] have demonstrated through experiments with α-glucose disaccharides that the order of stability of α-glucosidic links to acetolysis reagents is 1→2 > 1→3 > 1→4 ≫ 1→6 (see Fig.II.2.) which is the reverse of that found in partial acid hydrolysis (see Fig.III.$_{10}$).

FIGURE II.2. —— THE ACETOLYSIS VELOCITIES OF THREE α-GLUCOBIOSES.



① ISOMALTOSE.
② NIGEROSE.
③ KOJIBIOSE.

The increased stability to acetolysis of the secondary α-glucosidic links has been attributed to the greater steric hindrance to the attacking species - the solvated acetylium ion - at the glucosidic link formed through the less 'open' secondary hydroxyl positions compared with the primary hydroxyl position.[48]

These results show that, as a method of degrading α-glucosidic links, acetolysis is complementary to acid hydrolysis, and explain why partial acetolyses have enabled various workers to isolate fragments from dextrans containing secondary glucosidic linkages in greater yields than had been possible through partial acid hydrolysis experiments.

Through acetolysis it has been established that dextrans may contain many glucose units linked α-(1→2),[14,49] (1→3)[14,42] and (1→4)[43] in addition to those linked α-(1→6).

To investigate specifically any secondary links which may be present in the B-1299 dextrans, small amounts of each polysaccharide were partially degraded with acetolysis reagents as described in expt. 9(a).

The acetates formed were deacetylated and the rates of migration of the free sugars were compared with those of standard compounds on paper chromatograms eluted with solvent (a). The chromatograms were developed with spray reagents (a) and (c) (which gives a characteristic colour with each α-glucose disaccharide:[46] nigerose [α-(1→3), grey], maltose [α-(1→4), blue], kojibiose [α-(1→2), brown] and isomaltose [α-(1→6), green]).

In addition each fragment separated in solvent (a) was eluted from the paper chromatogram and subjected to electrophoresis in borate and

(after reduction) in molybdate electrolytes.

The results of these experiments are summarized in Table II.5. which indicated that each dextran gave (on acetolysis) a similar distribution of components with paper chromatographic and electrophoretic properties identical to those of glucose, nigerose, kojibiose, isomaltose and isomaltotriose. Some additional unidentified components were also observed but not a fragment corresponding to maltose [R_G (solvent (a)) 0.67, M_G (borate) 0.32, M_S (molybdate, on reduction) 0.46].

Table II.5. Chromatographic properties of the oligosaccharides produced by acetolysis of the B-1299 dextrans

Component	R_G solvent (a)	M_G borate	M_S* molybdate	Staining colour spray (c)	Staining intensity spray (a)	Possible identity
1	1.00	1.00	1.00	Green	10+	Glucose
2	0.48	0.70	0.70	Green	+	Isomaltose
3	0.26	0.60	0.60	Green	+	Isomaltotriose
9	0.75	0.71	0.00	Grey?	+	Nigerose
8	0.62	0.32	0.70	Brown	5+	Kojibiose
10	0.13	0.27	-	-	+	?
11	0.04	0.25	-	-	+	?

* After reduction with sodium borohydride.

These preliminary results indicate that each dextran contains in addition to the 1→6 link, a high percentage of 1→2 links with a smaller proportion of 1→3 links. Neither seemed to contain the 1→4 link.

A more complete examination of the fragments from acetolysis of B-1299S dextran appears in section IIIC.

E. An attempt to determine a ratio of 1→6 to non-1→6 linked glucose units by application of Nuclear Magnetic Resonance (NMR) spectroscopy to B-1299 dextrans

Paseka and Cragg[50] have examined the NMR spectra of deuterated dextrans produced by NRRL B-512 and B-742 micro organisms, and report that NMR spectroscopy may provide a particularly rapid means of determining the degree of branching, or more correctly the ratio of glucose units linked 1→6 to those linked non-1→6 in polysaccharides of this type.

Because accummulated chemical evidence[13b,14,22] suggested that the B-1299 dextrans contain considerable numbers of non-1→6 linked glucose units (see pages 3-9) successful application of the NMR spectroscopic technique to these dextrans would be especially valuable. Consequently, the method was examined in more detail and an attempt was made to extend it to the B-1299 dextrans.

In their initial experiments Paseka and Cragg[50] had used deuterated isomaltotriose as a model compound to aid peak assignments, because of its close structural similarity to B-512 dextran before investigating the spectra of the larger dextran molecules.

The spectrum which was obtained from the principally α-(1→6) linked B-512 dextran[51] is reproduced in Fig.II.3., and the assignments of the spectral peaks are shown in Table II.6.

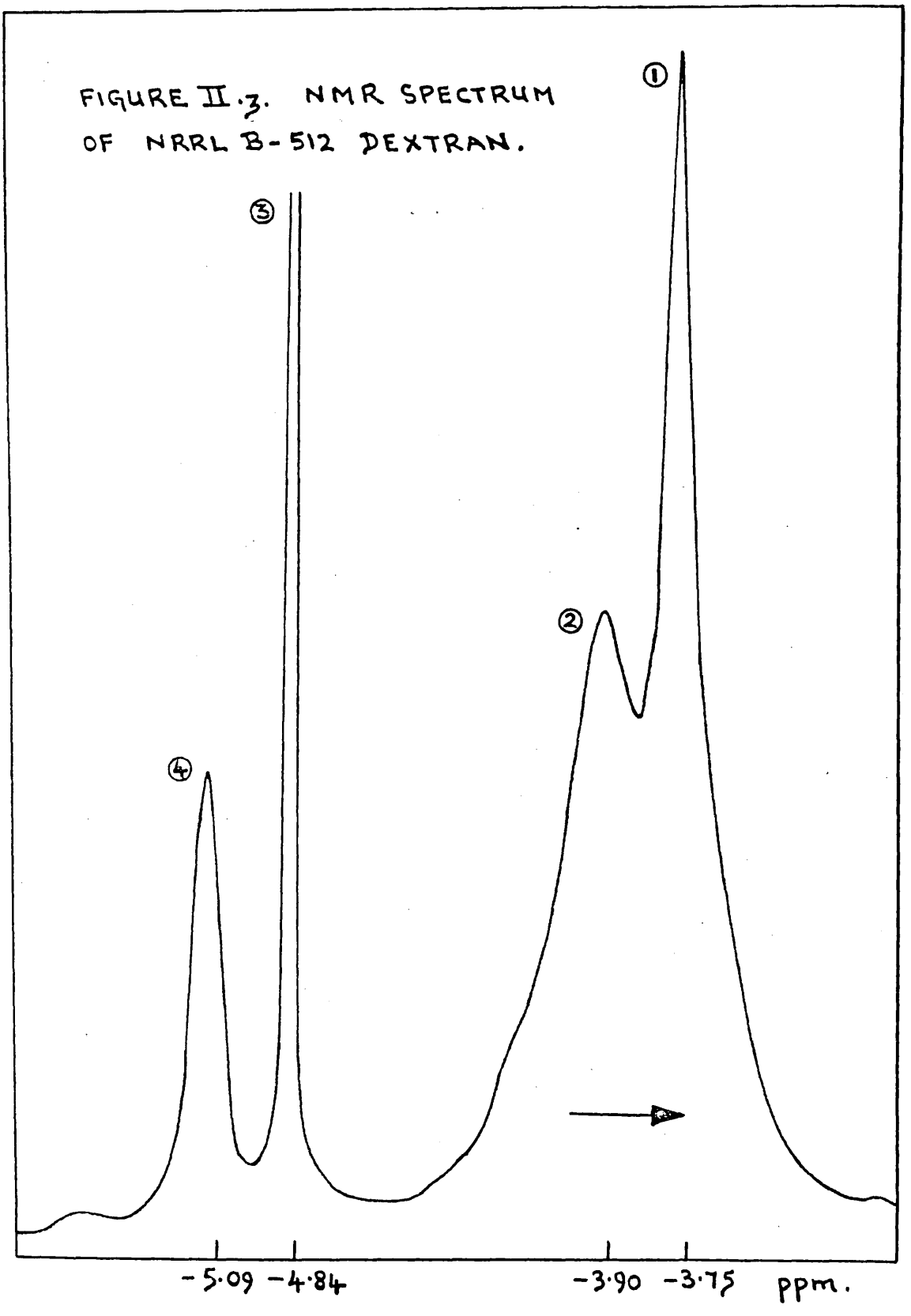


FIGURE II.3. NMR SPECTRUM OF NRRL B-512 DEXTRAN.

Table II.6. Assignments of absorption peaks in the NMR spectrum of deuterated B-512 dextran

Peak	Chemical shift (ppm)*	Protons responsible
1	-3.75	on C_5 and C_6 carbon atoms
2	-3.95	on C_2, C_3 and C_4 carbon atoms
3	-4.84	DOH
4	-5.09	on C_1 carbon atoms associated with the α-(1→6) glucosidic linkage

* relative to a TMS external standard, all shifts are to the left of the standard and are given a negative sign

When, however, the spectrum of the 1→3 branched[52] B-742 dextran was examined an additional peak 5 at -5.40 ppm was observed. This peak could only have been due to the presence of the non-1→6 linked units in the dextran, and therefore the proton responsible must have been either the one on the C_1 or C_3 carbon atoms involved in the α-(1→3) linkage.

Because peak 5 could only have arisen at the expense of peak 4, determination of the ratio of the sum of the areas under these peaks was a measurement of the ratio of the signal strengths from protons associated with the 1→3 and 1→6 links, and also represented the ratio of glucose units linked 1→3 (i.e. non-1→6) to 1→6 in this dextran. (As all the non-1→6 links formed branching points[52] this also represented the ratio of branched to unbranched units.)

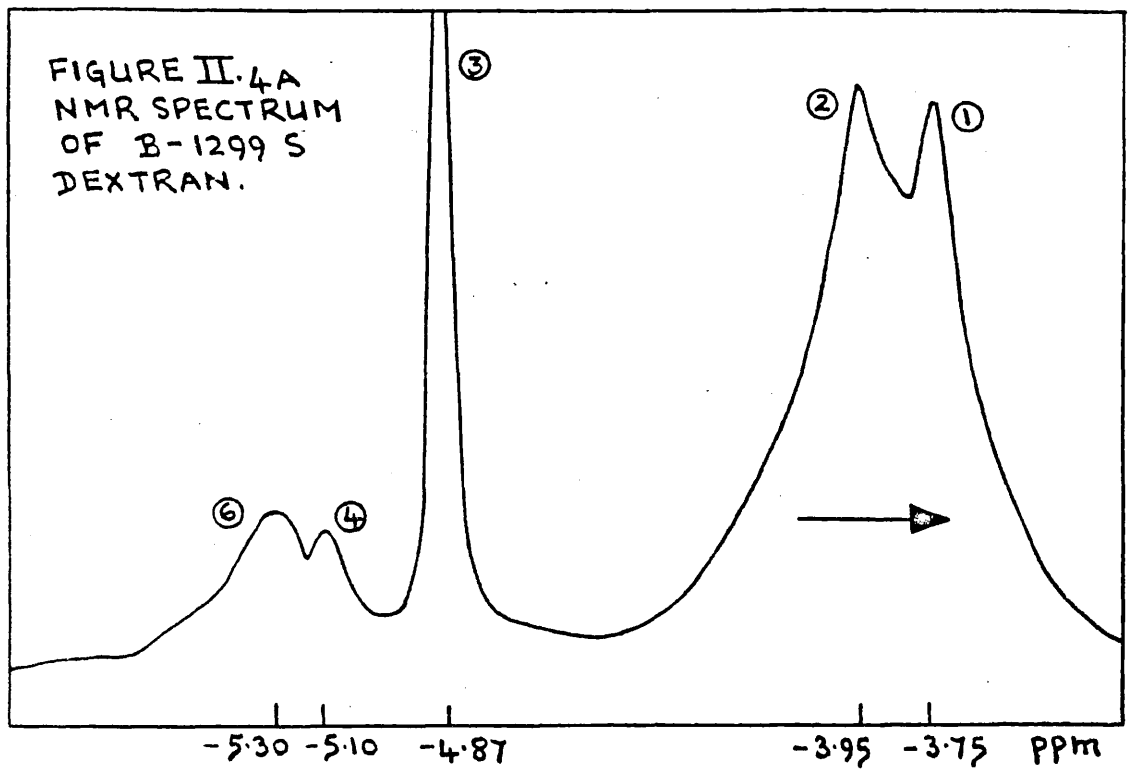


FIGURE II.4A
NMR SPECTRUM
OF B-1299 S
DEXTRAN.

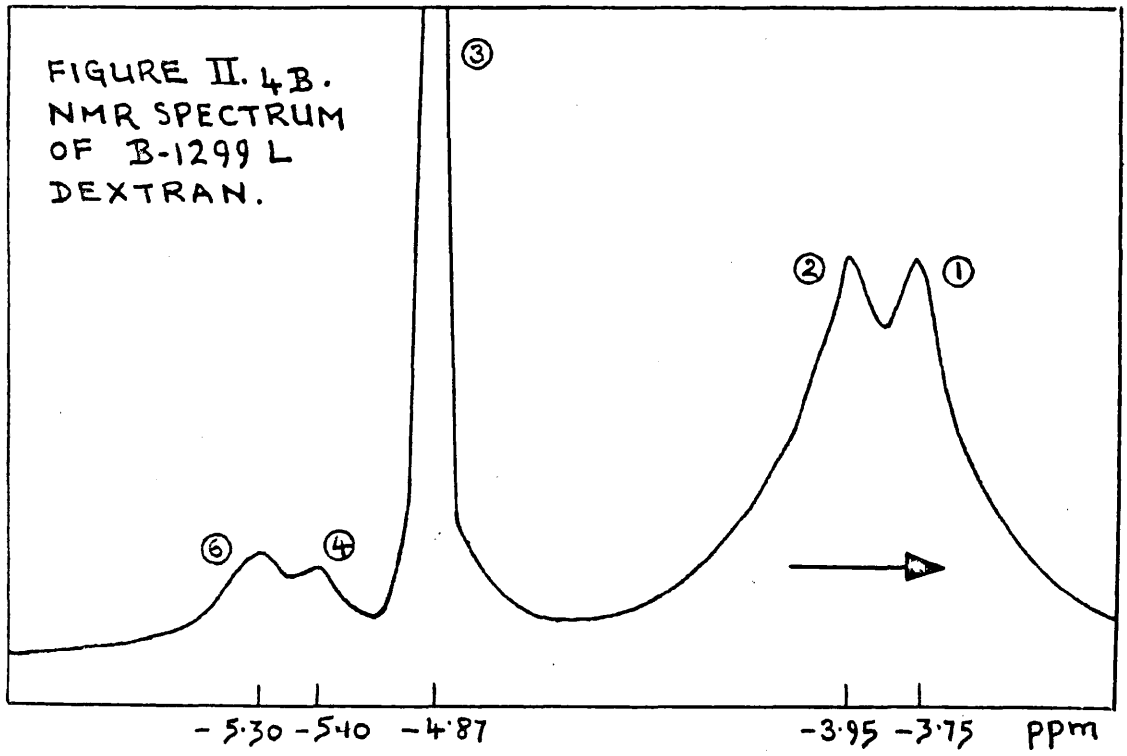


FIGURE II.4B.
NMR SPECTRUM
OF B-1299 L
DEXTRAN.

When this same ratio was calculated from methylation data[52] excellent agreement was obtained between the two values, which suggested that the NMR spectroscopic technique might well be applied to other dextrans containing non-1→6 links.

Samples of deuterated B-1299 dextrans were, therefore, prepared by the method outlined in experiment 10, and their NMR spectra were recorded on a Varian HA 60 IL instrument using a sweep width of 500 cps. The main features of these spectra are illustrated in Fig. II.4. A and B: each contained a peak 4 at -5.10 ppm (due to protons on carbon atom C_1 associated with the 1→6 link), and a peak 6 (probably due to protons on either the C_1 or C_2 carbon atom associated with the 1→2 link) at -5.30 ppm.

Unfortunately, signal 6 lay further upfield than had signal 5 in the spectrum of B-742 dextran. This resulted in overlapping of peaks 4 and 6 and made accurate determination of the areas under these peaks, and hence precise measurement of the ratio of 1→6 to non-1→6 links, impossible. Inspection of the spectra did, however, suggest that 1→6 and non-1→6 linked units might be present in equal proportions in each B-1299 polysaccharide.

A further difficulty encountered, in recording the B-1299 dextran spectra, was that of poor peak resolution, which might well have been due to the low solubility of the freeze dried polysaccharides in the deuterium oxide solvent.

In conclusion then it would seem that the NMR spectroscopic technique presented here cannot be directly applied to dextrans containing

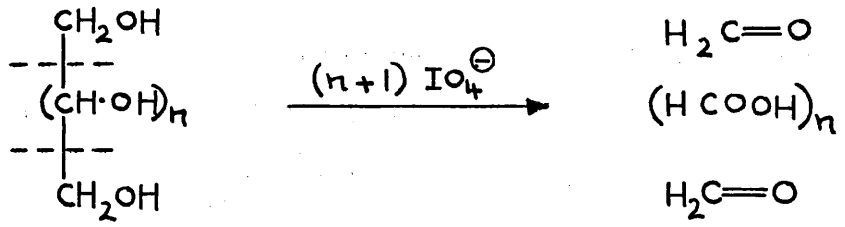
1→2 linked glucose units, or which may be particularly insoluble in water (or deuterium oxide).

F. Periodate oxidation experiments: to establish the type and distribution of links in B-1299 dextrans

The Malaprade reaction[53] - in which 1,2 diols are oxidatively cleaved by periodate ion - has been particularly applied to determining the structures of carbohydrates because of their polyhydric nature.

The oxidation of a typical (polyhydroxylic) system is shown in Fig. II.5.

Fig.II.5.



--- Point of cleavage

When a dextran was oxidized with sodium meta-periodate,[13] it was found that - provided each oxidation took place under identical conditions - the amounts of periodate reduced and formic acid released, per anhydro glucose unit, in a given time were characteristically constant for that particular molecule. Furthermore, these experimental figures could be used to calculate an approximate distribution of linkages within the

1→2 linked glucose units, or which may be particularly insoluble in water (or deuterium oxide).

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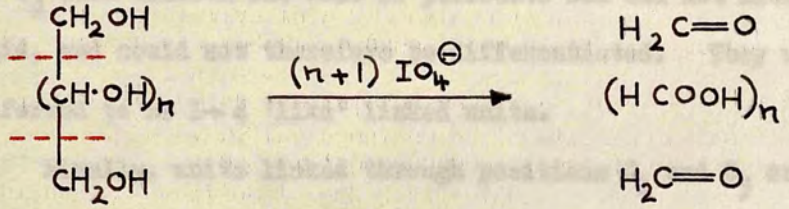

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dextran molecule.[13b]

The scheme of analysis, proposed by Jeanes,[13] however allowed only three groups of anhydro glucose units to be recognised, for which representative structures are shown in Fig. II.6.

Glucopyranose units linked through positions C_1 or C_1 and C_6 - which were referred to as 1→6 linked units - could be determined specifically, as each would reduce two moles of periodate in liberating one mole of formic acid, but glucopyranose units linked in other ways could be placed only in certain categories.

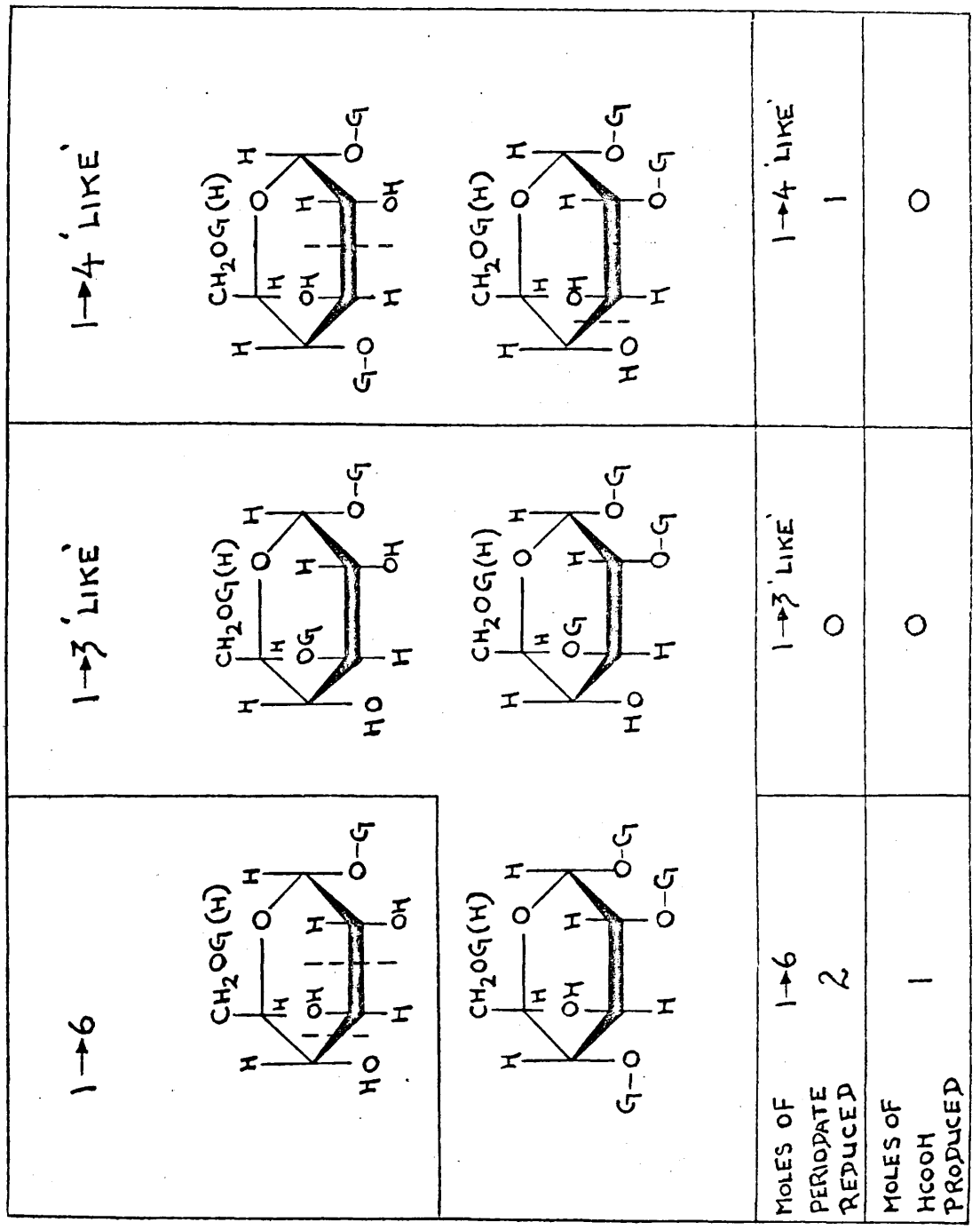
Those units linked through positions C_1 and C_2 or C_4 or C_1, C_6, C_2 or C_4 each reduced one mole of periodate and did not liberate formic acid, and could not therefore be differentiated. They were collectively referred to as 1→4 'like' linked units.

Finally, units linked through positions C_1 and C_3 or C_1, C_6 and C_3 could not be distinguished from certain units multiply linked through C_1, (C_6), C_4 and C_3 or C_1, (C_6), C_4 and C_2. These units were together referred to as 1→3 'like' linked units and remained unoxidized by periodate ion.

For polymers as large as native dextrans the effect of the reducing end unit can be neglected. Thus, the only units which liberate formic acid are the 1→6 linked units. The percentage of 1→6 linked units in the dextran will be equal to F.A x 100, when F.A. represents the number of moles of formic acid liberated per anhydro glucose unit of the dextran.

As two moles of periodate are reduced for each mole of formic acid liberated, any periodate in excess of 2 x F.A. must have been reduced by

FIGURE II.6.


1→6
1→3' LIKE
1→4' LIKE
MOLES OF PERIODATE REDUCED
1→6
2
1→3' LIKE
0
1→4' LIKE
1
MOLES OF HCOOH PRODUCED
1
0
0

1→4 'like' linked units and the percentage of the 1→4 'like' units will be equal to (P.R. - 2 F.A.) x 100, when P.R. represents the total periodate reduced per anhydro glucose unit of the dextran.

The percentage of 1→3 'like' linked units is the remainder when the sum of the 1→6 and 1→4 'like' linked units are subtracted from 100 per cent.

These calculations are summarized by the equations shown in Table II.7.

Table II.7.

Percentage of units linked:	Calculation involved:
1→6	F.A. x 100
1→4 'like'	(P.R. - 2 F.A.) x 100
1→3 'like'	100 - (sum of units linked 1→6 and 1→4 'like')

F.A. : formic acid liberated moles/mole A.G.U.
P.R. : periodate reduced moles/mole A.G.U.
A.G.U. : anhydro glucose unit.

Although this is a most rapid method for calculating the different kinds of linkages, it is not necessarily accurate: slight errors in the measurements of either the periodate reduced or formic acid liberated tend to be magnified in the final calculations.

The greatest source of error in the method could, however, come from over oxidation - that is the reduction of a greater number of moles of periodate than can be accounted for by the classical 1,2 diol splitting action of periodate ion shown in Fig. II.5. - which would result in an

increased content of units linked 1→6 or 1→4 'like' at the expense of the 1→3 'like' linked units, when the linkages values are calculated from the equations shown in Table II.7.

The method of analysis was applied to the B-1299 dextrans as follows:

A known weight of each dextran (dried to a constant weight and corrected for ash content) was dissolved (B-1299 S) or suspended (B-1299 L) in distilled water and oxidized with an excess of sodium metaperiodate, equivalent to 2.2 moles per anhydro glucose unit (AGU) of the dextran.

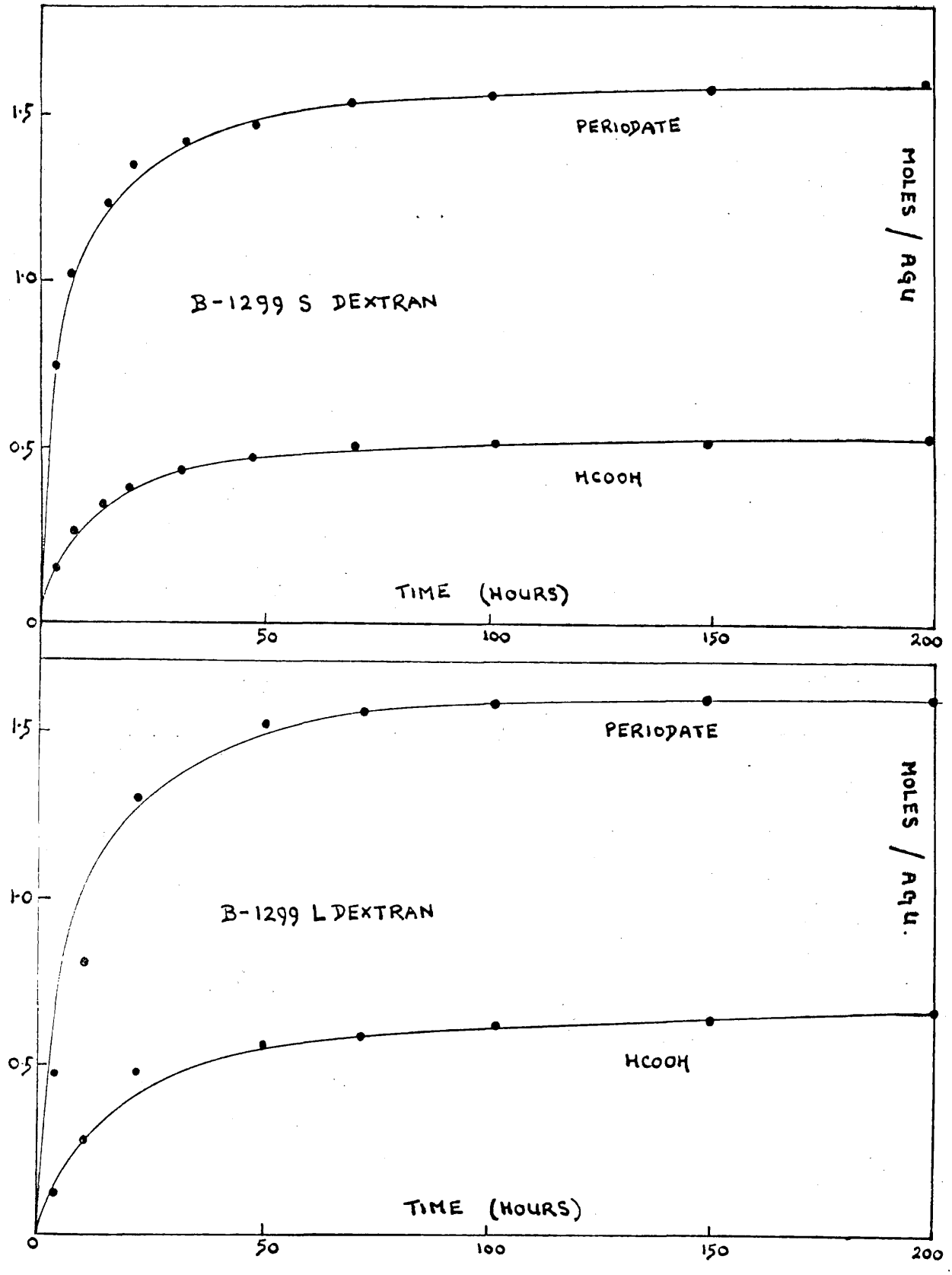
At regular intervals the periodate reduced and formic acid liberated were measured (expt. 11) and the results, expressed as periodate uptake (mole/mole AGU) and formic acid (HCOOH) liberated (mole/mole AGU), are shown in Table V.9. (page183) and represented graphically in Fig. II.7.

The oxidation curves shown in Fig.II.7. do not attain limiting values even after 200 hours, which is a probable indication that - under the conditions used - over oxidation of the dextrans has occurred.

Jeanes,[13] in similar oxidations of B-1299 dextrans observed that limiting values were not reached after 150 hours oxidation and, in the absence of these, used arbitrary 72 hour oxidation results to calculate the distribution of linkages shown on page 4.

A possibly more satisfactory approach, adopted here, was made by extrapolating the curves back to zero time and calculating the percentages of linkages from the extrapolated figures. The evaluations of the different types of linkages obtained from these extrapolated figures are shown in Table II.8., and are in close agreement with the values

FIGURE II.7. PERIODATE OXIDATIONS OF B-1299 DEXTRANS.



previously reported by Jeanes et al.[13]

Table II.8. The results of periodate oxidations of B-1299 dextrans

Dextran	Moles/mole AGU		% AGU linked		
	Periodate reduced	HCOOH released	1→6	1→4 like	1→3 like
B-1299 S	0.50	1.49	50	49	1
B-1299 L	0.55	1.52	55	42	3

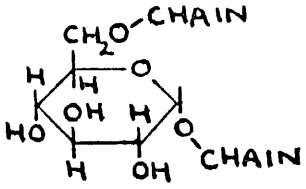
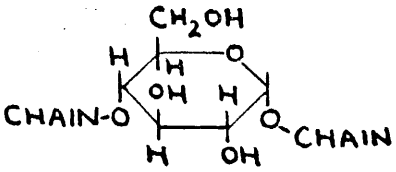
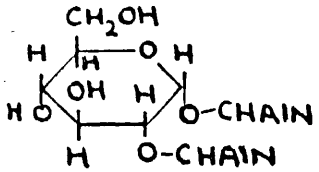
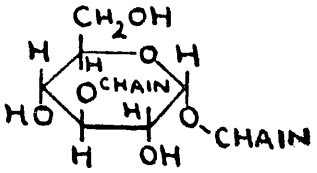
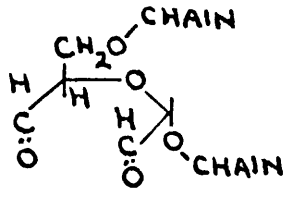
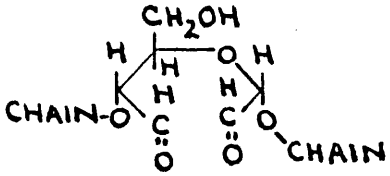
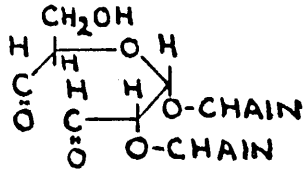
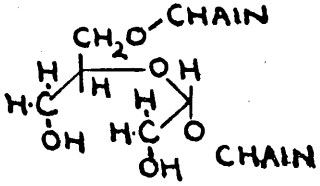
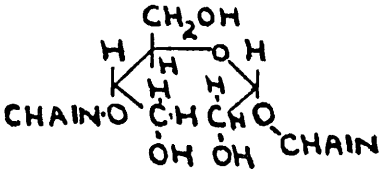
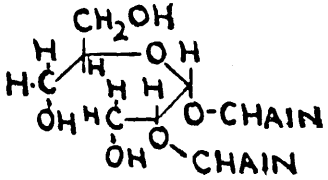
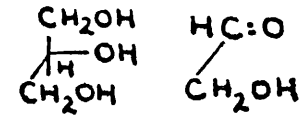
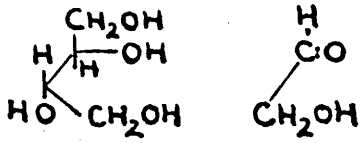
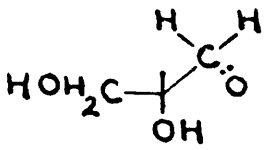
To establish the identity of the 1→4 'like' linked units, fragments from the periodate oxidized dextrans, other than formic acid, were examined.[54]

In the scheme of fragmentation indicated in Fig. II.8. erythritol is obtained as the characteristic fragment from a periodate oxidized 1→4 linked A.G.U. and glyceraldehyde from an oxidized 1→2 linked A.G.U.

Only periodate oxidized terminal non-reducing or 1→6 linked A.G.U. give equal molar quantities of glycerol and glycollic aldehyde, whilst glucose can only arise from an unoxidized 1→3 'like' linked A.G.U.

Identification and quantitative estimation[54] of these various fragments allows a ratio of the various kinds of glucosidic linkages to be calculated which has the advantage of being less affected by over oxidation reactions (see page 31) than that calculated from the amounts of periodate reduced and formic acid liberated by the dextran.

FIGURE II.8. ORIGIN OF FRAGMENTS FROM PERIODATE OXIDIZED B-1299 DEXTRANS.

	1→6 LINKED	1→4 LINKED	1→2 LINKED	1→3 LINKED
ANHYDROGLUCOSE UNIT				
PERIODATE OXIDIZED UNIT				NO CHANGE
REDUCED UNIT				NO CHANGE
CHARACTERISTIC FRAGMENT.	 GLYCEROL OR GLYCOLAL	 ERYTHRITOL OR GLYCOLAL	 GLYCERAL	GLUCOSE

Each of the B-1299 dextrans was oxidized with sodium metaperiodate, reduced with sodium borohydride and the resulting dextran polyalcohol isolated as a freeze dried powder. The experimental details and polyalcohol yields are given in expt. 12(a).

A small quantity of each polyalcohol was then completely hydrolysed with dilute sulphuric acid (expt. 12(b)). After neutralisation, the fragments in the hydrolysates were fractionated on paper chromatograms eluted with solvents (c) and (d) respectively, and also subjected to electrophoresis in borate electrolyte. The fragments were in each case located with spray reagent (a).

Comparison of the migration rates of the fragments from the polyalcohols with those of reference compounds spotted on the same chromatograms and electrophoretograms (Table II.9.) indicated that glyceraldehyde from 1→2 linked A.G.U. was present in each hydrolysate, but not erythritol from a 1→4 linked A.G.U. The absence of 1→4 linked A.G.U. in the dextrans established the important fact that the glucose present in the hydrolysates of both polyalcohols must have arisen from unoxidised units linked through the C_3 position (see Fig. II.8.).

The molar ratio of the glycollic aldehyde, glyceraldehyde and glucose was determined (expt. 12(c)) by using specific colorimetric reactions, and the results were used to calculate the percentages of units in each dextran linked 1→6, 1→2 and 1→3, which are shown in Table II.10.

Table II.9. Fragments from the complete hydrolysis of the B-1299 dextran polyalcohols

Component	R_G solvent (c)	R_G solvent (d)	M_G borate	Staining Intensity spray (a)	Possible Identity
12	3.7 diffuse spot	2.05 diffuse spot	1.18) 0.57)	2+	Glycollic aldehyde
13	1.47-3.20 streak	1.88 diffuse spot	0.78	+	Glyceral
14	2.30	1.73	0.50	+	Glycerol
1	1.00	1.00	1.00	+	Glucose

Erythritol has an R_G (solvent (c)) 1.70, R_G (solvent (d)) 1.43, M_G (borate) 0.75.

Table II.10. The ratio of the fragments from hydrolysis of B-1299 dextran polyalcohols

Dextran polyalcohol	Molar ratio of:			% AGU linked:		
	Glycolal	Glyceral	Glucose	1→6	1→2	1→3 Like
B-1299 S	0.56	0.36	0.07	56	36	7
B-1299 L	0.49	0.32	0.19	49	32	19

The figures in Table II.10. show that there are large percentages of secondary (i.e. 1→2 and 1→3) glucosidically linked units within each B-1299 dextran, which could be an indication of highly branched structures. The most significant difference between the dextrans appears to be the

higher percentage of glucose units linked through the C_3 position in the less water soluble B-1299 L polysaccharide. This is in agreement with the suggestion made by Jeanes,[55] that decreasing water solubility can be correlated with increasing percentage of 1→3 linkages in dextrans; although in the absence of molecular fractionation data the contribution of molecular size cannot be assessed.

The fragment 1-o-α-D-glucopyranosyl glycerol has been obtained as a product from Smith Degradation,[56] i.e. the mild acid hydrolysis of the polyalcohols, of four dextrans:[27,42,49] such a fragment could only have arisen from a situation in the native dextran, where 1→3 and 1→6 linked glucose units are adjacent (see Fig.II.9. - structures III and IV).

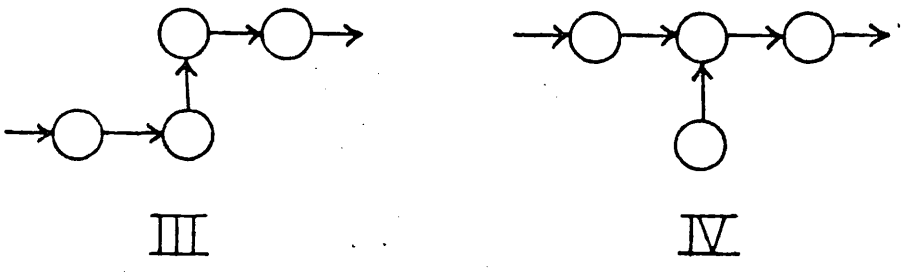
This indicated the possibility that 1→3 and 1→6 linked glucose units in the B-1299 dextrans (identified by periodate oxidation, acetolysis and acid hydrolysis experiments) might exist in a similar arrangement.

An experiment was therefore carried out to determine whether the glucopyranosyl glycerol fragment could be obtained from the polyalcohols of these dextrans.

In expt. 12(d), each of the B-1299 dextran polyalcohols was partially hydrolysed with dilute sulphuric acid and, after neutralising the hydrolysates, the liberated fragments were fractionated on paper chromatograms eluted with solvent (c) and subjected to electrophoresis in borate electrolyte.

The separated components were located with spray reagents (a) and (b) (specific for reducing groups) and the results - which were essentially

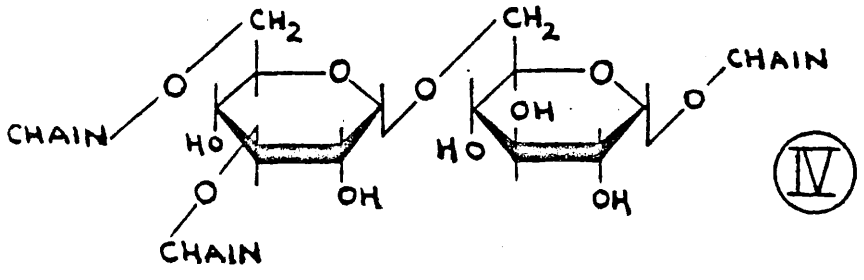
FIGURE II.9. ——— FRAGMENTS FROM THE MILD ACID HYDROLYSIS OF DEXTRAN POLYALCOHOLS.



III IV

→ 1→6 LINK, ↑ 1→3 LINK.

○ GLUCOPYRANOSYL UNIT.

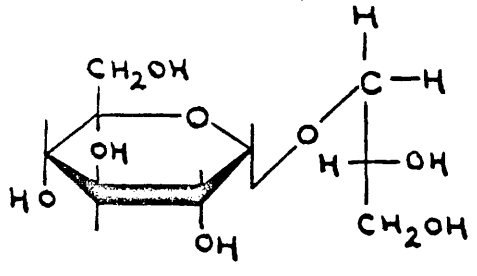


IV

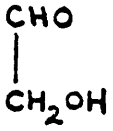
(i) OXIDATION WITH SODIUM METAPERIODATE.

(ii) REDUCTION.

(iii) MILD ACID HYDROLYSIS.



1-O-α-D-GLUCOPYRANOSYL GLYCEROL.



GLYCOLLIC ALDEHYDE

the same for both dextran polyalcohols - are summarized in Table II.11.

Comparison of the migration rates of the fragments from each hydrolysate with standard reference compounds indicated that a fragment corresponding to glucopyranosyl glycerol [R_G (solvent (c)) 1.0, M_G (borate) 0.12][27] was absent from both polyalcohol hydrolysates: from which it was concluded that the arrangements of glucose units shown in Fig. II.9. were unlikely to be present in either B-1299 dextrans.

Table II.11. Fragments from mild hydrolysis of the B-1299 dextran polyalcohols

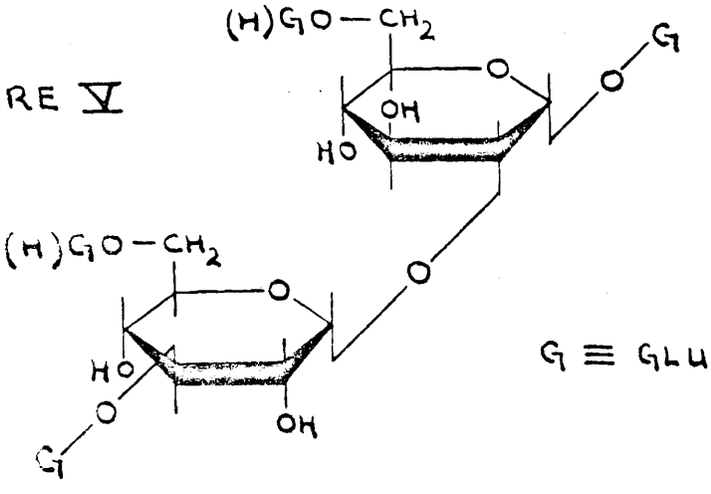
Component	R_G solvent (c)	M_G borate	Staining intensity spray (a)	Reaction spray (b)	Possible Identity
12	3.60 (diffuse spot)	1.20) 0.60)	+	+	Glycollic aldehyde
13	1.50-3.30 (streak)	0.80	+	+	Glyceraldehyde
14	2.30	0.50	4+	-	Glycerol
15	2.30	0.35	+	+	Glucosyl glyceraldehyde?

A reducing fragment [positive reaction with spray (b), R_G (solvent (c)) 2.30, M_G (borate) 0.35] which had not previously been reported was, however, present in each B-1299 polyalcohol hydrolysate.

Although no more detailed characterisation was attempted, the absence of glucose from the hydrolysate suggested that this fragment may well have been a glucosyl glyceraldehyde from an arrangement in the B-1299 dextrans where a 1→3 and a 1→2 linked glucose unit are adjacent (see Fig.II.10. -

FIGURE II.10 ——— FRAGMENTS FROM B-1299 DEXTRAN POLYALCOHOLS.

STRUCTURE V

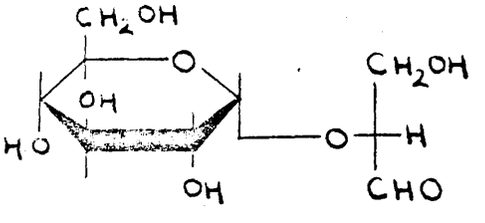


G ≡ GLUCOSE UNIT.

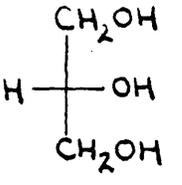
(i) OXIDATION WITH SODIUM META PERIODATE.

(ii) REDUCTION.

(iii) MILD ACID HYDROLYSIS.



2-O-α-D-GLUCOPYRANOSYL GLYCERALDEHYDE



GLYCEROL

structure V).

Such an arrangement, if present, could provide a possible explanation for the unusual serological reactivity towards α-(1→2) and α-(1→3) specific antibodies reported by Kabat[17-21] (see pages 5-9).

G. Methylation experiments: to determine the type and degree of branching in B-1299 dextrans

Periodate oxidation experiments of the kind described in experiment 12 may indicate the presence of secondary linked glucose units in the dextran but provide no information about the positions of these units within the molecule.

In addition, only in certain circumstances, i.e. the absence of 1→4 or 1→2 linked units, may the presence of 1→3 linked units be established.

Both these points may, however, be resolved through methylation studies on the dextran. In general the technique of polysaccharide methylation involves the metherification of all the free hydroxyl groups in the molecule, complete hydrolysis of the resulting methylated polysaccharide and characterisation and quantitative estimation of the partially methylated monosaccharide sugars released: each of which is characteristic of some structural feature of the parent polysaccharide.

The structures of many dextrans have been examined by the methylation experiment, from which it has been established that in general:

1) all dextrans are branching polysaccharides; the branching occurring through glucose units linked 1→2,[42] 1→4[43] or 1→3[51]

2) the predominant units in dextrans appear to be linked 1→6[43,51,57]

3) secondary linked units (i.e. 1→2, 1→3 or 1→4 linked) rarely (if at all) occur at non-branching points of the molecules.[49,57,58]

Of the procedures available for methylating the B-1299 dextrans[10] the one which proved to be most satisfactory was that due to Hodge and Karjala.[59] In this method the dextran is suspended in boiling liquid ammonia (-34°C) and treated first with sodium metal, to form the sodium derivative, and finally with methyl iodide to complete the methylation.

Complete details of the procedure, as applied to the B-1299 dextrans, are outlined in experiment 13(a).

After four or five treatments with the methylating agents, the partially methylated dextrans were extracted with chloroform to give products with methoxyl contents of 41.5% (B-1299 L) and 41.7% (B-1299 S) - each corrected for ash content - which had only small absorptions in the infra red spectrum at 3,200-3,700 cm^{-1}; characteristic of free hydroxyl groups.[60]

As a fully methylated glucan would have a methoxyl content of 45.6%, the methoxyl contents of the methylated B-1299 dextrans were considered sufficiently close to the theoretical value for a detailed examination of their hydrolysis products to be made.

Examination of the methylated B-1299 dextrans. Each methylated dextran was initially hydrolysed with sulphuric acid[64] (expt. 13(b)) and the syrups obtained from the neutralised hydrolysates were fractionated on paper chromatograms eluted with solvent (e).

Separated components were located with spray reagent (b), and, after elution from chromatograms, were subjected to electrophoresis in borate electrolyte.

The results of these experiments appear in Table II.12., in which each component was identified by comparison of its migration rate with that of a standard methyl glucose (Table II.13.), run wherever possible on the same chromatograms and electrophoretograms.

A comparison of Tables II.12. and II.13. indicates that component 16, from each methylated B-1299 dextran, could be 2,3,4,6 tetra-o-methyl glucose, component 17 - 2,3,4 tri-o-methyl glucose and component 18 - 2,4,6 tri-o-methyl glucose. Component 19 appears to be a mixture: principally of 3,4 di-o-methyl glucose, but containing traces of 2,3 di-o-methyl glucose and 2,4 di-o-methyl glucose.

Small amounts of mixed mono-o-methyl glucoses (component 20) and glucose (component 1) were also identified.

Small quantities of each methylated dextran were then methanolysed (expt. 13(c)) and the methyl glucosides released were separated by vapor phase chromatography on butan-1-4-diol succinate polyester (column I) and polyphenyl ether (m-bis [m-phenoxy phenoxy] benzene) (column II) stationary phases.[61]

The retention times (see page 157) of the separated components are recorded in Table II.14.

Each component was identified by comparison of its retention times with those of the standard methyl, o-methyl glucosides in Table II.13. which were chromatographed wherever possible on the same columns as the methanolysis mixtures.

Comparison of Tables II.13. and II.14. confirm the previous identification of methylated sugars 16, 17 and 18. Unidentified peaks

Table II.12. Chromatographic properties of methyl glucoses produced by hydrolysis of the methylated B-1299 dextrans

Methylated component	R_{TMG} solvent (e)	M_G borate	Staining colour spray (b)	Staining intensity spray (b)	Possible* Identity
16	1.00	0.00	Red	2+	2,3,4,6 tetra-
17	0.89	0.00	Yellow-brown	3+	2,3,4 tri-
18	0.84	0.00	Red		2,4,6 tri-
19	0.68	0.31	Yellow-brown	2+	3,4 di-
		0.15	Yellow	Trace	2,3 di-
		0.06	Orange	Trace	2,4 di-
20	0.40	0.28	Brown	Trace	2, or 4,mono-
		0.79	Brown		3, or 6,mono-
1	0.17	1.00	Yellow-brown	Trace	Glucose

* of o-methyl glucose

Table II.13. Chromatographic properties of some methyl glucoses

o-methyl glucose	R_{TMG} solvent (e)	MG borate	Staining colour spray (b)	Methyl glucoside, RT TMG			
				Column I		Column II	
2,3,4,6 tetra-	1.00	0.00	Red	1.00	1.43	1.00	1.33
2,3,4 tri-	0.90	0.00	Orange	2.50	3.52	1.34	1.83
2,3,6 tri-	0.89	0.00	Red-brown	3.21	4.21	1.75	2.30
3,4,6 tri-	0.86	0.30	Yellow-brown	2.95	3.50	1.73	2.30
2,4,6 tri-	0.84	0.00	Red	3.10	4.70	1.71	2.40
2,3 di-	0.68[62]	0.12[63]	Yellow-brown	-	-	2.46	3.22[61]
3,4 di-	0.65[62]	0.31[63]	Yellow-brown	-	-	-	-
2,4 di-	0.64[62]	0.05[63]	Orange	-	-	2.30	3.22[61]
3 or 6 mono-	0.40[62]	0.80[63]	-	-	-	-	-
2 or 4 mono-	0.40[62]	0.23[63]	-	-	-	-	-
Glucose	0.18	1.00	Yellow-brown	-	-	-	-

in the vapor phase chromatograms, of each methanolysed methylated dextran, could be due to the o-methyl glucoside of 3,4 di-o-methyl glucose (component 19), which had not previously been examined by this technique.

The remainder of each methylated dextran was then hydrolysed with sulphuric acid[64] (expt. 13(d)) and a portion of each syrup was fractionated on a paper chromatogram.

The molar ratio of the separated methyl glucoses was determined by the method of hypoidite oxidation[65] (expt. 13(e)(i)). However, as it proved impossible to completely resolve the two tri-o-methyl glucoses using solvent (e), the tri-o-methyl glucoses were combined and estimated.

The experimental ratio obtained, together with the calculated degree of branching (of the methylated B-1299 dextrans), are shown in Table II.15.

The degree of branching was based on both the tetra-o-methyl glucose unit from the non-reducing terminal end units of the methylated dextrans and the di-o-methyl glucose unit which represented the branching glucose unit in the methylated dextrans.

As the degree of branching (of each methylated dextran) calculated from both the molar ratios of the tetra- and the di-o-methyl glucose units was nearly equal, the small proportions of mono-o-methyl glucose and glucose units appear to be the products of incomplete methylation of all the free hydroxyl groups of the native dextrans.

The remainder of the syrups from the methylated dextran hydrolysates were applied to cellulose columns which were eluted with a butanol/

Table II.14. The retention times (RT $_{TMG}$) of o-methyl glucosides from methanolysed methylated B-1299 dextrans

Methyl glucoside of component	RT $_{TMG}$[x]				Possible identity +
	Column I		Column II		
16	1.00 L	1.43 L	1.00 M	1.37 VL	2,3,4,6 tetra-
17	2.51 M	3.54 L	1.37 VL	1.88 L	2,3,4 tri-
18	3.00 S	4.65 M*	1.72 S	2.35 Sh	2,4,6 tri-
19	8.00 L	9.60 T	2.50 L	-	?
	-	-	3.40 T	-	2,3 or 2,4 di-

KEY

VL	very large peak
L	large peak
M	medium peak
S	small peak
Sh	shoulder
T	trace

* S from B-1299 S dextran

\+ of o-methyl glucoside

x see page 157.

petroleum ether mixture (expt. 13(f)).

Each separation was followed polarimetrically and by paper chromatography (solvent (e)): which indicated an almost complete separation of the four major components 16, 17, 18 and 19 previously identified.

The resolution of the tri-o-methyl sugars 17 and 18 allowed an approximate value for their molar ratio to be calculated (expt. 13(e)(ii)), which was based on the polarimetric curves shown in Fig. V.5. (page201). The results, summarized in Table II.16., again indicate that the degree of branching based on both the tetra-o-methyl glucose unit and the di-o-methyl glucose unit are almost equal.

More detailed characterisations of the methylated sugars (separated on the cellulose columns) are outlined in experiments 13(g) (component 16), 13(h) (component 17), 13(i) (component 18) and 13(j) (component 19) - in which each component was identified by:

1) comparison of its migration rate and staining properties (spray (b)) in paper chromatography and borate electrophoresis with authentic methylated sugars
2) comparison of the retention times of the methyl glucoside derivatives with those of standard methyl, o-methyl glucosides on two stationary phases (see expt. 13(c))
3) being crystallised or converted to a crystalline derivative whose physical properties were compared with those of standard methyl glucoses.

The results of these experiments appear in Table II.17. and, for comparison, the properties of some methyl glucoses are given in Table II.18.

Table II.15. The molar ratio of the methyl glucoses from the methylated B-1299 dextrans

Dextran	Molar ratio of o-methyl glucose					Molar % of o-methyl glucose					Branching %*
	Tetra	Tri	Di	Mono	Glucose	Tetra	Tri	Di	Mono	Glucose	
	16	17/18	19	20	1	16	17/18	19	20	1	
B-1299 S	1.00	1.08	1.01	0.22	-	30	33	30	7	-	(i) 30 (ii) 30
B-1299 L	1.00	1.30	1.09	0.22	-	28	36	30	6	-	(i) 28 (ii) 30

* (i) calculated from tetra-o-methyl glucose unit

(ii) calculated from di-o-methyl glucose unit

Table II.16. The molar ratio of the methyl glucoses from the methylated B-1299 dextrans

Dextran	Molar ratio of o-methyl glucoses				Molar percentage of o-methyl glucoses				Branching % *
	Tetra	2,3,4 Tri	2,4,6 Tri	Di	Tetra	2,3,4 Tri	2,4,6 Tri	Di	
	16	17	18	19	16	17	18	19	
B-1299 S	1.00	0.76	0.20	0.94	34	26	7	32	(i) 34 (ii) 32
B-1299 L	1.00	0.86	0.53	1.17	28	24	15	31	(i) 28 (ii) 31

* (i) calculated from tetra-o-methyl glucose unit

(ii) calculated from di-o-methyl glucose unit

Comparison of the periodate oxidation data (Table II.10.) and the methylation results (Tables II.15. and II.16.) indicate that the B-1299 dextrans are highly branched molecules: the branching occurring through glucose units linked at the C_1, C_2 and C_6 positions (see Fig. II.11). The slight differences between the percentages of 1→2 linked units when calculated from the periodate oxidation and methylation experiments may be due to incomplete methylation of the dextrans or degradation during the methylation reaction, but in any case the figures (see Table II.16.) would indicate a slightly higher degree of branching in the more water soluble B-1299 S dextran.

The trace component, migrating in borate electrophoresis as 2,3 di-o-methyl glucose probably arises from under methylation as erythritol, also characteristic of a 1→4, 6 linked glucose unit, was not observed as a fragment from hydrolysis of the B-1299 dextran polyalcohols.

In contrast, the trace of component migrating in borate electrophoresis as 2,4 di-o-methyl glucose could be equally a product of incomplete methylation or have come from branched glucose units - linked 1→3, 6 - in the methylated dextrans. However, when the results of the periodate oxidation and methylation experiments are again compared, it appears that almost all the glucose units linked through the C_3 position give rise to 2,4,6 tri-o-methyl glucose (Fig. II.11.) and do not therefore constitute branching points in these dextrans.

This is a particularly interesting observation, for the secondary glucosidic linkages in dextrans so far examined have almost always formed branching points.[43,49,51]

Table II.17. Properties of -o-methyl glucoses from hydrolysis of the methylated B-1299 dextrans

Methylated component		Sugar								'Anilide'	
		R_{TMG} solvent (e)	M_G borate	RT TMG* column II	Mpt °C	Mixed Mpt °C	$[\alpha]_D$	OCH_3,%	Yield+ (gm)	Mpt °C	OCH_3,%
From B-1299 S Dextran	16	1.00	0.00	1.00) 1.36)	84-86	83-85	+ 98° + 83°	51.4	1.50	-	-
	17	0.89	0.00	1.34) 1.86)	Syrup	-	-	-	1.30	138-140	32.7
	18	0.84	0.00	1.63) 2.30)	117-120	117-120	+ 95° + 74°		0.10	-	-
	19	0.68	0.29	2.43	110-112	-	+ 74°	29.3	1.10	168-169	21.1
From B-1299 L Dextran	16	1.00	0.00	1.00) 1.35)	84-86	82-85	+ 99° + 83°	51.1	1.03	-	-
	17	0.89	0.00	1.34) 1.88)	Syrup	-	-	-	0.70	-	-
	18	0.84	0.00	1.71) 2.42)	118-121	117-121	+ 99° + 73°	39.1	0.20	-	-
	19	0.68	0.30	2.43	111-113	-	+108° + 74°	29.4	0.60	-	-

* of o-methyl glucoside + see experiment 13(f)

Table II.18. Properties of some o-methyl glucoses and their N-glucosylamine 'anilide' derivatives

o-methyl glucose	Chromatographic and physical properties of the sugar							Properties of the 'anilide' derivative			
	R_{TMG} solvent (e)	M_G borate	RT TMG* column II	Mpt °C	$[\alpha]_D$	OCH_3 %	Reference	Mpt °C	OCH_3 %	$[\alpha]_D$	Reference
2,3,4,6 tetra	1.00	0.00	1.00) 1.33)	83-85	+92° +84°	52.5	66	134-135	40.0	224 65°	70
2,3,4 tri-	0.90	0.00	1.35) 1.83)	syrup	+71.9°	41.9	51	145-146	31.2	-	74
2,3,6 tri-	0.89	0.00	1.75) 2.30)	114-116	+67°	41.9	66,67	syrup	31.2	-	67,68
2,4,6 tri-	0.84	0.00	1.71) 2.40)	120-121	+111° +75°	41.9	66,67	162-166	31.2	-113°	67
3,4,6 tri-	0.86	0.30	1.73) 2.30)	76-77	+92° +77°	41.9	67	syrup	31.2		68
2,3 di-	0.68[62]	0.12[63]	2.46)[61] 3.20)	85-87	+82° +48°	29.8	43	134	21.9	-	69
2,4 di-	0.64[62]	0.05[63]	2.30)[61] 3.20)	128-130	+76.5°	29.8	51	syrup	21.9	-	
3,4 di-	0.65[62]	0.30[63]	-	113	+76°	29.8	71,72	171-173	21.9	-	72

* of the -o-methyl glucoside

FIGURE II.11. ORIGIN OF FRAGMENTS FROM METHYLATED B-1299 DEXTRANS.

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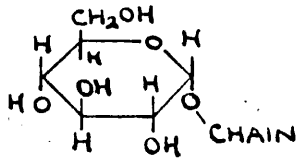
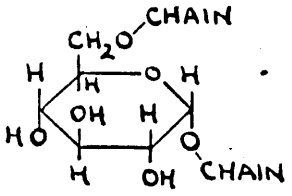
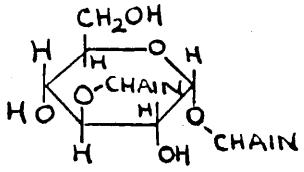
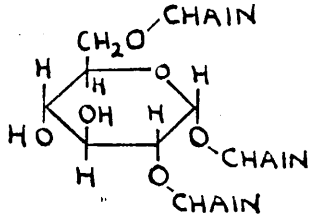
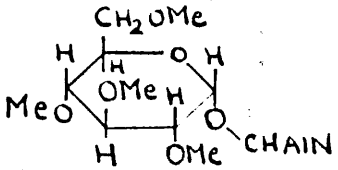
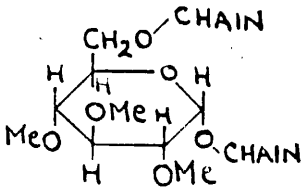
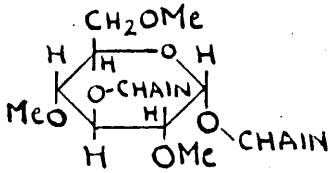
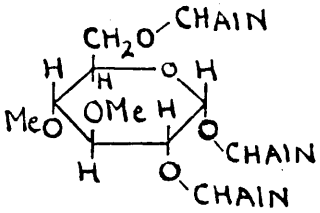
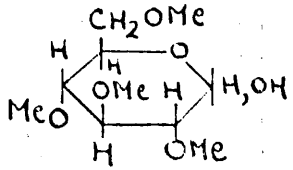
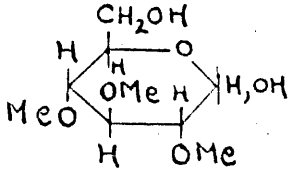
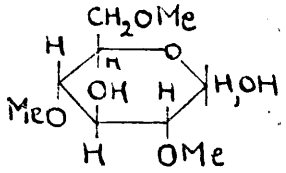
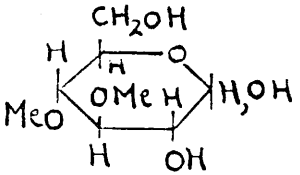
ANHYDROGLUCOSE UNIT				
METHYLATED UNIT				
CHARACTERISTIC METHYL SUGAR				
	2,3,4,6 TETRA-O-METHYL GLUCOSE	2,3,4 TRI-O-METHYL GLUCOSE	2,4,6 TRI-O-METHYL GLUCOSE	3,4 DI-O-METHYL GLUCOSE

Among the few exceptions appear to be the dextrans elaborated by NRRL B-1298[49] and NCIB-2706[57] micro-organisms, in which 1→3 linked glucose units may also occur at non-branching points, as traces of 2,4,6 tri-o-methyl glucose and its o-methyl glucoside derivative have been obtained from the methylated polysaccharides.

Also Wallenfels reports[58] that the dextran produced by an unspecified micro-organism contains 1→4 linked intrachain units because vapor phase chromatography has indicated that 2,3,6 tri-o-methyl, methyl glucoside is a component of the methanolysate from the methylated dextran.

However, only very small quantities of each of these fragments have been obtained which may be an indication that they are the products of incomplete methylation of the dextrans concerned rather than representative of structural features in these molecules.

In Table II.19. the results of the periodate oxidation and methylation experiments are compared and, from an average of these experimental figures, a repeating unit, i.e. the smallest number of glucose units which could contain all the structural features present in the polysaccharide, in their correct proportions, is proposed for each of the B-1299 dextrans.

Inspection of the proposed repeating units indicates that they are very similar: addition of two 1→3 linked glucose units to the repeating unit of B-1299 S dextran gives the repeating unit of B-1299 L dextran.

Table II.19. Summary of the results from periodate oxidation and methylation experiments with B-1299 dextrans

Dextran	Glucose units, linked	Method of Determination				Average per cent of each unit	Composition of a possible repeating unit
		Periodate* oxidation		Methylation*			
		1	2	3	4		
B-1299 S	Terminal non-reducing	50	56	30	34	32	5
	1→6			33	26	26	4
	1→3	1	7		6.5	6·5	1
	1→2,6	49	36	30	32	32	5
	Unaccounted for	-	-	7	-	3	-
B-1299 L	Terminal non-reducing	55	49	28	28	28	5
	1→6			36	24	24	4
	1→3	3	19		15	17	3
	1→2,6	42	32	30	31	31	5
	Unaccounted for	-	-	6	-	-	-

* see (1) Table II.8. (2) Table II.10 (3) Table II.15 (4) Table II.16.

It could well be that the more water soluble B-1299 S dextran is an intermediate in the synthesis of the less soluble B-1299 L dextran. If this is so the question of whether the B-1299 S and B-1299 L dextrans are really to be regarded as separate polysaccharides is again raised.

Possible sequences of the glucose units within each of the proposed repeating units, which take account of all the information available for the B-1299 dextrans, are shown in Fig. II.12.

Each, for example, is based on a skeleton of 1→6 linked glucose units, whilst 1→3 linked glucose units have been placed adjacent to 1→2 linked glucose units rather than to 1→6 linked units.

The branches of the repeating unit represented by structure VI consist of one or two glucose units and in this respect closely resemble the structures proposed for the 1→3 branched and 1→4 branched dextrans produced by NRRL B-1375 and NRRL B-1415 micro-organisms.[27,43,75,76]

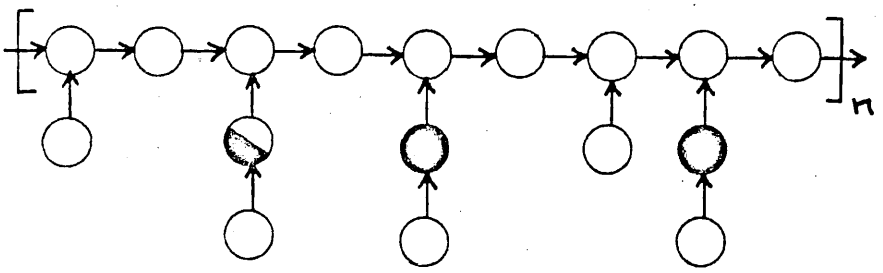
In contrast, a majority of branches of the unit represented by structure VII are two or more glucose units in length and may be compared with the structure proposed for the dextran produced by NCIB-2706 micro-organism[57] and (acid degraded) L. mesenteroides B-512 dextran.[77]

Finally structure VIII contains two structural features not so far reported in dextrans, they are:

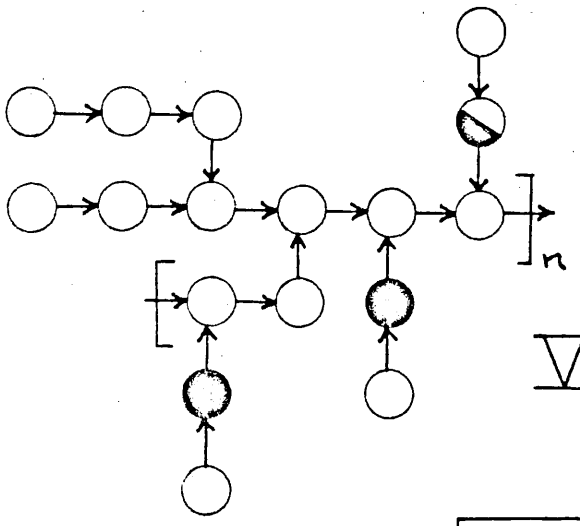
1) branches consisting of a single 1→6 linked glucosyl unit
2) secondary (i.e. 1→2 and 1→3) linked glucose units in adjacent intra chain positions.

It is particularly interesting to note the diverse arrangements of the differently linked glucose units which may be accommodated in the

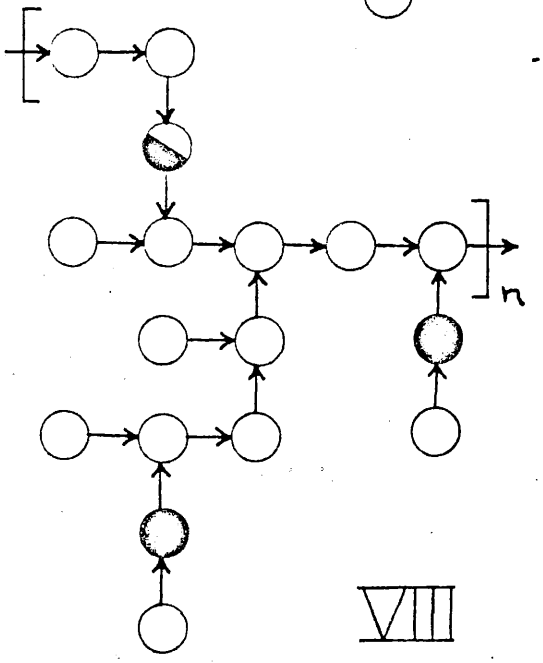
FIGURE II.12 — POSSIBLE REPEATING UNITS FOR THE B-1299 DEXTRANS.



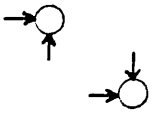
VI



VII



VIII

	UNIT, LINKED
	1→6
	1→2, 6
	1→3
	1→3 B-1299L ONLY

repeating units proposed in Table II.19. and emphasises the fact that the two B-1299 dextrans could have totally different detailed constructions even though their repeating units are 'statistically' similar.

In the following Section III of the thesis the repeating unit of B-1299 S dextran will be re-examined in somewhat greater detail.

III. Further investigations into the structure of a water soluble dextran produced in cultures of Leuconostoc mesenteroides NRRL B-1299

More detailed information about the sequential arrangements of glucose units in B-1299 S dextran - which comprises 95% of the total polysaccharide from L. mesenteroides B-1299 cultures - was obtained by characterising oligosaccharides produced from chemical and enzymic degradation experiments.

In the task of separating and characterising these oligosaccharides particular use was made of paper electrophoresis in sodium borate and sodium molybdate electrolytes.

Therefore, before proceeding to discussion of the results of experiments in Section III, some aspects of oligosaccharide electrophoresis in each of these electrolytes will be considered.

A. Electrophoresis of some oligosaccharides in sodium borate and molybdate electrolytes

Relationships between the structures of the α-D glucose oligosaccharides and oligosaccharide alcohols, and their abilities to complex with borate and molybdate ion respectively, have been established by Foster[63] and Weigel.[80]

In summarizing this work particular emphasis will be placed on those α-D glucose oligosaccharides (or oligosaccharide alcohols) which may be obtained from degraded dextrans.

(i) Electrophoresis in sodium borate electrolyte

Boric acid acts as a Lewis acid, rather than a proton donor, accepting the electron pair of a base to form the stable anion $B(OH)_4^{\ominus}$[63].

At alkaline pH this anion is capable of forming a weak negatively charged complex with many neutral sugars and their derivatives: application of a potential difference to the anionic complex results in migration towards the anode.

Foster considers[73] that, in general, the magnitude of the net charge and hence the rate of migration of the sugar complex will depend upon two factors, these are:

(a) the number of contributions of the ring (either pyranose or furanose) and open chain forms of the sugar (or its derivative) which can interact with the borate ion

(b) the strengths of the interactions at each complexing centre.

Polarographic studies[78] on solutions of sugars under equilibrium conditions have revealed the presence of reducible (probably aldehydo) forms, which increase rapidly with increase of pH. It seems probable that the open chain or aldehydo forms of the sugars provide a significant contribution to the borate complex.

If the aldehydo forms of the sugars adopt the favoured zig-zag shape of a carbon atom chain then several possibilities for the interaction of borate ions with the various pairs of hydroxyl groups along the open carbon chain must exist and the limiting factor will be the distance the borate ion can span in forming a complex.

In this respect there is an analogy with the formation and stability of acetal rings[64] which are regulated by the distances separating the various pairs of hydroxyl groups involved in ring formation.

If the letters α and β refer to the relative positions of the

hydroxyl groups along the chain in the aldehydo form, and C and T refer to cis and trans, the orders of stability are β C, β, α T, α, β T and α C.

Experiments with many sugars[78] led to the conclusion that for D-glucose and its derivatives effective interactions of the borate ions with ring or open chain forms of the reducing sugar in equilibrium at pH 10.0 are limited to three possibilities.

These, in preferred order, involve the β C hydroxyl groups at C_2 and C_4 in the aldehydo form (IX) the cis hydroxyl groups at C_1 and C_2 in the (furanose or pyranose) ring forms (X), and the β hydroxyl groups at C_4 and C_6 in the aldehydo form (XI).

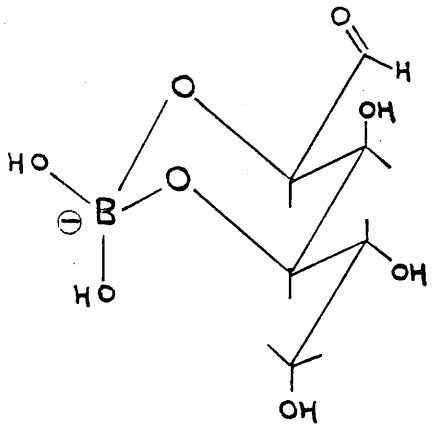
From the migrations of the α-D glucose disaccharides[78] (Table III.1.) it may be seen that with respect to M_G values (see page 157) the oligosaccharides fall into three groups where:

(a) interaction at IX and X are possible, leading to the high M_G values of isomaltose and nigerose

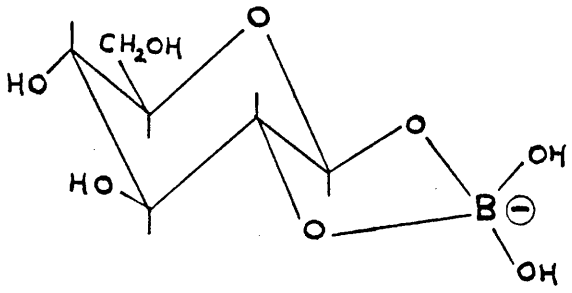
(b) interaction at X only is possible, leading to the moderate M_G value of maltose

(c) interaction at XI only is possible, leading to the moderate M_G value of kojibiose.

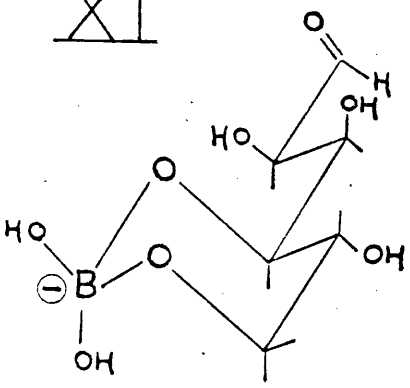
IX



X



XI



XII

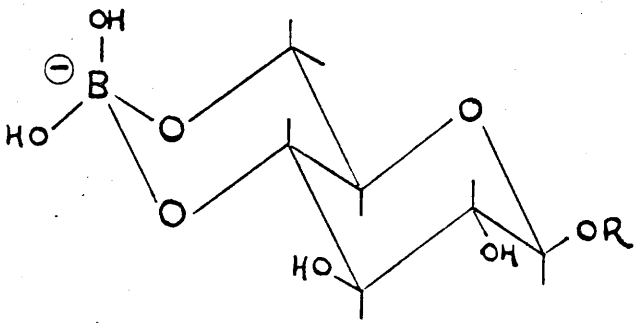


Table III.1. The migration rates of α-D-glucose disaccharides in sodium borate electrolyte[78]

Sugar	Link	M_G
Isomaltose	α-(1→6)	0.69
Nigerose	α-(1→3)	0.69
Maltose	α-(1→4)	0.32
Kojibiose	α-(1→2)	0.32

The D-glucose oligosaccharides may present a special case where interaction at C_4 and C_6 of the pyranose ring (XII) could occur.[79] The contribution to the overall M_G value can be judged to be small from the M_G value of α-methyl glucopyranoside (0.10) but may become rather more consequential in the case of branched oligosaccharides.

In general though it would appear that the rate of migration of the D-glucose oligosaccharides are principally controlled by the positions at which the remainder of the molecule (the non-reducing moiety) is attached to the reducing glucose unit. Oligosaccharides of equal molecular size terminating with an α-(1→6) or α-(1→3) link to the reducing unit could be expected to migrate much faster than those terminating in either an α-(1→2) or an α-(1→4) link to the reducing end unit.

(ii) Electrophoresis in sodium molybdate electrolyte

In acid solution (pH 5.0) molybdates are able to form anionic complexes with certain polyhydroxy compounds,[82] but due to the formation of several isopolyacid ions at acid pH, the form of the complexing species was not initially apparent.[81]

As D-glucopyranosides do not migrate in sodium molybdate the reduced α-D glucose disaccharides were used to investigate the effects of substituents on complexing within the hexitol molecule.

The migrations of the α-D glucopyranosyl hexitols[83] are shown in Table III.2. The term M_S value is defined on page 57.

Table III.2. The migration rates of some α-D glucopyranosyl hexitols in sodium molybdate electrolyte

Sugar alcohol	Link	M_S
Isomaltitol	α-(1→6)	0.70
Kojiblitol	α-(1→2)	0.70
Maltitol	α-(1→4)	0.45
Nigeritol	α-(1→3)	0.05

Analysis of the molybdate/substituted hexitol complex[82] indicated that substituted glucitols fall into three groups.

(a) The 2 or 6 substituted glucitols, which form a complex in which the sugar alcohol/molybdenum atom ratio is 1:2, have high M_S values.

(b) The 4 substituted glucitols, which form a complex in which the sugar alcohol/molybdenum atom ratio is 1:1, have medium M_S values.

(c) The 3 substituted glucitols, which do not appear to form a stable complex, have zero M_S values.

Angus and Weigel,[82] from potentiometric titrations of the molybdate - hexitol complex have concluded that the complexing molybdate anion is in all cases $Mo_2O_7^{2\ominus}$.

Periodate oxidation of the essentially similar, though more stable, tungstate complexes[83] has indicated that, where a complex is formed containing a hexitol/molybdenum atom ratio of 1:2, four adjacent hydroxyl groups are involved in the complexing.

Migration of isomaltitol (or any isomaltodextrinol) can be satisfactorily explained by formation of a complex XIII, involving the 1,2,3,4 (α,αT,αT) tetritol arrangement, and that of kojibiitol by formation of a complex XIV involving the 3,4,5,6 (αT,αC,α) tetritol grouping, each with a single $Mo_2O_7^{2\ominus}$ anion.[82]

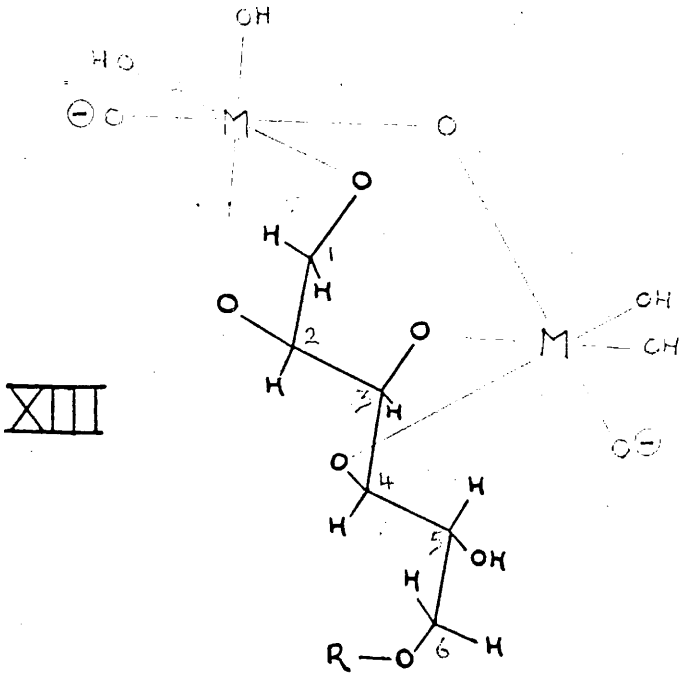
The migration of maltitol (or a maltodextrinol) is accounted for by formation of complex XV involving the 1,2,3 (α,αT) triol system of 2 molecules of maltitol and a single $Mo_2O_7^{2\ominus}$ anion.[82] Nigeritol possessing only an (αC,α) triol arrangement is incapable of complex formation.

In general then two factors may influence the overall migrations of the alcohols of the α-D-glucose oligosaccharides, they are:

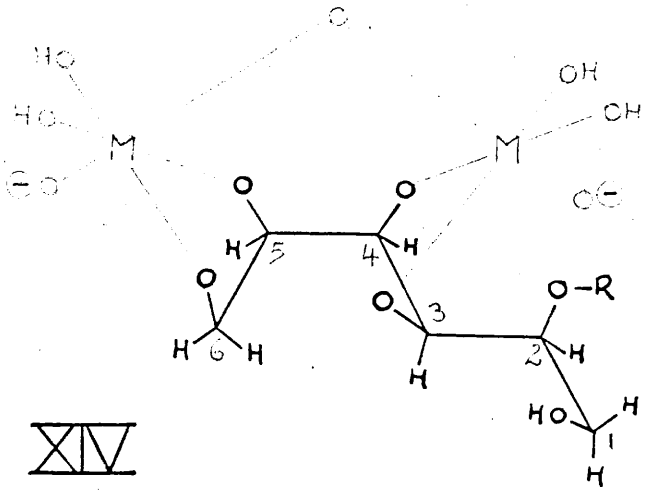
(a) The position at which the remainder of the molecule (non-reducing moiety) is attached to the hexitol unit.

(b) The size of, though not the types of α-linkages within, the glucosyl portion of the molecule.

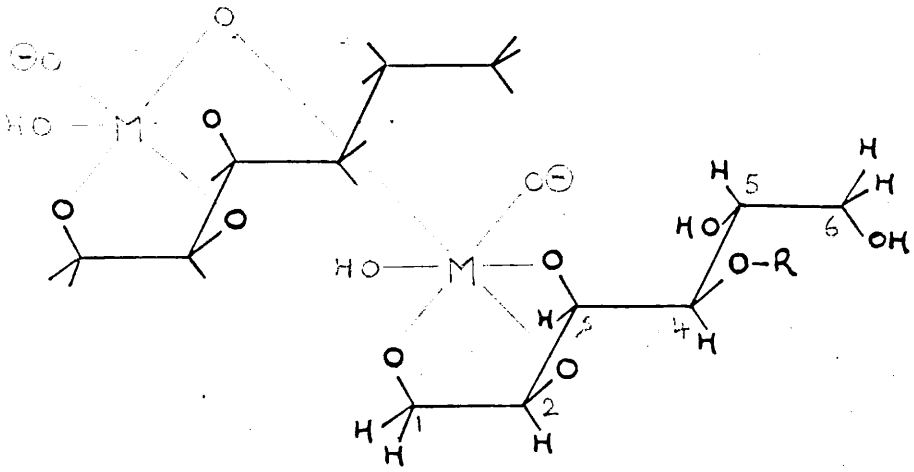
As a rule α-D-glucose oligosaccharide alcohols of equal molecular size, terminating in either a 2 or 6 substituted glucitol unit, can be expected to have almost identical and greater migrations in molybdate electrophoresis than the equivalent alcohols terminating in a 4 substituted glucitol unit. Any oligosaccharide alcohol terminating in a



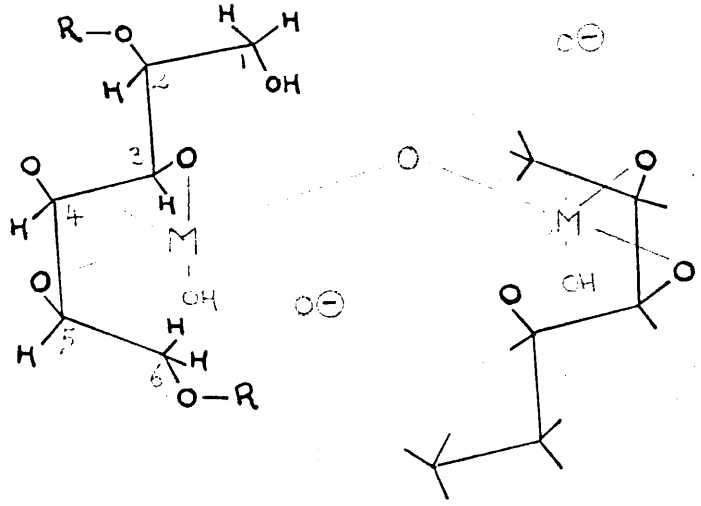
XIII



XIV



XV



XVI

3 substituted glucitol unit will have zero migration.

The reduced branched oligosaccharides of α-D glucopyranose fall into two groups.

(a) When the branch is not attached to the hexitol portion it cannot affect complexing and will, therefore, have a minimal effect on the alcohol migration.

(b) When the branch is attached to the hexitol portion, the molecule may be regarded as a di (or multiply) substituted glucitol.

In this case the 4,6 di substituted glucitol will migrate, having an M_S value equal to that of the maltodextrinol of equivalent molecular size.

With one possible exception - the 2,6 di substituted glucitol - all the other di (or multiply) substituted glucitols cannot be expected to migrate as they do not possess the required sequence of three or four correctly disposed hydroxyl groups.

The 2,6 di-substituted glucitol may be capable of forming a weak complex with molybdate ion, for experiments with models show that when held in a certain conformation (XVI) the distances between the (αT, αC) hydroxyl groups on C_3, C_4 and C_5 can approximate to that of the 4-substituted glucitol (XV).

When held in conformation (XVI) interactions between eclipsed groups must increase the internal energy of the hexitol molecule. However, it is probable that this increase of internal energy is not so great as to be a factor preventing formation of a stable molybdate complex, for interactions of a similar magnitude must be present in the 2-substituted

glucitol - which does form a stable molybdate complex - when this molecule is held in the conformation (XIV) required for complexing with molybdate.

Further, the identical migration rates of the 2- and 6-substituted glucitols (Table III.2.) may be construed as indicating that an equally stable molybdate complex is formed in each case; even though the molybdate complexed 6-substituted glucitol is free of eclipsed groups (XIII).

B. Investigation of the structures of the oligosaccharides produced by partial acid hydrolysis of the B-1299 S dextran

It was suggested in Section IIC that the series of isomaltose homologues is liberated by partial acid hydrolysis of the B-1299 polysaccharides; from which it was concluded that each B-1299 polysaccharide contains sequences of 1→6 linked glucose units.

However, the identification of these oligosaccharides rested directly upon their paper chromatographic migrations and staining properties, and a more complete characterisation seemed desirable.

The object of the present experiment was, therefore, to re-examine in greater detail the oligosaccharides from the acid hydrolysed B-1299 S dextran.

B-1299 S dextran (22gm. - weight uncorrected for ash or moisture contents) was partially hydrolysed with dilute sulphuric acid (expt. 8(a)). The hydrolysis products were initially fractionated on a charcoal column (see Fig. V.2B. and Table V.1.) and then re-separated on Whatmann papers.

The final yields of the separated components are shown in Table III.3., together with their degrees of polymerisation (expt. 8(c)), specific

rotations (expt. 8(d)) and paper chromatographic and electrophoretic migration rates.

The values for the optical rotations are in good agreement with those reported for the series of isomaltose homologues:[41] isomaltose (+120°), isomaltotriose (+142°), isomaltotetraose (+153°), isomaltopentaose (+160°) and isomaltohexaose (+163°).

However, to investigate more fully whether a homologous series[47] existed, the degree of polymerisation of each oligosaccharide component was plotted against its R_M value[41] (see page 157), and its molecular rotation[41] (molecular weight times specific rotation). The results are shown in Fig. III.1. In each case a straight line indicated that all the oligosaccharides, with the exception of component 8, belong to a homologous series. The first member of the series (component 2) was converted to the crystalline β-octa acetate derivative[41] (expt. 8(e)) which had a melting point of 139-141°C, undepressed by authentic isomaltose β-octa acetate.

In expt. 8(f) the oligosaccharide components 2 to 7 were incubated with dextranase - α-(1→6) glucosidase - and the degradation patterns were compared with those of authentic isomaltose homologues.

The degradation pattern of each oligosaccharide (Table III.4.) was found to be identical to that of the isomaltose homologue of equivalent molecular size.

Component	Yield (gm.)	$[\alpha]_D^{20}$ (in water)	D.P.	R_G solvent (a)	M_G (borate)	M_S* (Molybdate)	Stain[46] spray (c)	Identity
1	4.8	+ 52°	-	1.00	1.00	1.00	Green	Glucose
2	0.6	+120°	1.9	0.56	0.71	0.73	Green	Isomaltose
8	0.04	-	2.1	0.65	0.33	0.70	Brown	Kojibiose
3	0.45	+141°	2.7	0.30	0.56	0.59	Green	Isomaltotriose
4	0.35	+150°	4.0	0.16	0.46	0.50	"	Isomaltotetraose
5	0.38	+153°	4.8	0.08	0.42	0.44	"	Isomaltopentaose
6	0.32	+162°	5.8	0.04	0.40	0.39	"	Isomaltohexaose
7	0.05	-	-	0.02	0.37	0.34	"	Isomaltoheptaose

* After reduction with sodium borohydride

Table III.3. Physical and chromatographic properties of oligosaccharides from acid hydrolysed B-1299 S dextran

FIGURE III.1. —— OLIGOSACCHARIDES FROM THE PARTIALLY HYDROLYSED B-1299S DEXTRAN.

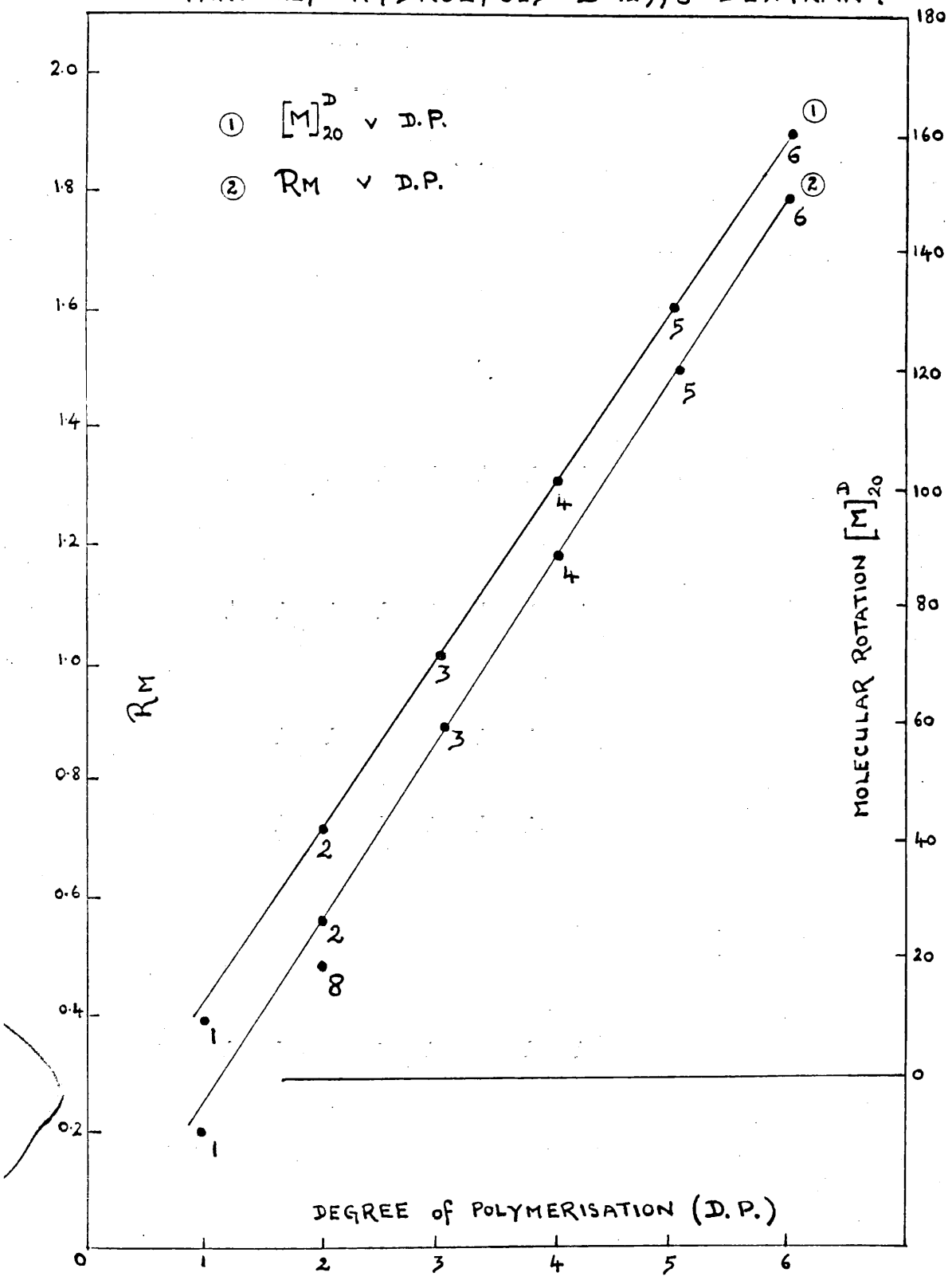


Table III.4. The results of dextranase digests of oligosaccharides from acid hydrolysed B-1299 S dextran

Substrate (component)	Degradation products, chromatographing as:						
	Glucose	IM_2	IM_3	IM_4	IM_5	IM_6	IM_7
2	-	2+	-	-	-	-	-
3	-	-	2+	-	-	-	-
4	+	2+	+	-	-	-	-
5	+	3+	3+	-	-	-	-
6	+	2+	+	-	-	-	-
7	+	3+	3+	-	-	-	-

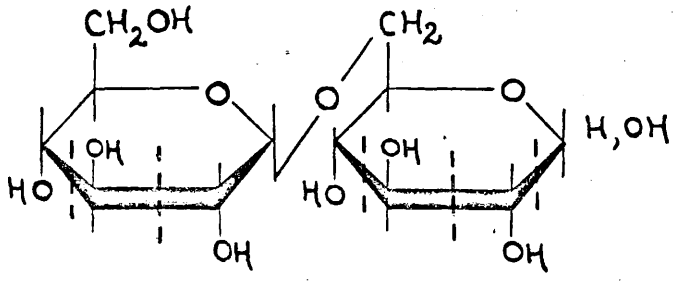
IM_2 isomaltose, IM_3 isomaltotriose, IM_4 isomaltotetraose, IM_5 isomaltopentaose, IM_6 isomaltohexaose, IM_7 isomaltoheptaose.

Further evidence that components 2 to 6 were a series of isomaltose homologues was obtained by oxidizing each component with sodium meta-periodate (expt. 8(g)).

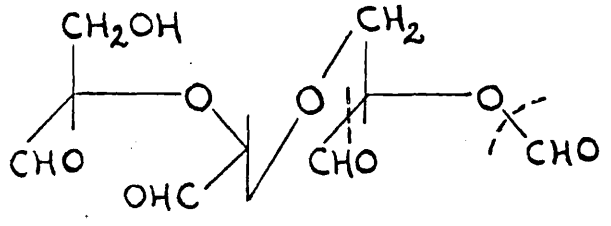
When fully oxidised by sodium periodate isomaltose has been reported[85] to reduce 6 moles of periodate and to liberate 5 moles of formic per mole of the sugar - a probable course of this oxidation is shown in Fig. III.2. - and isomaltotriose to reduce 8 moles of periodate and release 6 moles of formic acid per mole of the sugar.[85]

These experimental results enabled the amount of periodate reduced and formic acid liberated per mole of each isomaltose homologue to be calculated. The calculated values are shown in Table V.4. (page 176), and are based on the fact that each non-reducing anhydro glucose unit of an isomaltose homologue appears to reduce 2 moles of periodate and liberate 1 mole of formic acid, per mole of the unit.

FIGURE III.2. THE PERIODATE OXIDATION OF ISOMALTOSE.

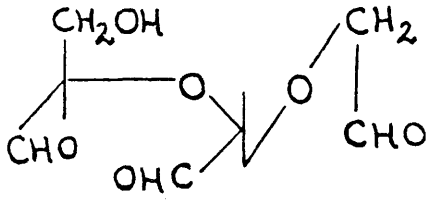


5 MOLES OF PERIODATE PER MOLE. ISOMALTOSE



+ 3 HCOOH.

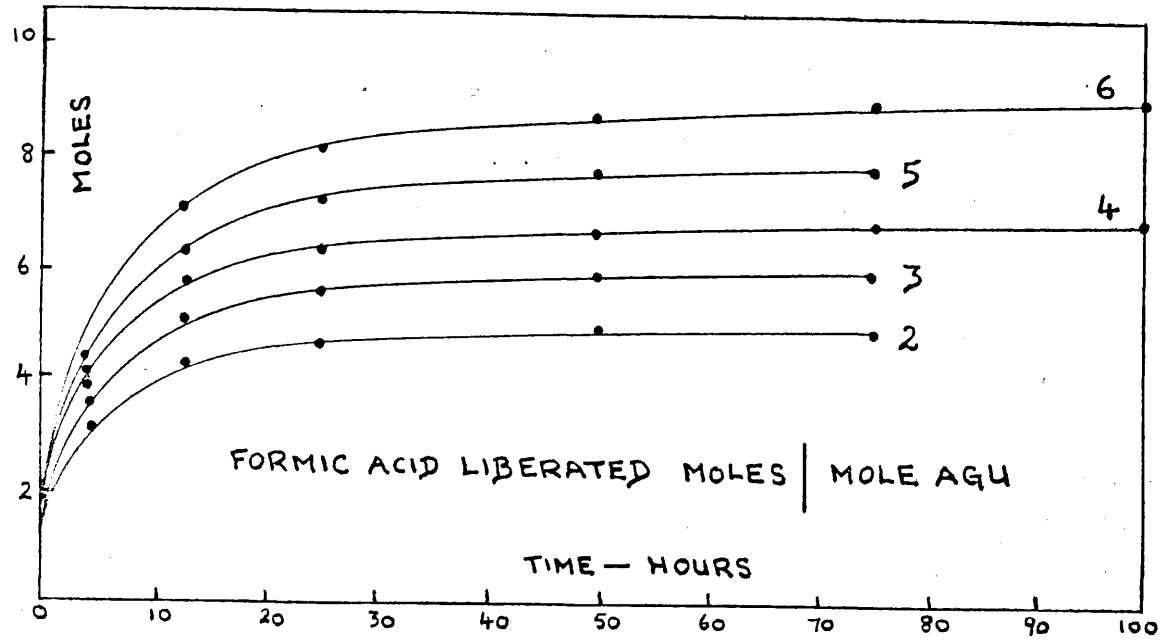
(1) HYDROLYSIS OF FORMATE ESTER.
(2) 1 MOLE OF PERIODATE.

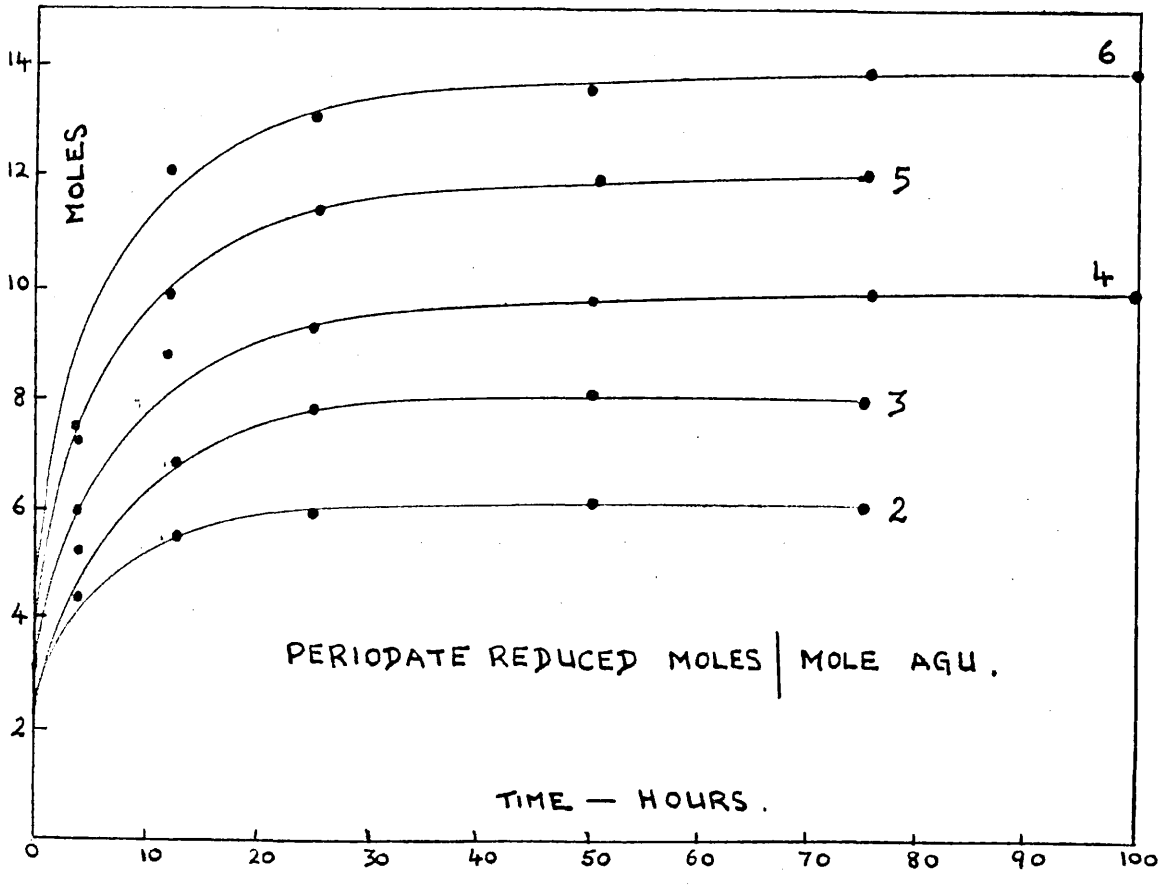


+ 2 HCOOH.

TOTAL (per MOLE of ISOMALTOSE)
Periodate reduced 6 MOLES.
Formic acid released 5 MOLES.

FIGURE III.3. RESULTS OF THE PERIODATE OXIDATIONS OF OLIGOSACCHARIDES FROM B-1299S DEXTRAN.





The results obtained from the periodate oxidation of oligosaccharide components 2 to 6 (expt. 8(g)) are shown in Table V.5. and are illustrated in Fig. III.3.

The amounts of periodate reduced and formic acid released are in good agreement with those calculated for isomaltose, isomaltotriose, isomaltotetraose, isomaltopentaose and isomaltohexaose.

The results of the investigations in this section of the thesis confirm the earlier suggestion that components 2 to 7 were respectively isomaltose and a series of higher homologues.

Component 8, which did not fit into this homologous series, was identified from its paper chromatographic and electrophoretic migration rates as kojibiose.

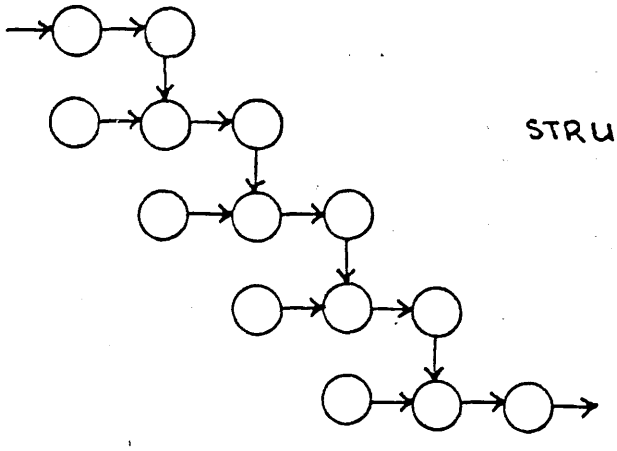
The presence of the isomaltose homologues in the hydrolysate indicates that the B-1299 S dextran must contain sequences of at least 7 α-(1→6) linked glucose units.

In terms of an overall structure it may be concluded that B-1299 S dextran is unlikely to be composed primarily of sequences of alternating 1→2 and 1→6 linked glucose units (Fig.III.4., structure XVII) but could consist of chains of 1→6 linked glucose units branching at intervals through the C_2 position (Fig. III.4., structure XVIII).

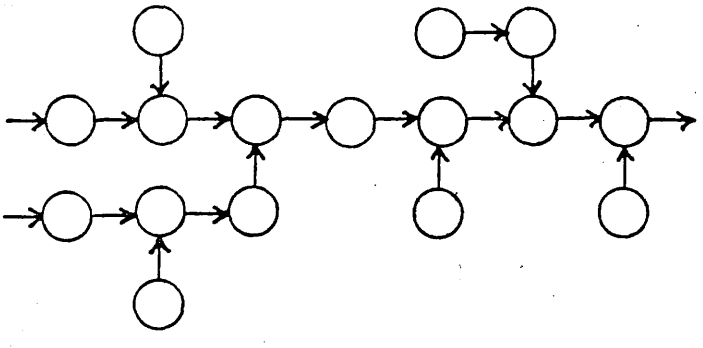
C. An investigation of the structures of oligosaccharides produced by acetolysis of the B-1299 S dextran

It was reported in Section II.D that the B-1299 dextrans yielded, on acetolysis, fragments having paper chromatographic and electrophoretic migration rates consistent with those of kojibiose, nigerose and isomaltose.

FIGURE III.4. POSSIBLE STRUCTURES FOR B-1299 S DEXTRAN INVESTIGATED IN ACID HYDROLYSIS EXPERIMENTS.



STRUCTURE XVII



STRUCTURE XVIII

→ 1→6 LINK.

↑↓ 1→2 LINK

○ GLUCOPYRANOSE UNIT.

The present acetolysis of B-1299 S dextran was conducted in order that these oligosaccharides could be more fully characterised and other, as yet unidentified, higher oligosaccharides could be examined.

B-1299 S dextran (30gm. - weight uncorrected for ash or moisture contents) was degraded with acetolysis reagents (expt. 9(b)) and, after deacetylation, the products were separated on a carbon-celite column (see Fig. V.2A. and Table V.6.) and refractionated on Whatman No.3 papers where necessary.

The final yields of the components 1, 2, 8 and 9 - which were the major products of the acetolysis - are shown in Table III.5., together with their degrees of polymerisation (expt. 9(e)), specific rotations (expt. 9(f)) and paper chromatographic and electrophoretic migration rates.

In addition, components 1 and 8 were crystallised from aqueous ethanol and converted into their β -penta acetate (expt. 7(d)) and β -octa acetate (expt. 9(d)) derivatives respectively. The melting points of these crystalline compounds are shown in Table III.5.

The amount of crystalline component 8, identified as kojibiose (2-o-α-D-glucopyranosyl D-glucopyranose) indicated that at least 20% of the glucosidic links of B-1299 S dextran were α-(1→2).

Syrupy component 2, which was slightly contaminated with component 8 (kojibiose), was identified by paper chromatography and electrophoresis as isomaltose (6-o-α-D-glucopyranosyl D-glucopyranose), which has already been obtained as a major product of the partial acid hydrolysis of B-1299 S dextran (see Section III.B.).

Table III.5. Properties of oligosaccharides from acetolysed B-1299 S dextran

Component	Yield (gm.)	R_G+	M_G borate	M_G* molybdate	Stain spray (c)	D.P.	Mpt °C	$[\alpha]_D$ (H_2O)	Acetate derivative	Identity
1	5.3	1.00	1.00	1.00	green	-	138 -143	+ 51°	β-pentaacetate mpt 130-131°	Glucose
2	0.06	0.50	0.72	0.70	green	2.0	syrup	-	-	Isomaltose
8	4.2	0.63	0.31	0.72	brown	1.9	187 -189	+137°	β-octaacetate mpt 117-121°	Kojibiose
9	0.06	0.72	0.70	0.00	grey?	1.9	syrup	+100°	-	Nigerose

+ solvent (a), * after reduction

Kojibiose has a reported[49] mpt. 186-187°C, $[\alpha]_{D(H_2O)}$ + 136.5°

Kojibiose β-octaacetate has a reported[49] mpt. 118°C

Nigerose has a reported[49] $[\alpha]_{D(H_2O)}$ + 128°

The small amount of syrupy component 9 which could not be crystallised - probably due to slight contamination with component 8 - had a high positive specific rotation and paper chromatographic and electrophoretic migration rates consistent with those of nigerose (3-o-α-D-glucopyranosyl D-glucopyranose).

This indicated that a small portion of the glucosidic links of B-1299 S dextran could be α-(1→3).

The crystalline disaccharide nigerose has, however, been isolated from unfractionated B-1299 dextrans in 2% yield,[14] and it is possible that in this instance the culturing conditions influenced the Leuconostoc organism to produce greater amounts of the less water soluble B-1299 L dextran which seems to contain a much higher percentage of 1→3 links than the B-1299 S dextran (see Section II.F. and II.G.).

Unfractionated, higher oligosaccharides (total weight 1 gram) were also eluted from the carbon-celite column (expt. 9(b)), and were subsequently fractionated by chromatography on Whatman No.3 papers and electrophoresis in sodium borate electrolyte (expt. 9(c)).

Each of these components was partially characterised by methods of partial acid hydrolysis, paper chromatography and electrophoresis in sodium borate[63] and molybdate[80] electrolytes: the degree of polymerisation (see expt. 9(e)) of each component is shown in Table III.6., together with its chromatographic and electrophoretic migration rates and specific staining properties.

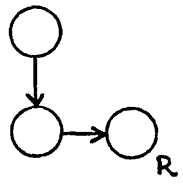
Table III.6. Chromatographic properties of some oligosaccharides from acetolysed B-1299 S dextran

Component	D.P.	R_G solvent (a)	M_G borate	M_G* molybdate	Staining intensity spray (a)	Colour spray (c)
21	3.0	0.38	0.27	0.57 0.12	3+ +	brown
3	2.9	0.31	0.61	0.58		green
22	4.1	0.30	0.24	0.51 0.10	3+ +	brown
23	3.7	0.23	0.51	0.48		green
24	3.9	0.23	0.22	0.50 0.10	2+ 3+	brown

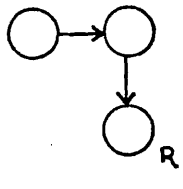
* after reduction with sodium borohydride

Component 21 was a trisaccharide whose rate of migration on paper chromatograms (solvent (a)) was greater than that of isomaltotriose (R_G 0.30). Partial acid hydrolysis of component 21 gave degradation products having migration rates identical to those of glucose, kojibiose and isomaltose.

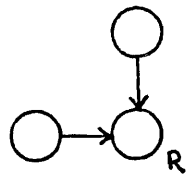
This would indicate that component 21 could be a mixture of isomeric trisaccharides with structures XIX, XX and XXI.



XIX



XX



XXI

KEY

→ 1→6 LINK

↓ 1→2 LINK

O_R REDUCING GLUCOSE UNIT.

In borate electrophoresis (Section III.A(i)) trisaccharide XIX would have an M_G value (see page 63) similar to that of isomaltotriose (M_G 0.60) and give a green stain in spray reagent (c), characteristic of an oligosaccharide with a 6-substituted reducing glucose unit.[46] Trisaccharides XX and XXI would have much lower M_G values, similar to that of maltotriose (M_G 0.20), and give a brown stain - characteristic of an oligosaccharide with a 2-substituted reducing glucose unit - with spray reagent (c).

The experimental results shown in Table III.6., therefore, indicate that component 21 cannot have the structure XIX.

In molybdate electrophoresis (Section III.A(ii)) the reduced trisaccharide XX would have a high M_S value (see page 66), similar to isomaltotri-itol (M_S 0.59), expected of a reduced trisaccharide terminating in a 2 or 6 substituted glucitol unit.

In contrast, reduced trisaccharide XXI would have a much lower M_S value expected of a reduced trisaccharide terminating in a 2,6 di-substituted glucitol unit (see page 71).

The experimental evidence summarized in Table III.6. indicates that component 21 must be a mixture of trisaccharides XX and XXI. Trisaccharide XX appears to be the major constituent of this mixture.

It is also conceivable that kojitriose[86] (2-o-α-D-glucopyranosyl -2-o-α-D-glucopyranosyl -D-glucopyranose) could be present in this mixture as the chromatographic and electrophoretic properties of this compound have not as yet been sufficiently reported,[86] and are probably similar to those of component 21.

Component 3 was a trisaccharide whose migration (solvent (a)) and staining (spray (c)) properties were identical to those of isomaltotriose.

Partial acid hydrolysis of component 3 gave degradation products having paper chromatographic migration rates (solvent (a)) identical to those of glucose and isomaltose. The degradation product chromatographing as isomaltose, after elution from a chromatogram, had an M_G value in borate electrophoresis consistent with that of isomaltose and, after reduction, an M_G value in molybdate electrophoresis equal to that of isomaltitol.

Trisaccharide 3 must therefore be isomaltotriose, which has already been identified as a major product of the partial acid hydrolysis of B-1299 S dextran (see Section III.B.).

Component 22 was a tetrasaccharide whose paper chromatographic migration rate (solvent (a)) was the same as that of isomaltotriose, but component 22 gave a brown stain with spray reagent (c), and could be separated from isomaltotriose by borate electrophoresis.

Partial acid hydrolysis of component 22 gave the degradation products shown in Table III.7.

Table III.7. Chromatographic properties of the degradation products from component 22

Degradation product	R_G solvent (a)	M_G borate	M_G* molybdate	Identity
22.1	1.00	-	-	Glucose
22.2	0.62	0.30	0.69	Kojibiose
22.3	Streak	0.70	-	Isomaltose
	0.50 → 0.36	0.28		Mixed trisaccharides

* After reduction with sodium borohydride

From the figures in Table III.7. degradation product 22.1 was identified as glucose and 22.2 as kojibiose.

The product 22.3, which streaked on the paper chromatogram, appeared to be a mixture containing isomaltose, component 21 and possibly a third component with an R_G value intermediate between these two.

It seems possible that this third component is the trisaccharide kojitriose,[86] for the indirect evidence presented below suggests that kojitriose would have an R_G value between that of isomaltose and component 21.

In solvent (a) the trisaccharides isomaltotriose, panose[87] and maltotriose have R_G values of 0.31, 0.40 and 0.49 respectively.

Isomaltotriose contains two α-(1→6) links, panose a single α-(1→6) and a single α-(1→4) link and maltotriose two α-(1→4) links.

The calculated R_M values of the trisaccharides are: isomaltotriose (0.35), panose (0.18) and maltotriose (0.02) - indicating that the differences in R_M values between isomaltotriose and panose, and panose and maltotriose are almost equal.

If the series of trisaccharides, isomaltotriose, component 21 (a 1→2/1→6 linked trisaccharide) and kojitriose (a 1→2 linked trisaccharide), are now considered, by analogy, the differences in the R_M values between isomaltotriose and component 21 and between component 21 and kojitriose should be nearly equal (although these differences will not be the same as that of the earlier series).

The R_G values of isomaltotriose and component 21 in solvent (a) are 0.31 and 0.38 respectively, indicating a difference of 0.135 in their calculated R_M values. If 0.135 also represents the difference of R_M value between component 21 and kojitriose the latter would have an R_G value of 0.45 - and could therefore be a constituent of degradation product 22.3.

The presence of isomaltose and kojibiose in the hydrolysate of component 22 indicate that the latter contains only 1→6 and 1→2 linked glucose units.

If kojitriose is present in the same hydrolysate this would further indicate that two of the glucosidic links were 1→2.

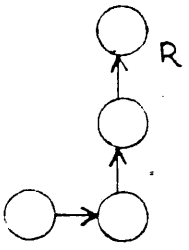
The migration rate of component 22 (R_G 0.30) also appears to indicate that this tetrasaccharide contains two 1→2 and only a single 1→6 link, for enzymic degradation of B-1299 S dextran (Section III.D(iii)) yielded two isomeric tetrasaccharides XXVIII and XXIX with R_G values (solvent (a)) of 0.24, which when oxidized with periodate proved to contain two 1→6 and a single 1→2 linkage.

If component 22 does indeed contain two 1→2 links, then it could be a mixture of the isomeric tetrasaccharides XXII, XXIII, XXIV, XXV, XXVI and XXVII.

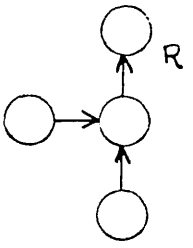
The experimental results shown in Table III.6. indicate that component 22 could not have the structure XXVII, for this tetrasaccharide would have a high M_G value in borate-electrophoresis, similar to that of isomaltotetraose (M_G 0.50) and would stain green with spray reagent (c).[46]

The other isomers would have lower M_G values, similar to that of maltotetraose (M_G 0.24) and give a brown stain with spray reagent (c) (characteristic of an oligosaccharide with a 2-substituted reducing glucose unit) and are therefore all possible.

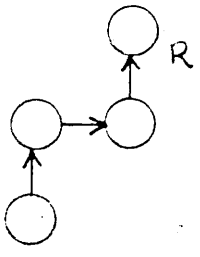
In molybdate electrophoresis the reduced tetrasaccharides XXII, XXIII and XXIV would have high M_B values - similar to isomaltotetraitol (M_B 0.50) - expected of reduced tetrasaccharides terminated by a 2 or 6



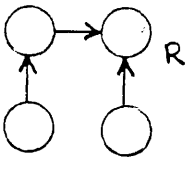
XXII



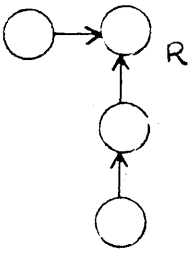
XXIII



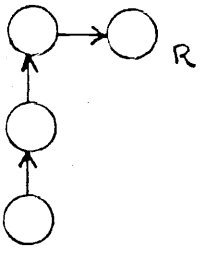
XXIV



XXV



XXVI



XXVII

→ 1→6 LINK

↑ 1→2 LINK

O_R REDUCING GLUCOSE UNIT.

substituted glucitol unit. In contrast the reduced tetrasaccharides XXV and XXVI would have the lower M_G values expected of reduced tetrasaccharides terminated by a 2,6 di substituted glucitol unit.

The experimental results in Table III.6. show that, on reduction, component 22 migrates with M_G values of 0.51 and 0.10, and could in consequence be a mixture of tetrasaccharides XXII, XXIII, XXIV, XXV and XXVI. The relative intensities of the spots on the molybdate electrophoretogram would, however, suggest that tetrasaccharides XXII, XXIII and XXIV are the major oligosaccharides in this mixture.

Component 23 was a tetrasaccharide, whose rate of migration on paper chromatograms eluted with solvent (a) was marginally greater than that of isomaltotetraose (R_G 0.22).

Partial acid hydrolysis of component 23 gave the degradation products shown in Table III.8.

Table III.8. Chromatographic properties of the degradation products from component 23

Degradation product	R_G solvent (a)	M_G borate	M_G* molybdate	Identity
23.1	1.00	-	-	Glucose
23.2	0.60	0.30	0.68	Kojibiose
23.3	0.50	0.68	0.68	Isomaltose
23.4	0.35	0.56 0.23	-	Mixed trisaccharides

* After reduction with sodium borohydride

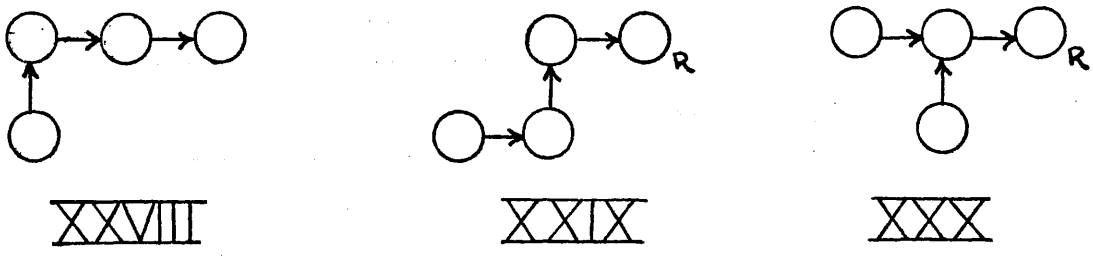
From the figures in Table III.3. degradation product 23.1 was identified as glucose, 23.2 as kojibiose and 23.3 as isomaltose.

The presence of kojibiose and isomaltose in the hydrolysate of component 23 indicate that the component contains only 1→2 and 1→6 linked glucose units.

The chromatographic migration rate of component 23 (R_G 0.23) was identical to that of the isomeric tetrasaccharides XXVIII and XXIX, isolated from an enzymic hydrolysate of the B-1299 S dextran (Section III.D(iii)).

Periodate oxidation of tetrasaccharides XXVIII and XXIX had indicated that they contained two 1→6 linkages and a single 1→2 link, and it seems probable that a similar ratio of the glucosidic links is present in component 23.

Component 23 gave a green stain with spray (c), and had a high M_G value in borate electrophoresis - similar to isomaltotetraose (M_G 0.50) - from which it was concluded that the component could be a mixture of isomeric tetrasaccharides XXVIII, XXIX and XXX, each of which terminates in a 6-substituted reducing glucose unit.



XXVIII XXIX XXX

→ 1→6 LINK

↑ 1→2 LINK

O_R REDUCING GLUCOSE UNIT.

Tetrasaccharides XXVIII and XXIX were also isolated from enzymic hydrolysates of B-1299 S dextran. A more complete characterization is described in Section III.D(iii).

Component 24 was a tetrasaccharide which migrated slightly faster than isomaltotetraose (R_G 0.22) in solvent (a).

Partial acid hydrolysis of component 24 gave the degradation products shown in Table III.9.

Table III.9. Chromatographic properties of the products from degradation of component 24

Degradation product	R_G solvent (a)	M_G borate	M_G* molybdate	Identity
24.1	1.00	-	-	Glucose
24.2	0.60	0.30	0.69	Kojibiose
24.3	0.50	0.70	0.69	Isomaltose
24.4	0.35	0.61	0.52	Mixed trisaccharides
		0.26	0.11	

* After reduction with sodium borohydride

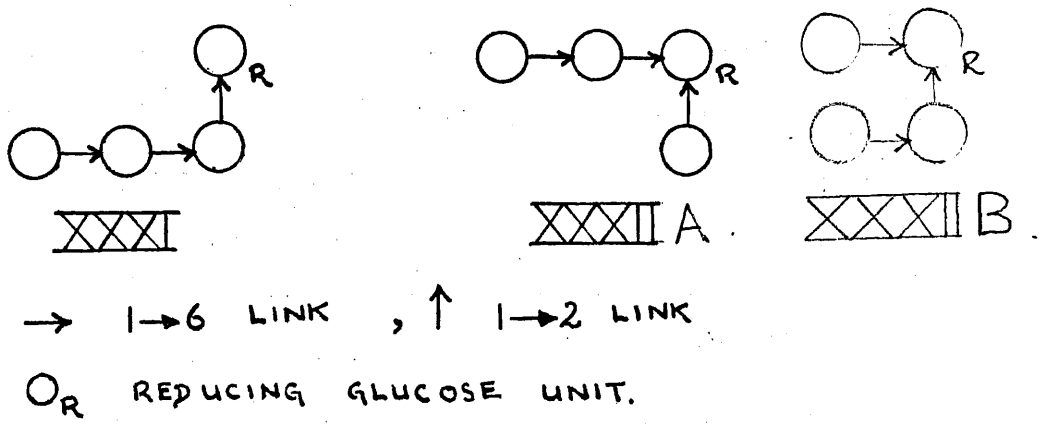
The results summarized in Table III.9. indicate that component 24.1. is glucose, 24.2. is kojibiose and 24.3 is isomaltose. The presence of kojibiose and isomaltose in the hydrolysate indicate that component 24 contains only 1→2 and 1→6 linked glucose units.

The chromatographic migration rate of component 24 in solvent (a) was identical to that of isomeric tetrasaccharides XXVIII and XXIX which were isolated from the enzymically degraded B-1299 S dextran (see Section III.D (iii)).

As periodate oxidation experiments had indicated that tetrasaccharides XXVIII and XXIX contained two 1→6 and a single 1→2 linkage it was concluded that component 24 contained these links in a similar ratio.

In borate electrophoresis, however, component 24 had a much lower M_G value - similar to that of maltotetraose (M_G 0.24) - than either tetrasaccharides XXVIII or XXIX, and gave a brown stain with spray reagent (c).

Component 24 must, therefore, be terminated by a 2-substituted reducing glucose unit, and in consequence could only be a mixture of tetrasaccharides with structures XXXI or XXXII.



In molybdate electrophoresis the reduced tetrasaccharide XXXI would have a high M_G value - similar to isomaltotetraitol (M_G 0.50) - expected of a reduced tetrasaccharide terminating in a 2 or 6 substituted glucitol unit.

Reduced tetrasaccharide XXXII, however, would have a much lower M_G value expected of a reduced tetrasaccharide terminated by a 2,6 di-substituted glucitol unit.

The experimental results, summarized in Table III.6. indicate that component 24 must be a mixture of tetrasaccharides XXXI and XXXII. Tetrasaccharide XXXII, however, appears to be the major constituent of this mixture.

On the basis of the linkage to the glucose unit at the reducing end of the molecule these higher oligosaccharides from acetolysed B-1299 S dextran may be divided into the following groups.

(a) Isomaltotriose, and tetrasaccharides XXVIII, XXIX and XXX, which are terminated by a 6-o-substituted reducing glucose unit.

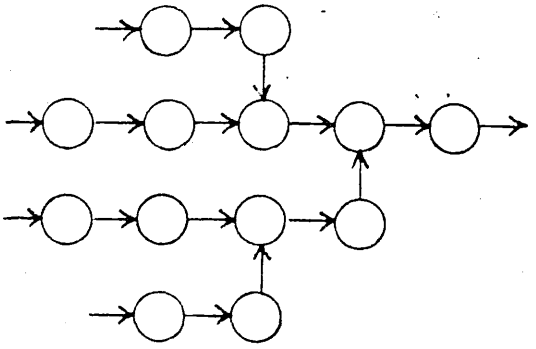
(b) Trisaccharide XX, and tetrasaccharides XXII, XXIII, XXIV and XXXI, which are terminated by a 2-o-substituted reducing glucose unit.

(c) Trisaccharide XXI, and tetrasaccharides XXV, XXVI and XXXII, which are terminated by a 2,6 di-o-substituted reducing glucose unit.

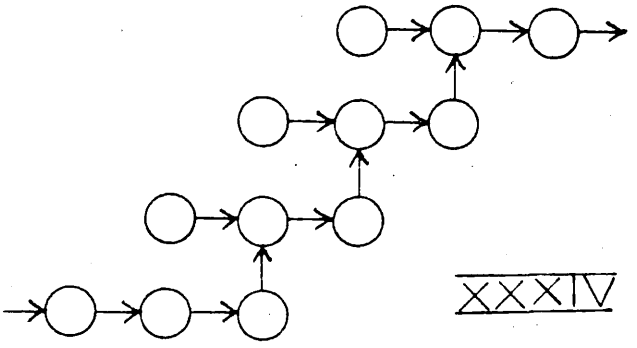
Although the oligosaccharides falling into group (c) are further evidence that B-1299 S dextran has a branched structure, only members of group (b) have any important new structural significance: for fragments of this kind could only have arisen from an arrangement where a 1→2 link in the B-1299 S dextran is in an intra chain position.

It has already been established (Section II.G.) that all the 1→2 links of B-1299 S dextran form branching points, and it is therefore likely that this dextran contains segments of the kind represented by structures XXXIII and XXXIV in Fig. III.5. It would appear, from a consideration of the results of partial acid hydrolysis experiments (Section III.B.), that structural segment XXXIII is rather more important than XXXIV.

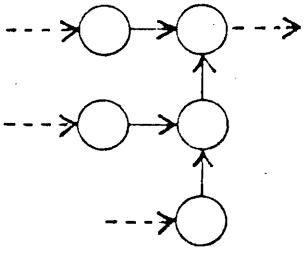
FIGURE III·5.



XXXIII



XXXIV



XXXV

→ 1→6 LINK

↑↓ 1→2 LINK

---> α— LINKAGE

○ GLUCOPYRANOSE UNIT

The absence of a homologous series of oligosaccharides based on either kojibiose or nigerose indicate that extended sequences of secondary linked glucose units do not occur in the B-1299 S dextran.

Examination of the tetrasaccharide component 22 did suggest, however, that two 1→2 links may be adjacent in this dextran - giving rise to a segment of the kind shown in Fig. III.5., structure XXXV.

The presence of intra chain 1→2 links in the B-1299 S dextran is particularly interesting, as few dextrans so far examined appear to contain non 1→6 links within the main chain.

Exceptions so far reported are B-1299 S dextran - from which the trisaccharide 3-o-isomaltosyl-D-glucose has been isolated[42] - and the 1→3 branched NCIB-2706[57] and B-512[77] dextrans. Significantly, B-1298 dextran[49] - which is similar to B-1299 S dextran in that α-(1→2) links form many of the branching points - failed, on acetolysis, to give higher oligosaccharides terminated by a 2-o-substituted reducing glucose unit, and so the branches would appear to consist of single α-(1→2) linked glucosyl units.

D. <u>The enzymic degradation of B-1299 S dextran to investigate the arrangements of the glucose units at the branching points</u>

Tsuchiya (1952)[89] reported that certain species of <u>Penicillium</u> moulds, when cultured on NRRL B-512 dextran (95% α-(1→6) linked), produced exocellular enzymes (dextranases) which degraded this and other dextrans to give isomaltose and isomaltotriose in high yields. The culture fluids from these moulds were, however, found to possess only a limited capacity to hydrolyse dextrans with high degrees of branching.

Later, Weigel[90] and co-workers were able to induce selected *Penicillium* moulds to grow in media containing the essentially unbranched *S. Bovis*[92] dextran, and so to produce dextranases virtually uncontaminated by other carbohydrases.

The exocellular dextranases of *P. lilacinum* (NRRL 896, IMI 79197) and *P. funiculosum* (NRRL 1132, IMI 79195) were shown to hydrolyse *S. Bovis* dextran, isomaltodextrins and modified isomaltodextrins containing not less than three α-glucosidic linkages.[90] The dextran of *L. mesenteroides* NRRL B-1375 (an α-(1→3) branched dextran) was degraded to glucose, isomaltose, isomaltotriose and a complex mixture of 'branched' oligosaccharides.[76]

To explain these findings Hutson and Weigel proposed[91] an 'action pattern' for each dextranase, which is summarized as follows:

(a) The smallest linear (non-branching) system hydrolysed by either dextranase was of the type represented by structure XXXVI in figure III.6.

It had a single point of hydrolysis at the central glucosidic linkage, in contrast to the slightly larger system, represented by structure XXXVII, which had two equivalent points of hydrolysis.

(b) The most readily hydrolysed system, realised in an unbranched dextran, consisted of a long chain of α-(1→6) linked glucose units.

(c) The structures of the 'branched' oligosaccharides obtained from NRRL B-1375 dextran,[76] shown in figure III.7., indicated that, within this system, certain α-(1→6) linkages were resistant to the action of the dextranases by virtue of their proximity to the α-(1→3)

branching linkage. These resistant linkages are shown in figure III.8.

However, slow hydrolysis of these oligosaccharides when incubated for long periods with dextranase suggested that the resistance was not total.

These dextranase 'action patterns'[91] enabled the structures of oligosaccharides obtained from dextranase digests of the α-(1→4) branched NRRL B-1415 dextran to be correctly predicted;[75] results which also indicated that the 'action patterns' were unaffected by the types of -linkages forming the branching points.

It was significant that the branches of the oligosaccharides - shown in figure III.7. - consisted of a single glucosyl unit. Indeed this fact was the first indication[76] that in the parent dextrans the branches might also consist of a single gluconyl unit - later proved by an unambiguous method.[27]

Thus the oligosaccharides arose from the enzymic hydrolysis of α-(1→6) links of the main chain.

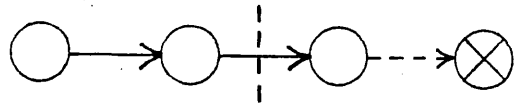
It was now of interest to investigate the effect of dextranases on the structure of L. mesenteroides NRRL B-1299 S dextran.

The results of these investigations are discussed under the following headings.

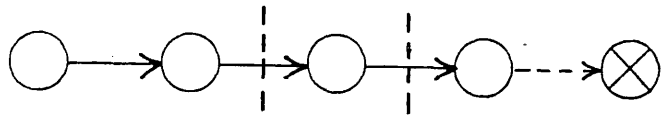
(i) Initial failure to degrade native B-1299 S dextran with two dextranases.

(ii) The debranching of native B-1299 S dextran by controlled acid hydrolysis.

FIGURE III·6. —— THE ACTION OF DEXTRANASES ON TWO LINEAR SYSTEMS.



XXXVI



XXXVII

○ GLUCOSYL UNIT.

⊗ GLUCOSE, FRUCTOSE, SORBITOL, METHYL GROUP.

→ $\alpha-(1\rightarrow6)$ GLUCOSIDIC LINK.

---→ $\alpha-$ GLUCOSIDIC LINK

--- PRINCIPLE POINT OF HYDROLYSIS.

FIGURE III.7. —— OLIGOSACCHARIDES PRODUCED BY THE ACTION OF PENICILLIUM DEXTRANASES ON NRRL B-1375 AND NRRL B-1415 DEXTRANS

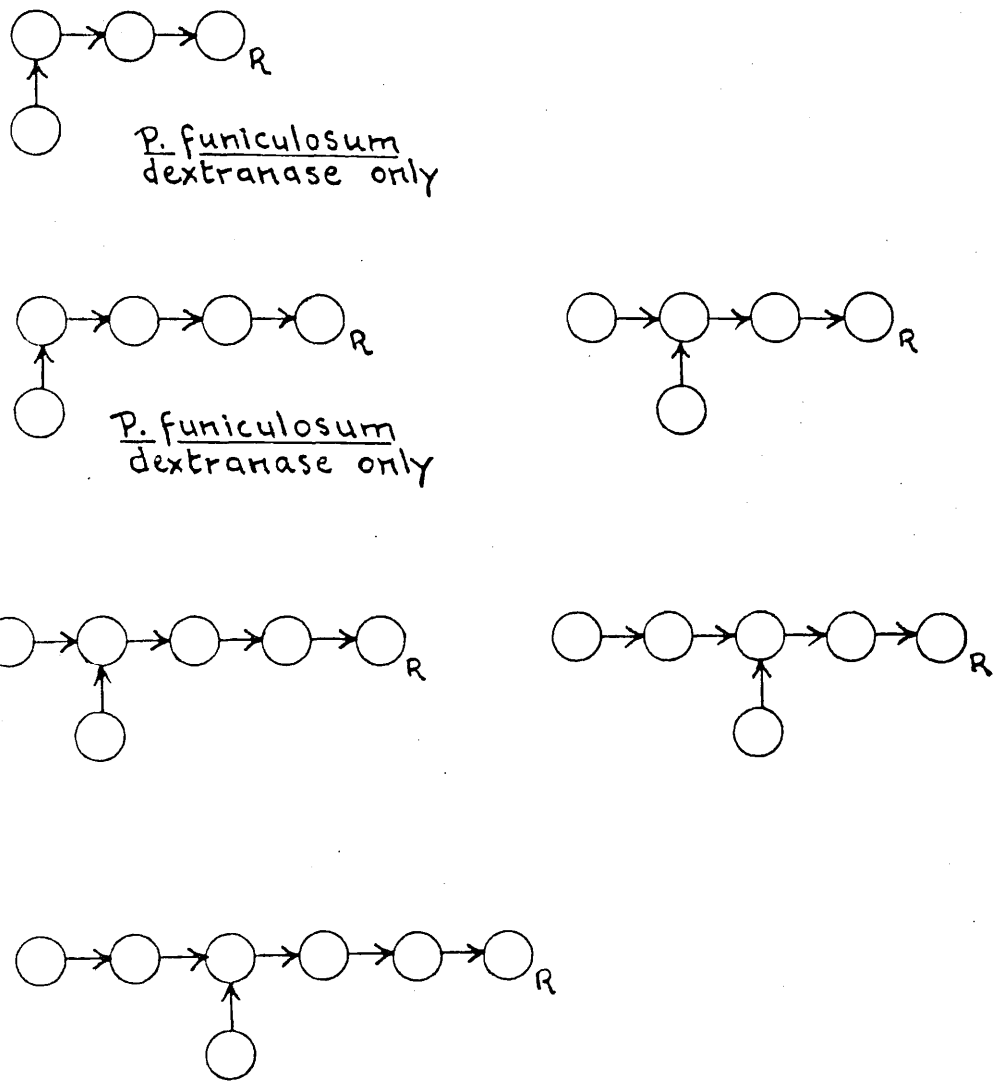
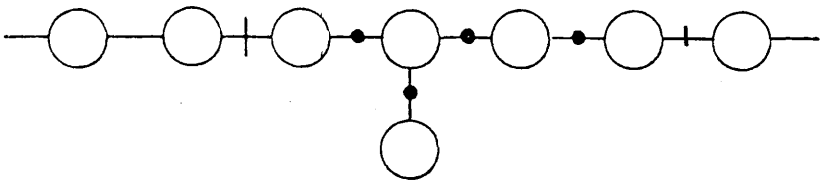
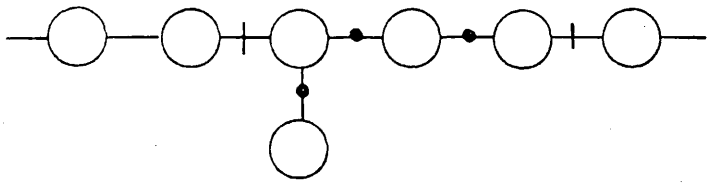


FIGURE III·8. DEXTRANASE RESISTANT LINKS IN AN α-(1→3) BRANCHED DEXTRAN.



P. LILACINUM DEXTRANASE.



P. FUNICULOSUM DEXTRANASE

○ GLUCOSYL UNIT , — α-(1→6) LINK.

| α-(1→3) LINK , —•— RESISTANT LINK IN DEXTRAN AND OLIGOSACCHARIDES.

+ ADDITIONAL RESISTANT LINK IN OLIGOSACCHARIDES.

(iii) The characterisation of oligosaccharides produced by the action of P. lilacinum dextranase on acid degraded B-1299 S dextran.

(iv) Summary of results in Section III.D., and some general conclusions.

(i) Initial failure to degrade native B-1299 S dextran with two dextranases

The dextranase from P. funiculosum (NRRL 1132) was obtained from the departmental collection. The P. lilacinum (NRRL 896) dextranase was prepared in the usual way[90] by culturing P. lilacinum mould in a medium containing S. Bovis dextran as the sole source of carbohydrate (expt. 14 (a)).

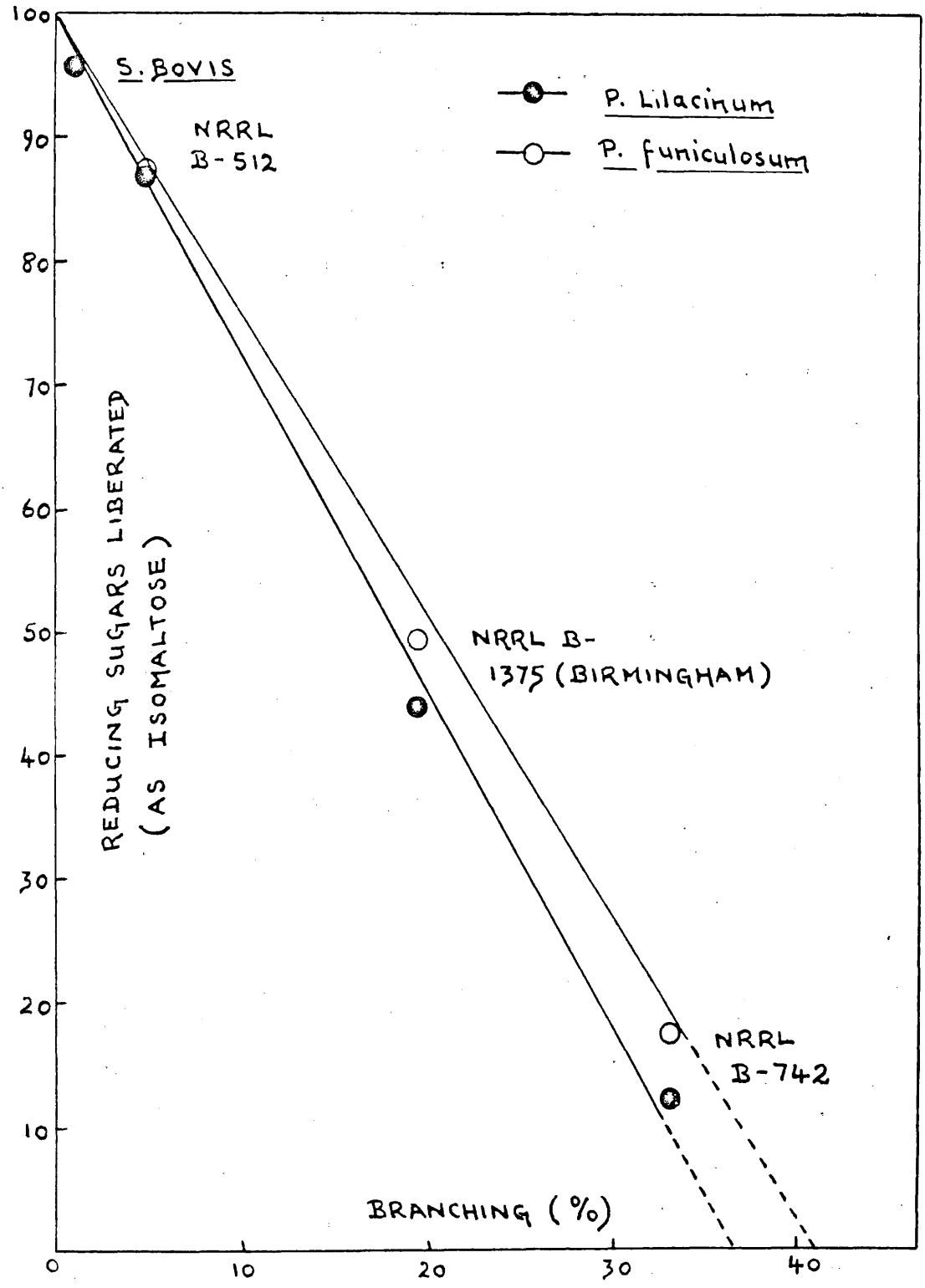
The S. Bovis dextran had been prepared according to the method of Bailey[92] (expt. 14(b)) and had 95% of 1→6 links, when these were determined from periodate oxidation data[13] (expt. 14(c)).

Each of the dextranases readily hydrolysed S. Bovis, NRRL B-1375 and NRRL B-1415 dextrans in standard digests, to give products chromatographing as glucose, isomaltose, isomaltotriose and higher 'branched' oligosaccharides[75,76] (expt. 14(d)).

The P. lilacinum (extract) had an activity of 0.12 dextranase units/ml., and the P. funiculosum (freeze dried) of 1.25 dextranase units/mg.; when 1 dextranase unit is the amount of enzyme which will liberate 1 mg. equivalent of isomaltose monohydrate in 1 hour at 40°C from S. Bovis dextran (Tsuchiyas' definition).[89] Details of the determination of the activities are given in experiment 14(e).

While the P. funiculosum dextranase was able to release traces of oligosaccharides, the P. lilacinum preparation was unable to affect any degradation of the native B-1299 S dextran (or of the native B-1299 L

FIGURE III.9. —— ACTION OF DEXTRANASES ON FOUR DEXTRANS



dextran).

This failure to affect appreciable degradation of B-1299 S dextran is attributed to the very high degree of branching of the molecule (page 52), as earlier experiments[90] had indicated that degradation of a dextran by these dextranases would be unlikely if the molecule had a degree of branching in excess of 41% (P. funiculosum) or 37% (P. lilacinum).

Fig. III.9. summarises this work in which the 'ease' of enzymic hydrolysis (expressed in terms of the amount of isomaltose equivalent liberated, compared with the amount which could theoretically be released from the α-(1→6) linkages present) is compared with the degree of branching of the dextran.

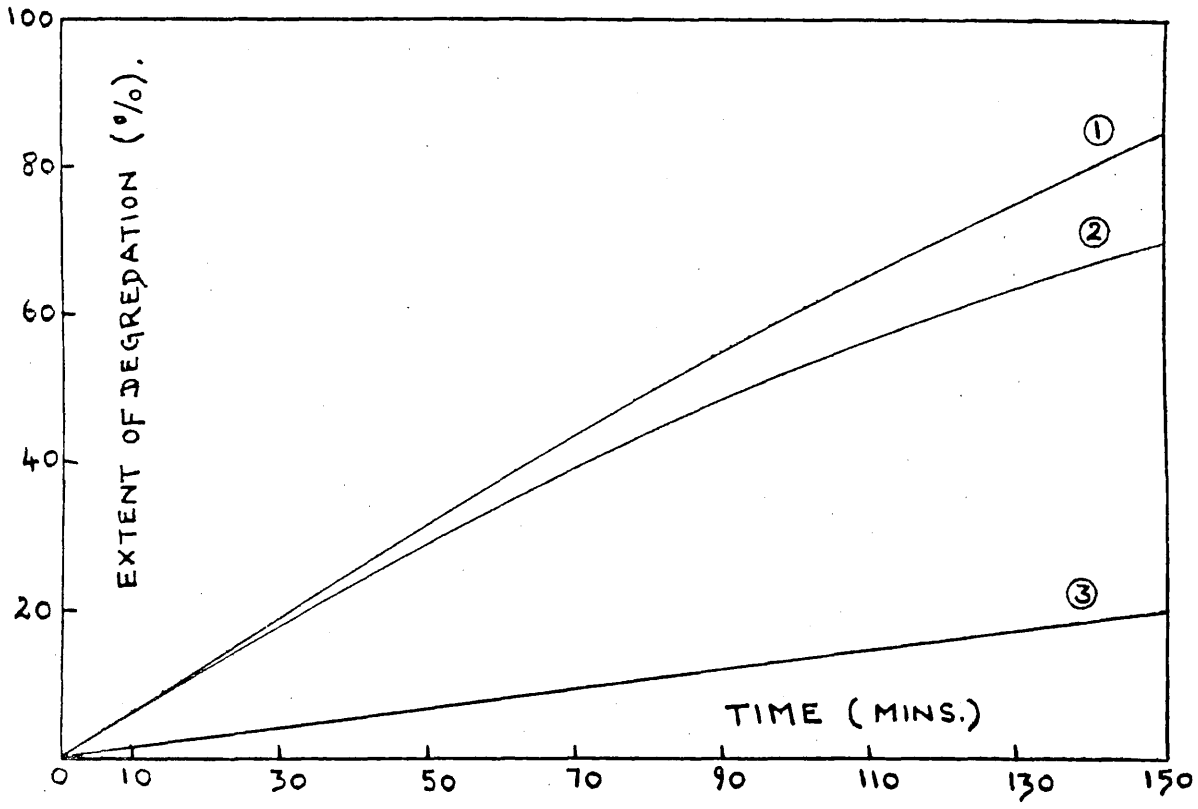
It was apparent from this evidence that before either of the dextranases could extensively degrade B-1299 S dextran a considerable debranching of the structure would be necessary.

(ii) The debranching of native B-1299 S dextran by controlled acid degradation

Methylation and acetolysis experiments (sections II.G. and III.C.) had shown that the branching in native B-1299 S dextran occurred exclusively through α-(1→2) linked glucose units, although a small percentage of α-(1→3) linked units were situated at non-branching points. Together these dextranase resistant linkages accounted for 40% of the total glucosidic linkages in this dextran.

In addition, partial acid hydrolysis of the dextran (Section III.B.) indicated that the molecule contained sequences of α-(1→6) linked glucose units - which probably formed the 'skeleton' of the molecule.

FIGURE III·10 — HYDROLYSIS VELOCITIES OF THREE α-GLUCOBIOSES, WITH N-SULPHURIC ACID AT 85 °C.



① KOJIBIOSE
② NIGEROSE
③ ISOMALTOSE

Wolfrom[39] and Matsuda,[40] experimenting independently with α-glucose disaccharides, have demonstrated that the rates of acid hydrolysis of the α-(1→2) and α-(1→3) linkages are considerably greater than that of an α-(1→6) link (Fig. III.10.).

This indicated the possibility of achieving the necessary debranching by a mild hydrolysis of the B-1299 S dextran.

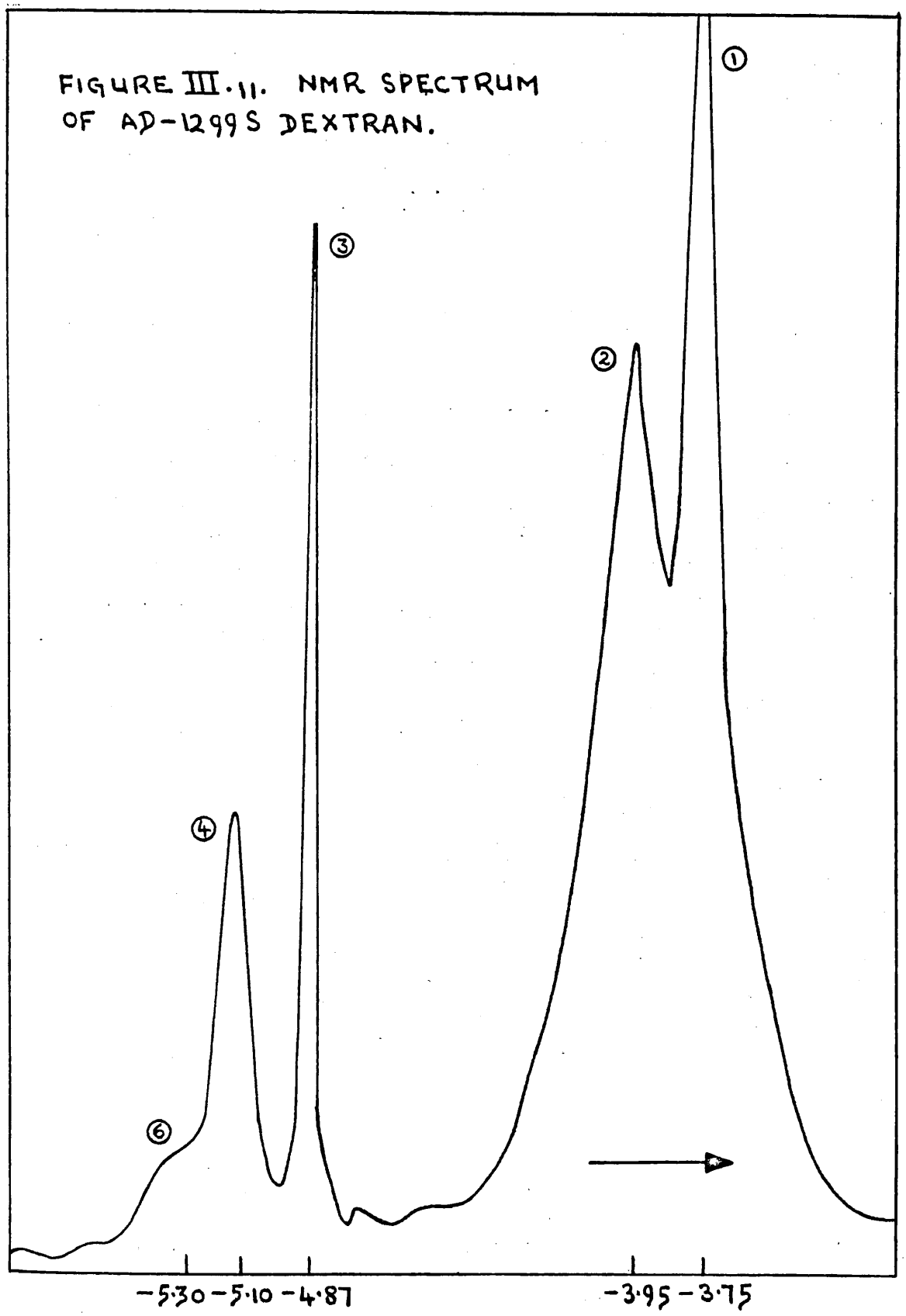
In experiment 14(f)(i) - using as a guide for the hydrolysis conditions the values given in Fig. III.10. - samples of B-1299 S dextran were hydrolysed with dilute acid for different periods of time, to produce a series of variously degraded dextrans. It was noted that, as the time of hydrolysis increased, the return of degraded dextran decreased as shown in Fig. V.6., but the more highly degraded of these dextrans gave the greater yields of oligosaccharides (estimated visually from chromatograms) after incubation with each of the dextranases (expt. 14(d)).

Both of these factors were considered when selecting conditions for a larger scale acid degradation of B-1299 S dextran. In expt. 14(f)(ii), the B-1299 S dextran (200 gm.) was hydrolysed as before to yield degraded dextran (55 gm.), plus glucose and isomaltose homologues which were recovered from dialysates.

The degraded dextran, which will be referred to as AD-1299 S dextran, was oxidised with sodium metaperiodate (expt. 14(g)) and, from the experimental results, by applying the analysis described on pages 29-31,[13] it was calculated that 91% of the linkages were 1→6 and 9% 1→2.

In addition the NMR spectrum of the deuterated AD-1299 S dextran (Fig. III.11.) showed a greatly increased signal - at -5.10 ppm - from

FIGURE III.11. NMR SPECTRUM OF AD-1299S DEXTRAN.



protons associated with the 1→6 glucosidic linkage when compared with that of the native B-1299 S dextran (Fig. II.4.). The presence of 1→2 glucosidic linkages was indicated by the additional signal - a shoulder at -5.28 ppm to the main peak - which was not observed in the spectrum of the 1→6 linked B-512 dextran (Fig. II.3.).

(iii) The characterisation of oligosaccharides produced by the action of P. lilacinum dextranase on acid degraded B-1299 S dextran

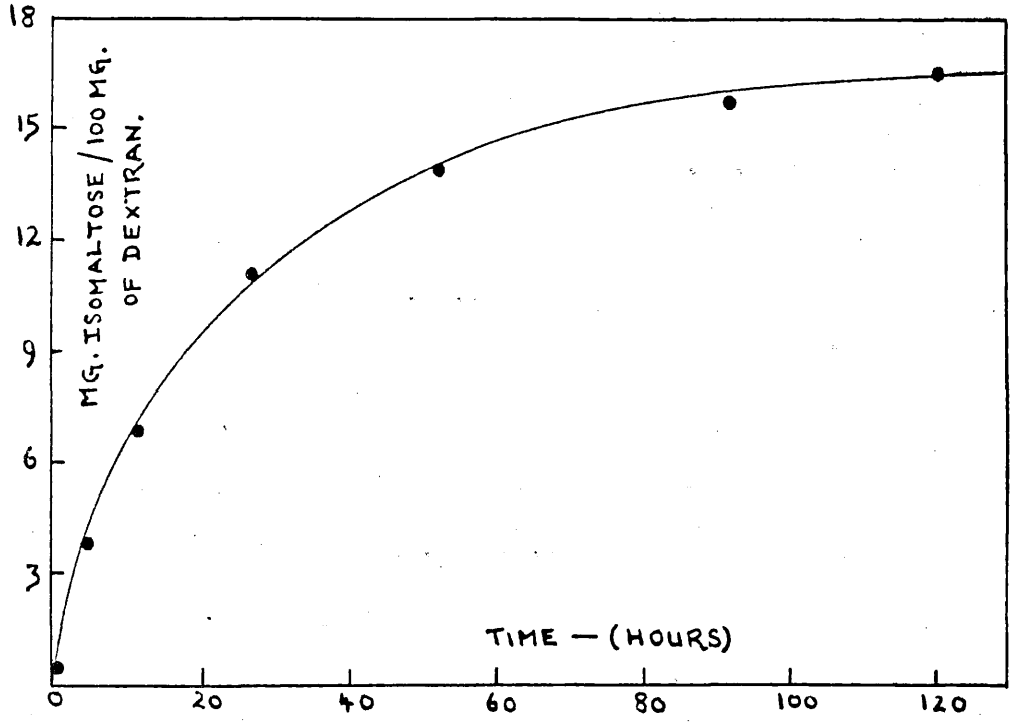
The AD-1299 S dextran was incubated with P. lilacinum dextranase (the one chosen for future study) to determine its 'degradability' in terms of the reducing power (as isomaltose) of the oligosaccharides liberated. The results are summarized in Table V.21. (see expt. 14(h)).

The absence of a limiting value for the released reducing power, which is seen clearly in Fig. II.12., is probably due to a slow hydrolysis of the dextranase resistant 1→6 linkages described in the 'action patterns' on page 97 .

It is particularly interesting that, if the AD-1299 S dextran is compared with the dextrans in Fig. III.9., it shows a far greater resistance to the P. lilacinum dextranase than could be anticipated from the numbers of 1→2 links present in the molecule. The amount of reducing sugars (estimated as isomaltose) liberated from AD-1299 S dextran (17%) is much smaller than that expected[90] (72%) from the results summarized in Fig. III.9., for a dextran containing 9% of 1→2 links.

This resistance was again demonstrated when paper chromatography of the products of AD-1299 S dextran-dextranase digests (expt. 14(d)) revealed large amounts of unresolved 'high molecular weight' material.

FIGURE III·12. —— LIBERATION OF REDUCING SUGARS (AS ISOMALTOSE) BY P. LILACINUM DEXTRANASE FROM AD-1299S DEXTRAN.



Some of the paper chromatographic properties of the resolvable oligosaccharides from these digests are shown in Table III.10..

Each component gave a green stain with spray reagent (c) characteristic of a 6-substituted reducing glucose unit. The major products, components 2 and 3, were chromatographically identical to isomaltose and isomaltotriose in solvent (b), but the higher oligosaccharides 25, 26, 27, 28 and 29 migrated marginally faster than the isomaltose homologues with degrees of polymerisation 4, 5, 6, 7 and 8 respectively.

When the R_M value (see page 157) of each of these components was plotted against a degree of polymerisation, provisionally assigned on the basis of the chromatographic migration rate, a straight line resulted - indicating the possibility that these oligosaccharides formed a homologous series distinct from the isomaltodextrins, with which they are compared in Fig. III.13..

FIGURE III.13.

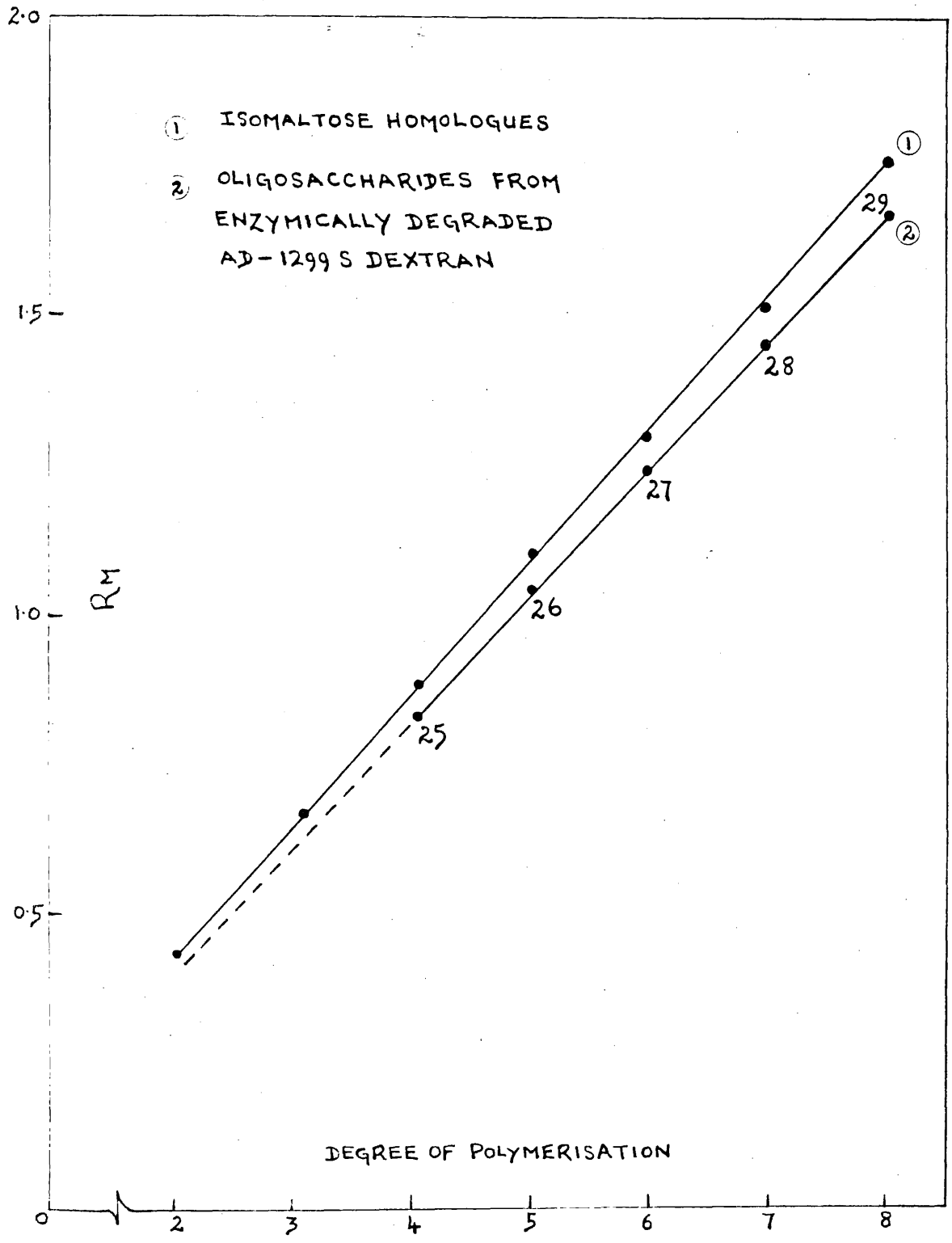


Table III.10. Oligosaccharides produced by enzymic degradation of AD-1299 S dextran

Component	R_G solvent (b)	R_M	Staining colour spray (c)	Staining intensity spray (a)	Possible identity
1	1.00	-	-	+	Glucose
2	0.74	0.45	green	4+	Isomaltose
3	0.55	0.70	green	3+	Isomaltotriose
25	0.41	0.95	green	+	'Branched' trisaccharide
26	0.27	1.10	green	1½+	'Branched' pentasaccharide
27	0.18	1.30	green	+	'Branched' hexasaccharide
28	0.12	1.55	green	+	'Branched' heptasaccharide
29	0.07	1.75	green	+	'Branched' octasaccharide

Sufficient quantities of these 'enzymic' oligosaccharides for structural analysis were obtained by incubating the AD-1299 S dextran (50 grams) with P. lilacinum dextranase (expt. 14(i)).

The oligosaccharides were separated on a carbon column, and components 25, 26 and 27 were refractionated on paper chromatograms. The yields of these oligosaccharides are given in Table V.22.

Some properties of components 1, 2, 3 (21), 25, 26 and 27 are listed in Table III.11., including the degrees of polymerisation (see expt. 15(a)) and optical rotations (see expt. 15(b)).

Table III.11. Properties of some oligosaccharides produced by enzymic degradation of AD-1299 S dextran

Component	D.P.	$[\alpha]_{D}^{20}$ (equilibrium)	R_G solvent (b)	M_G borate	M_S* molybdate
1	-	-	1.00	1.00	1.00
2	1.9	+120°	0.74	0.70	0.70
3	2.9	-	0.56	0.60	0.58
21		-	0.60	0.27	0.12
25	4.0	+157°	0.42	0.50	0.46
26	5.0	+169°	0.26	0.45	0.41
27	5.9	+171°	0.16	0.41	0.38

* After reduction with sodium borohydride

Although components 25, 26 and 27 were chromatographically homogeneous in solvent (b), heavily spotted borate electrophoretograms revealed traces of components having M_G values of 0.24 (in component 25), 0.21 (in component 26) and 0.20 (in component 27). Also, heavily spotted molybdate electrophoretograms revealed traces of reduced components with M_S values of 0.11 (in reduced component 25), 0.10 (in reduced component 26) and 0.09 (in reduced component 27).

The partial acetolysis experiments (Section III.C.) had indicated that such electrophoretic behaviour was typical of oligosaccharides having a 2, 6 di-o-substituted glucose unit at the reducing end of the molecule.

Because of the small amounts of these materials present no attempt was made to remove them from the major isomers, but reference is made

to them later (Section III.D(iv)). The characterisation of the components 1, 2, 3, (21), 25, 26 and 27 was complete as follows.

Component 1, had chromatographic properties identical to those of glucose and, after crystallisation from aqueous ethanol, had a mpt. of 138-143°C, undepressed by authentic D-glucose.[36]

The presence of a small amount of glucose in the hydrolysate was not unexpected as the 'action pattern'[91] predicts that this monosaccharide will be released in small quantities when P. lilacinum dextranase is used to degrade a dextran.

Component 2, had chromatographic properties and an optical rotation consistent with isomaltose. On oxidation with sodium metaperiodate (expt. 15(c)) the disaccharide reduced 6 moles of periodate in liberating 5 moles of formic acid per mole of the sugar. These figures are identical to those previously reported[85] for isomaltose.

The dextranase 'action pattern'[91] predicts that a large quantity of isomaltose will be liberated when a dextran is degraded by P. lilacinum dextranase.

Component 3, had at first appeared to be chromatographically homogeneous on paper chromatograms eluted with solvent (b) but, when this component was separated on carbon-celite and then rechromatographed in solvent (b), heavily spotted chromatograms revealed that component 3 was contaminated by a small amount of a second component which migrated faster on the chromatogram, with an R_G value identical to that of the trisaccharide component 21 obtained from acetolysis of B-1299 S dextran (Section III.C.).

In addition, component 3 was not homogeneous after borate

electrophoresis on heavily spotted electrophoretograms, being contaminated by a small amount of a slow migrating component, whose M_G value was identical to that of component 21.

After reduction component 3 proved to be non-homogeneous when subjected to molybdate electrophoresis; containing a small amount of a component whose M_G value was identical to that of reduced trisaccharide XXI - a constituent of the reduced component 21 (see Table III.6.).

From these results it was concluded that the trisaccharide fraction from the enzymically degraded AD-1299 S dextran contained two components; the major component 3 having chromatographic properties identical to those of isomaltotriose and a minor component having chromatographic properties identical to those of trisaccharide XXI of the mixture constituting component 21.

Components 3 and 21 were not separated, but the mixture was examined by partial acetolysis (expt. 15(d)) and by periodate oxidation (expt. 15 (c)).

The partial acetolysate contained components chromatographing as glucose, isomaltose and kojibiose (trace).

Periodate oxidation indicated that the mixed trisaccharides reduced an average of 7.7 moles of periodate in liberating 5.6 moles of formic acid per mole (see Table V.25. and Fig. III.14.). These figures were slightly lower than those reported for isomaltotriose[85] (8 moles of periodate reduced and 6 moles of formic acid liberated) but were consistent with the presence of a small amount of trisaccharide XXI in admixture with isomaltotriose.

The presence of large quantities of isomaltotriose was predicted by the 'action pattern'[91] of P. lilacinum enzyme, but the additional presence of trisaccharide XXI was not expected and for this reason is considered later (Section III.D.(iv)).

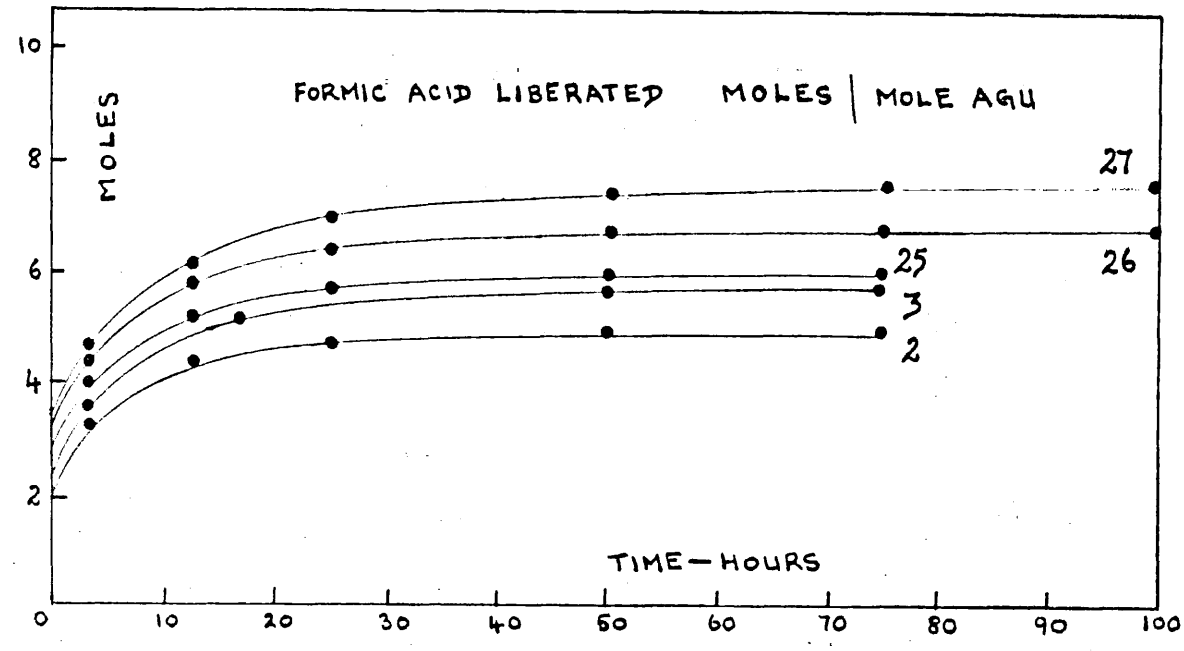
Component 25, had a paper chromatographic migration rate (solvent (b)) slightly greater than that of isomaltotetraose, but in borate electrophoresis, and (after reduction) in molybdate electrophoresis the two were inseparable.

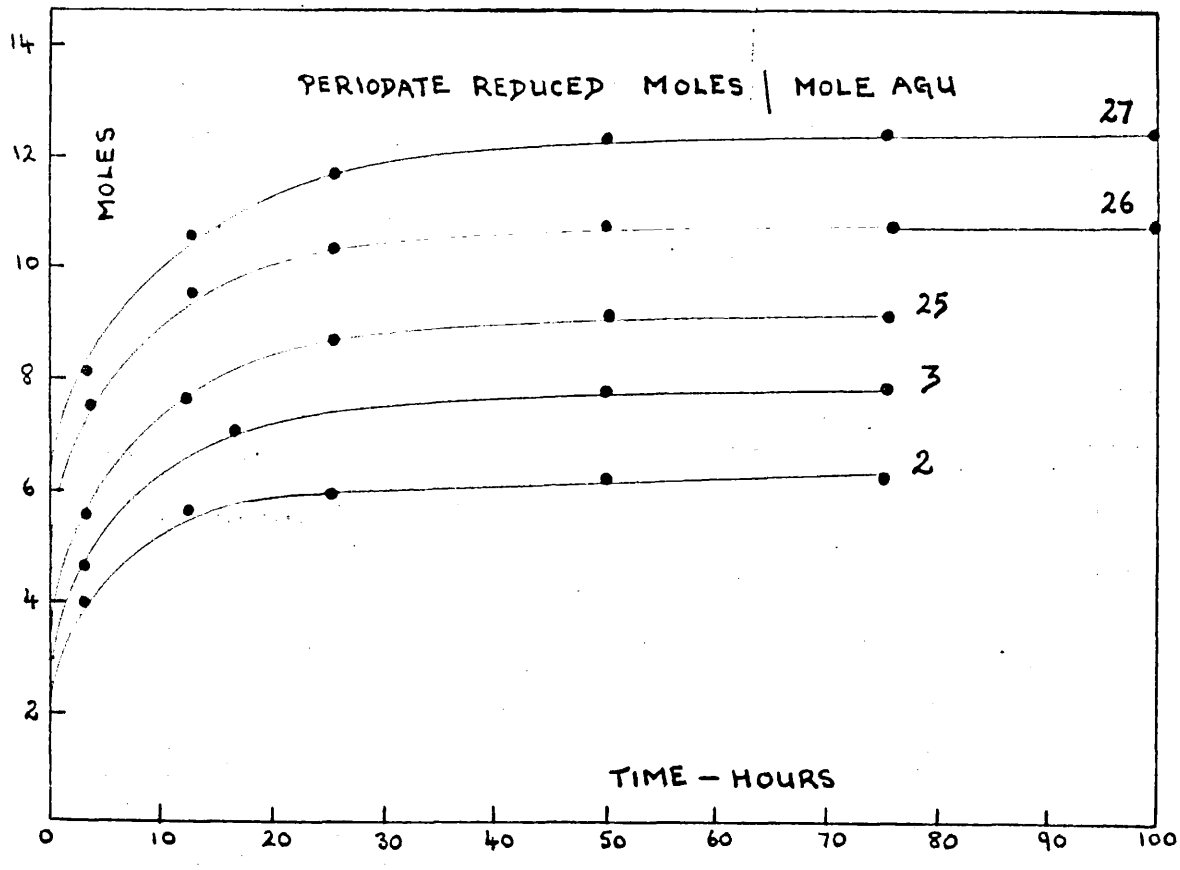
After incubation (expt. 15(e)) in a standard digest with P. lilacinum dextranase, however, component 25 was found to be only slightly degraded and could not therefore have been isomaltotetraose. On partial acid hydrolysis (expt. 15(f)) component 25 gave fragments which had paper chromatographic migration rates (solvent (b)) similar to those of glucose, isomaltose, kojibiose (trace) and isomaltotriose - results which indicate that the resistance to dextranase was due to the presence of the 1→2 link within an otherwise 1→6 linked molecule.

To determine the number of 1→2 linked units within the molecule, component 25 was oxidized with an excess of sodium metaperiodate (expt. 15(c)). The results of the oxidation, summarized in Table V.25. and Fig. III.14., show that 9 moles of periodate were reduced and 5.9 moles of formic acid were liberated per mole of the tetrasaccharide.

These figures, when compared with the 10 moles of periodate reduced and 7 moles of formic acid released per mole of isomaltotetraose indicate that each molecule of tetrasaccharide component 25 contains a single 1→2 glucosidic linkage.

FIGURE III. 14. —— RESULTS OF THE PERIODATE OXIDATIONS OF OLIGOSACCHARIDES FROM AD-1299S DEXTRAN





To establish whether the 1→2 link formed a branching point or not, component 25 was methylated by the Kühn[93] and Purdie[94] procedures (expt. 15(g)).

The methylated tetrasaccharide was then methanolysed and the methyl glucosides so formed were examined by vapour phase chromatography. The components located had the retention times (RT_{TMG}) - see page - shown in Table V.27. (page), and were identified by reference to standard compounds, chromatographed on the same columns, whose retention times are shown in Table V.26. (page).

The presence of 2,3,4 and 3,4,6 tri-o-methyl, methyl glucosides in the methanolysate showed that the major isomer (or isomers) comprising component 25 must have a linear structure.

Three isomers which fulfill both the requirements of the periodate oxidation and methylation data are illustrated in Fig. III.15.

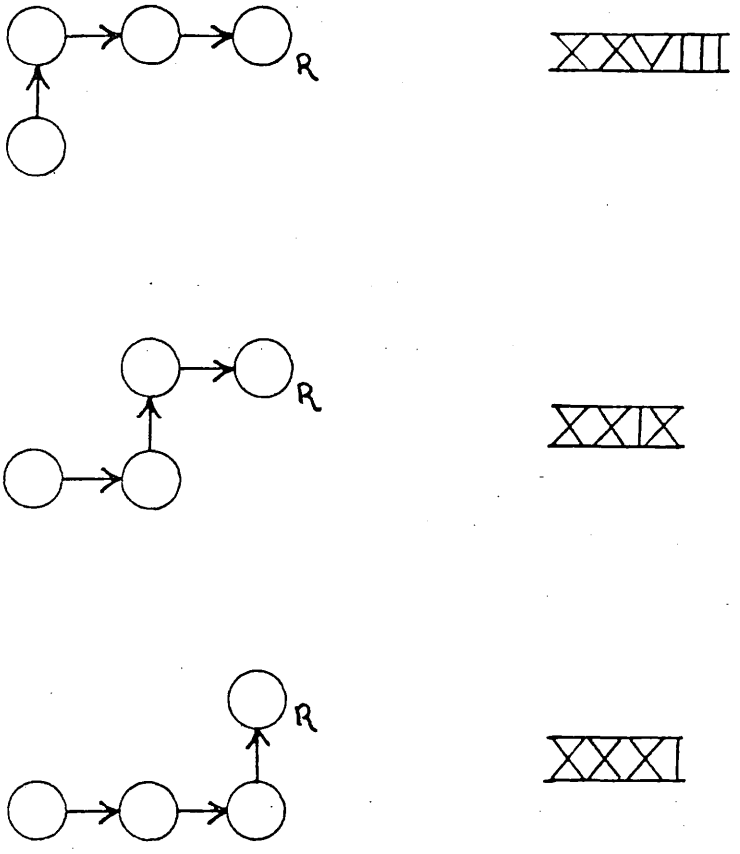
IsomerXXXI can, however, be ruled out for the migration rate in borate electrophoresis, and staining property (green with spray (c))[46] of component 25 indicated that the oligosaccharide had a 6-o-substituted glucose unit at the reducing end of the molecule.

The more difficult problem of establishing the presence of absence of isomers XXVIII and XXIX remained.

Weigel[75,76] has overcome a similar problem in locating the positions of 1→3 and 1→4 branched glucose units in otherwise 1→6 linked oligosaccharides (see Fig. III.7.), by subjecting the molecules to a process of 'reductive erosion'.

The technique involved a progressive fragmentation of the reduced

FIGURE III.15. —— POSSIBLE STRUCTURES FOR COMPONENT 25



→ 1→6 LINK

↑ 1→2 LINK

O_R REDUCING GLUCOSE UNIT

oligosaccharide and the separation and identification of the fragments by paper chromatography and electrophoresis in molybdate electrolyte.

An essential requirement of this method was, however, that a fragment containing a single 'branched' (secondary glucosidic) linkage should be separable in paper chromatography from the isomaltose homologue of equivalent degree of polymerisation.

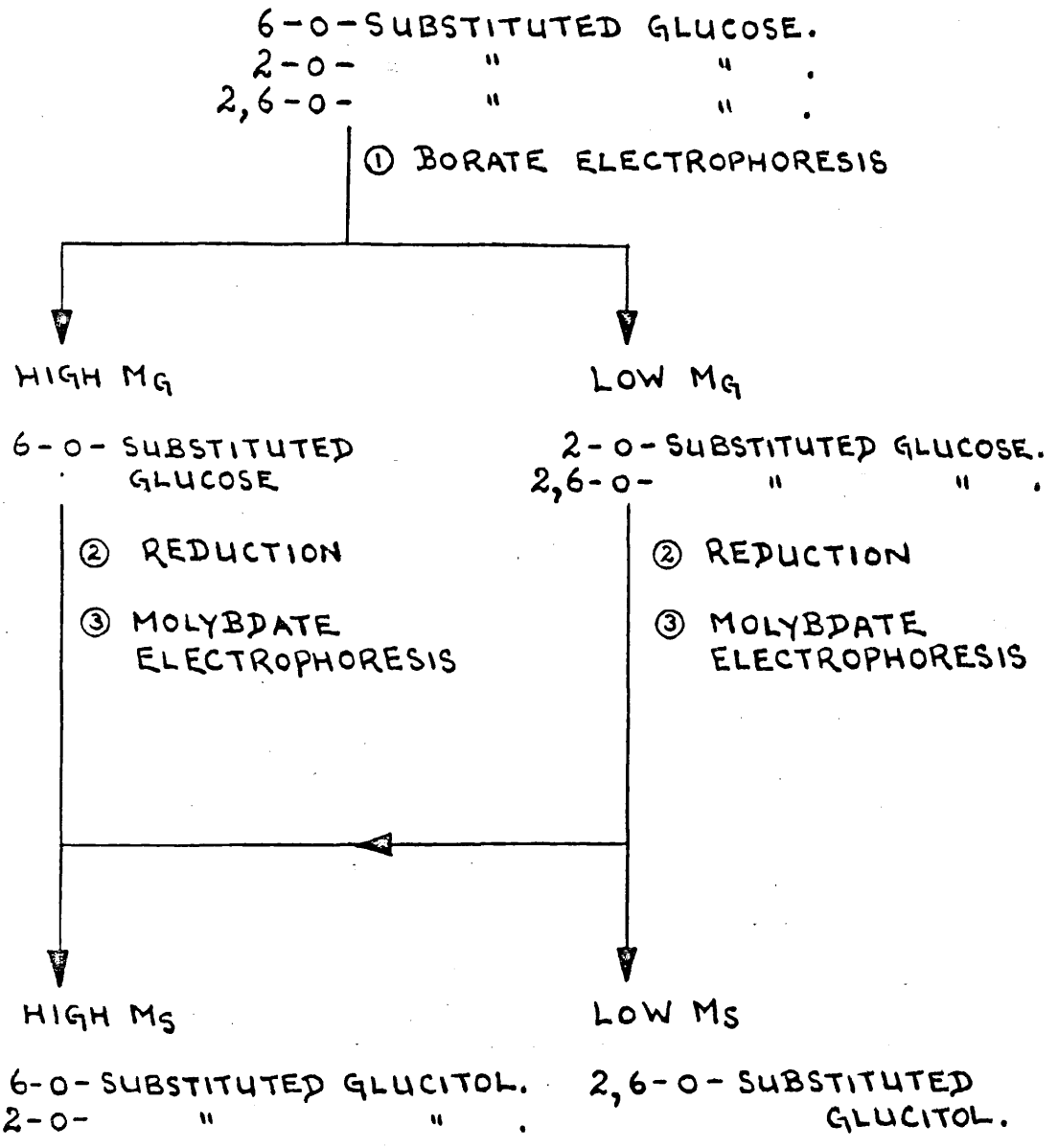
This requirement prevented the direct application of this method to component 25, for although 1→6 linked oligosaccharides containing a single 1→2 linkage do migrate at a faster rate than isomaltose homologues of equivalent degree of polymerisation, the difference is so slight that adequate resolution of mixtures of the two is not practically possible.

This approach did, however, suggest a similar scheme in which fragments from the degraded molecule were identified by electrophoresis, but which did not require separating isomaltose homologues from fragments containing the 1→2 linkage.

The basis for the method, discussed in Section III.A. rests on the fact that glucose oligosaccharides of equivalent molecular weight, terminating in either a 2-o-substituted, 6-o-substituted or a 2,6 di-o-substituted reducing end unit will behave differently in borate and (after reduction) in molybdate electrophoresis - differences which make possible the separations outlined in Fig. III.16.

After partial degradation (Fig. III.17.) the isomeric tetrasaccharides XXVIII and XXIX will each give common disaccharide fractions containing kojibiose and isomaltose, but the trisaccharide fractions will be different: degradation of isomer XXVIII will give rise to the

FIGURE III.16. —— SCHEME FOR SEPARATING OLIGOSACCHARIDES FROM AD-1299 S DEXTRAN



HIGH M_G $\geq$ 0.40 for hexasaccharide
LOW M_G $<$ 0.30 for hexasaccharide

HIGH M_S $\geq$ 0.40 for reduced hexasaccharide
LOW M_S $<$ 0.10 for reduced hexasaccharide

trisaccharide XIX and isomaltotriose, but isomer XXIX will produce trisaccharides XIX and XX.

Although these trisaccharides have essentially the same paper chromatographic migration rates isomaltotriose and trisaccharide XIX, which have 6-o-substituted reducing end units, will migrate faster in borate electrophoresis (M_G approx. 0.60) than trisaccharide XX (M_G approx. 0.28) which has a 2-o-substituted reducing end unit.

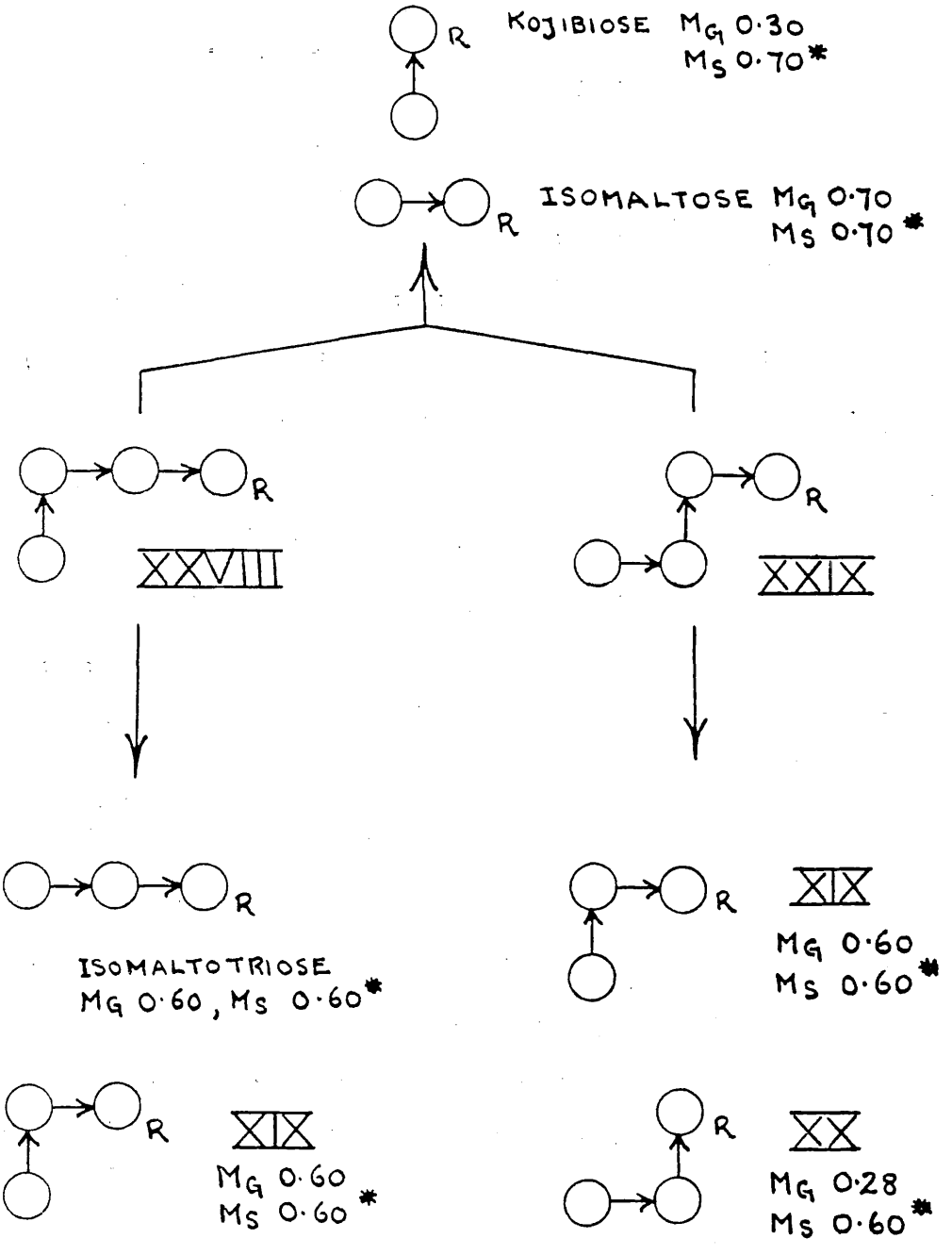
In practice location of two components (M_G approx. 0.60 and 0.28) in borate electrophoresis of the trisaccharide fraction, from partial degradation of component 25, will indicate the presence of trisaccharides XIX and XX which could only come from isomer XXIX. A single component (M_G approx. 0.60) in borate electrophoresis will indicate that only isomaltotriose and trisaccharide XIX from isomer XXVIII are present.

If component 25 is a mixture of isomers XXVIII and XXIX this will be reflected in a greater staining intensity of the trisaccharide component M_G approx. 0.60 when compared with that of the slower component M_G approx. 0.28 on the electrophoretogram.

The partial degradation of component 25 by acetolysis reagents is described in expt. 15(d). Partial acetolysis was chosen in preference to partial acid hydrolysis because of the greater yield of fragments containing the 1→2 linkage which could be expected[40] (see Section II.D.).

The deacetylated products were separated on paper chromatograms in solvent (b), and after elution the disaccharide and trisaccharide fractions were examined by electrophoresis in borate and molybdate electrolytes.

FIGURE III.17.



O_R REDUCING GLUCOSE UNIT.

→ 1→6 LINK, ↑ 1→2 LINK.

* After reduction.

The results of these experiments are shown in Table III.12.. The disaccharides isolated had chromatographic properties consistent with those of kojibiose and isomaltose, but borate electrophoresis of the trisaccharide fraction revealed two components M_G 0.61 and 0.27 which after reduction migrated as a single component M_S 0.58 - figures which indicate that trisaccharide XX (from isomerXXIX) was a component of this fraction.

The staining intensity of the component with M_G 0.61 was approximately three times greater than that of the slower component with M_G 0.27, from which it was concluded that isomer XXVIII was present in admixture with isomer XXIX.

Table III.12. Products of the partial acetolysis of component 25

Degradation product	R_G solvent (b)	M_G borate	M_S* molybdate	Identity
25.1	1.00 (3+)	-	-	Glucose
25.2	0.82 (+)	0.30	0.69	Kojibiose
25.3	0.74 (+)	0.70	0.69	Isomaltose
25.4	0.56 (2+)	0.61(3+)	0.58	Isomaltotriose, , Trisaccharide XIX
		0.27 (+)		Trisaccharide XX

* After reduction

The figures in parenthesis refer to the 'relative' staining intensities of the components with spray (a).

Although tetrasaccharides have been observed in the oligosaccharide patterns of native dextrans degraded by P. funiculosum[75,76] and Lactobacillus bifidus[95] dextranases they had been absent from similar

P. lilacinum[75,76] digests. The importance of the tetrasaccharides XXVIII and XXIX will therefore be considered later, with respect to both the B-1299 S dextran structure and the 'action pattern' of the P. lilacinum dextranase.

Component 26 had a slightly greater migration rate in solvent (b) than isomaltopentaose, from which it was inseparable by borate and (after reduction) molybdate electrophoresis.

Component 26 was, however, only slightly degraded by P. lilacinum dextranase (expt. 15(e)) and could not have been isomaltopentaose.

Fragments from the partial acid hydrolysis of component 26 (expt. 15(f)) had paper chromatographic migration rates similar to those of glucose, kojibiose (trace), isomaltose, isomaltotriose and 'isomaltotetraose'.

To establish with more certainty the identity of the 'isomaltotetraose', this component was eluted from the paper chromatogram and incubated with P. lilacinum dextranase (expt. 15(e)).

Paper chromatography (solvent (b)) revealed that approximately half the 'tetrasaccharide' had been degraded to products chromatographing as glucose, isomaltose and isomaltotriose (but not kojibiose). Reference to Fig. III.18. - which shows the only two dextranase degradable tetrasaccharides which could have been produced by acid hydrolysis of component 26 - indicates that these products could only have come from isomaltotetraose.

It would, therefore, appear that component 26 consists (in part) of isomers which had a backbone of four α-(1→6) linked glucose units to which an α-(1→2) linked unit is attached to cause the resistance to dextranase.

To determine whether each molecule contained only a single 1→2 link component 26 was oxidized with sodium metaperiodate (expt. 15(e)).

The results of the oxidation, summarized in Table V.25. and Fig. III.14., show that 10.82 moles of periodate were reduced and 6.85 moles of formic acid were released per mole of the pentasaccharide.

Comparison of these oxidation figures with those from isomaltopentaose (12 moles of periodate reduced and 8 moles of formic acid liberated) confirm that each molecule of pentasaccharide component 26 contains a single 1→2 linked glucose unit.

The proposed[91] 'action pattern' of P. lilacinum dextranase indicated the probability that component 26 had a branching structure. To obtain confirmatory evidence the pentasaccharide was methylated by the Kühn[93] and Purdie[94] procedures (expt. 15(g)).

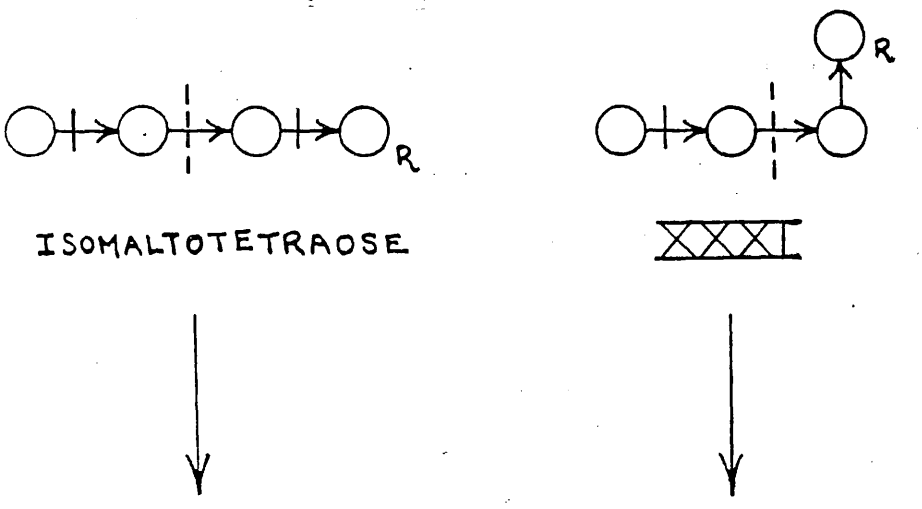
The methylated component 26 was methanolysed and the methyl glucosides liberated were examined by vapour phase chromatography.

The components located had the retention times shown in Table V.28. (page 225), and were identified by reference to standard compounds - chromatographed on the same columns - whose retention times are shown in Table V.26.

The presence of the methyl glucoside of 3,4,6 tri-o-methyl glucose, and absence of the methyl glucoside of 3,4 di-o-methyl glucose, indicated that the 1→2 link of component 26, contrary to the expectations of the 'action pattern'[91] formed a non-branching point.

The four isomeric pentasaccharides which, on the evidence of the periodate oxidation and methylation experiments, could comprise component

FIGURE III.18. THE ACTION OF P.LILACINUM DEXTRANASE ON TWO TETRASACCHARIDES.



ISOMALTOTETRAOSE

XXXI

① ISOMALTOSE *
② ISOMALTOTRIOSE
③ GLUCOSE

① ISOMALTOSE *
② KOJIBIOSE *
③ GLUCOSE
④ 2-O-α-ISOMALTOSYL D-GLUCOSE.

* MAJOR PRODUCT

→ 1→6 LINK

↑ 1→2 LINK

--- PRINCIPLE HYDROLYSIS POINT.

— SECONDARY HYDROLYSIS POINT.

26 are shown in Fig. III.19.

Isomer XXXXI can be ruled out, for the electrophoretic and staining properties of component 26 indicate that it has a 6-o-substituted reducing end unit. Isomer XXXVIII being the only remaining structure based on the isomaltotetraose system (identified in partial acid hydrolysates), must equally be present.

Further, the 'action pattern'[91] indicates that structure XXXVIII would be stable to the action of P.lilacinum dextranase.

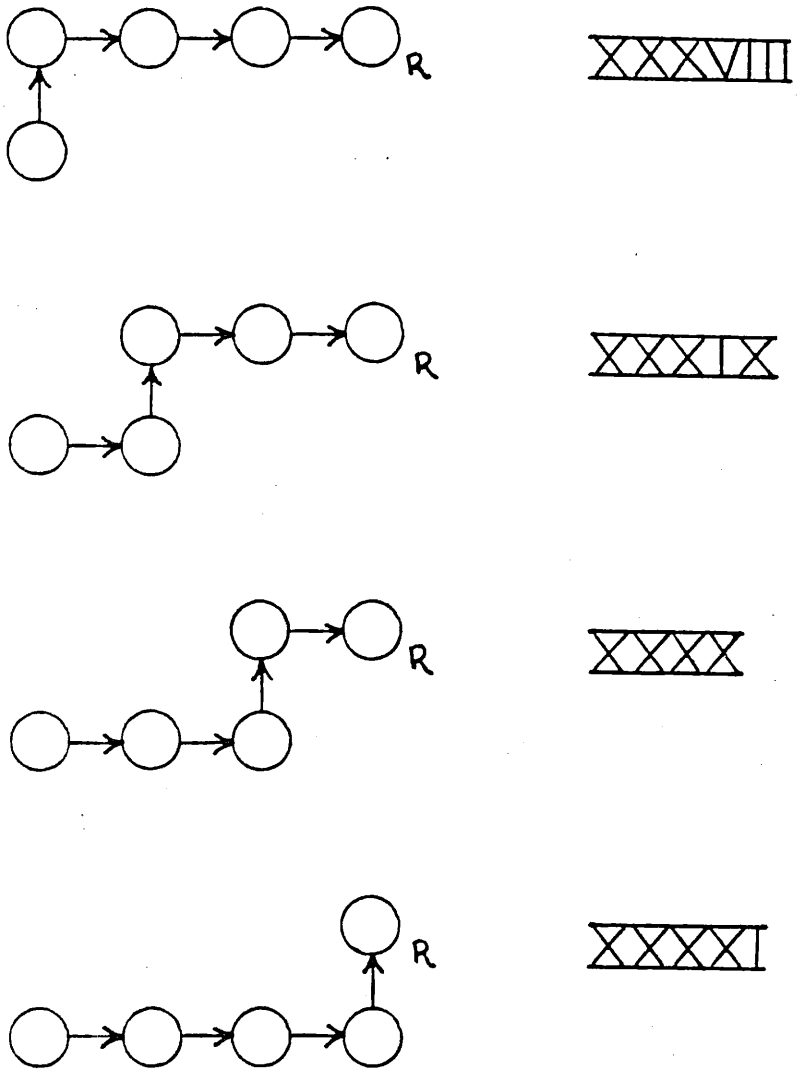
Two isomers XXXIX and XXXX remain which do not have the isomaltotetraose backbone. Of these isomer XXXIX could be expected to be resistant to hydrolysis by dextranase, but isomer XXXX has a structure which should be readily hydrolysed by dextranase (see Fig. III.21.).

A more detailed examination of the structure of component 26 was, however, possible by the scheme of degradation outlined in Fig. III.20.. By this, partial degradation of each isomeric pentasaccharide will give a common disaccharide fraction containing kojibiose and isomaltose, but the trisaccharide and tetrasaccharide fractions, although paper chromatographically homogeneous, will have different electrophoretic properties.

Isomer XXXVIII will give isomaltotriose and trisaccharide XIX which terminate in a 6-o-substituted reducing glucose unit, and will therefore have identical migration rates in borate electrophoresis.

Isomers XXXIX and XXXX will give the same three trisaccharides, one of which (trisaccharide XX) terminates in a 2-o-substituted reducing glucose unit and will have a low rate of migration in borate electrophoresis.

FIGURE III.19. ——— POSSIBLE STRUCTURES FOR COMPONENT 26.



→ 1→6 LINK.

↑ 1→2 LINK.

O_R REDUCING GLUCOSE UNIT.

Isomer XXXIX and/or XXXX will, therefore, be indicated by the presence of two components (M_G 0.60 and 0.28) in borate electrophoresis of the trisaccharide fraction.

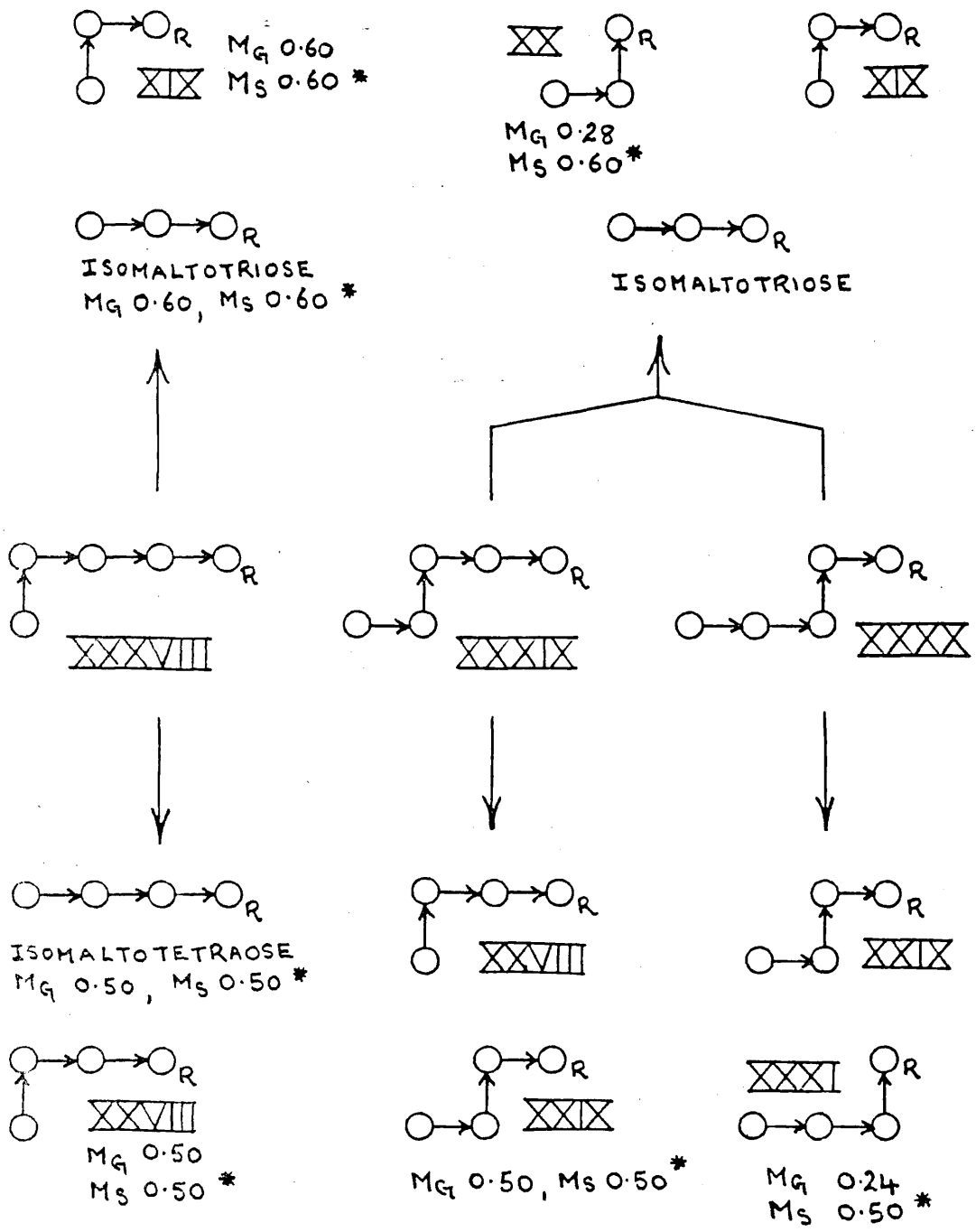
Examination of the tetrasaccharide fraction shows that isomers XXXVIII and XXXIX will give only tetrasaccharides terminating in a 6-o-substituted reducing glucose unit, which will migrate in borate electrophoresis as a single component whose M_G value will be similar to that of isomaltotetraose (M_G approx. 0.50).

Isomer XXXX will give tetrasaccharide XXXI which has a 2-o-substituted reducing end unit and will therefore have a much slower migration rate in borate electrophoresis. The presence of isomer XXXX will be indicated by two components (M_G approx. 0.50 and 0.24) in borate electrophoresis of the tetrasaccharide fraction. A limitation of this analysis is, however, that the presence of isomer XXXIX cannot be established in mixtures which also contain isomer XXXX.

The partial acetolysis of component 26 is described in expt. 15(d); the deacetylated products were fractionated on paper chromatograms and examined by borate and (after reduction) by molybdate electrophoresis. The results are summarized in Table III.13.

The disaccharides located had chromatographic properties identical to those of kojibiose and isomaltose, but borate electrophoresis of the trisaccharide fraction revealed two components (M_G 0.60 and 0.27), which after reduction migrated as a single component in molybdate electrophoresis (M_S 0.58) - figures which show that trisaccharide XX from isomer XXXIX and/or XXXX was present in this fraction.

FIGURE III.20.



→ 1→6 LINK , ↑ 1→2 LINK , O_R REDUCING GLUCOSE UNIT.

* After reduction

Table III.13. Products of the partial acetolysis of component 26

Degradation product	R_G solvent (b)	M_G borate	M_S* molybdate	Identity
26.1	1.00(2+)			Glucose
26.2	0.83(+)	0.30	0.70	Kojibiose
26.3	0.74(+)	0.70	0.70	Isomaltose
26.4	0.56(2+)	0.60(3+)	0.58	Isomaltotriose Trisaccharide XIX
		0.27(+)		Trisaccharide XX
26.5	0.40(4+)	0.50	0.47	Isomaltotetraose Tetrasaccharides XXVIII, XXIX

* After reduction

The figures in parenthesis refer to the relative staining intensities of the components with spray (a).

Examination of the tetrasaccharide fraction, however, revealed a single component (M_G 0.50) in borate electrophoresis, which after reduction had an M_S value of 0.47 in molybdate electrophoresis. This indicated that tetrasaccharide XXXI from isomer XXXX was absent from the tetrasaccharide fraction of component 26, which must, therefore, consist of a mixture of isomers XXVIII and XXXIX.

The importance of pentasaccharides XXXVIII and XXXIX will be considered later (Section III.D(iv)) with respect to both the B-1299 S dextran structure and the 'action pattern' of the P. lilacinum dextranase. Component 27 had a paper chromatographic migration rate slightly greater than isomaltohexaose from which it was inseparable in borate and molybdate electrophoresis.

Unlike isomaltohexaose, component 27 was only slightly degraded by P. lilacinum dextranase (expt. 15(e)).

Partial acid hydrolysis (expt. 15(f)) revealed components chromatographing as glucose, isomaltose, kojibiose (trace), isomaltotriose,

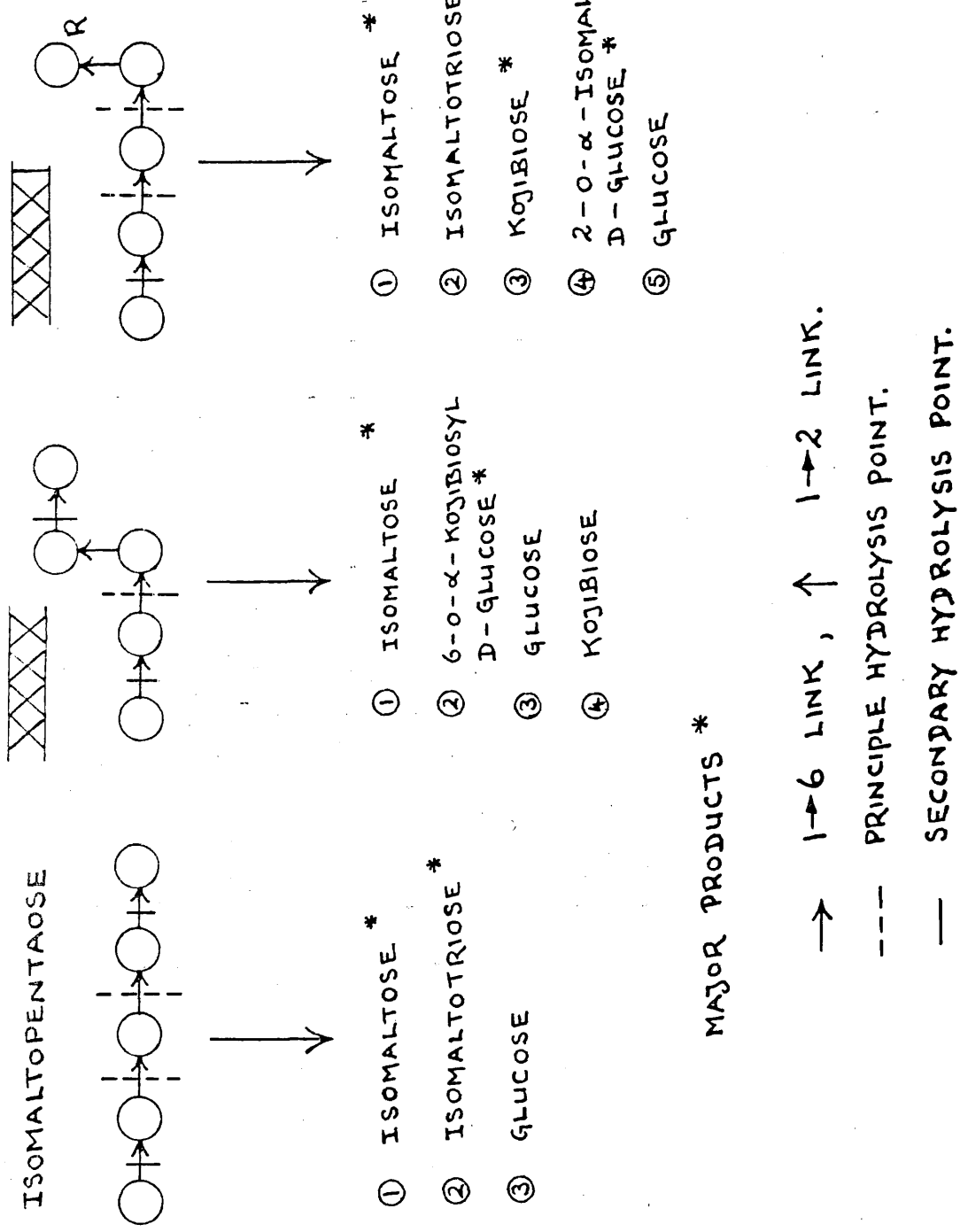
'isomaltotetraose' and 'isomaltopentaose'.

The component chromatographing as 'isomaltotetraose' was considerably degraded by dextranase (expt. 15(e)) and gave products which chromatographed as glucose, isomaltose and isomaltotriose (but not kojibiose). These products could only have come from isomaltotetraose (see Fig. III.18.). Component 27 must, therefore, contain compounds based on the isomaltotetraose unit. In a similar incubation with dextranase the component chromatographing as 'isomaltopentaose' was extensively degraded to give products chromatographing as glucose, isomaltose, and isomaltotriose (but not kojibiose). Figure III.21. shows the only three dextranase degradable pentasaccharides which could be produced by hydrolysis of component 27, from which it would seem that component 27, contained oligosaccharides based on an isomaltopentaose unit.

However, similarities in the chromatographic properties of each of the trisaccharides liberated from the pentasaccharides in Fig. III.21. make this identification by no means certain.

The number of 1→2 links in each molecule was determined by oxidizing component 27 with sodium metaperiodate (expt. 15(c)). The results - summarized in Table V.25. and Fig. III.14. - show that for each molecule of the hexasaccharide 12.6 moles of periodate were reduced and 7.6 moles of formic acid liberated. Under similar conditions isomaltohexaose reduced 14 moles of periodate in liberating 9 moles of formic acid, which indicated that component 27 consisted of a mixture of compounds, some of which probably contained a single and others two 1→2 links per molecule. This fact prevented a satisfactory structural analysis of the

FIGURE III.21.— THE ACTION OF P.LILACINUM DEXTRANASE ON THREE PENTASACCHARIDES.



hexasaccharide by the methods of methylation and partial acetolysis previously described. The following information was, however, obtained.

Component 27 was methylated as described in experiment 15(g) and, after methanolysis, the liberated methyl glucosides were examined by vapour phase chromatography. The results are shown in Table V.29. and the components were identified by reference to the standard methyl glucosides shown in Table V.26.

The major components correspond to 2,3,4,6 tetra- and 2,3,4 tri-o-methyl, methyl glucosides although a small amount of 3,4,6 tri-o-methyl, methyl glucoside also appeared to be present.

From these results it was concluded that component 27 contained primarily non-branching oligosaccharides.

Partial acetolysis of component 27 (expt. 15(d)) gave a disaccharide fraction containing kojibiose and isomaltose (Table III.14.), but borate electrophoresis of the trisaccharide fraction revealed two components (M_G 0.59 and 0.25) which after reduction migrated as two components (M_S 0.58 and 0.10) in molybdate electrophoresis.

The tetrasaccharide fraction also migrated as two components in borate (M_G 0.50 and 0.21) and, after reduction, in molybdate (M_S 0.50 and 0.09) electrophoresis.

The pentasaccharide fraction migrated as a single component in both borate (M_G 0.42) and (after reduction) in molybdate (M_S 0.42) electrophoresis.

These results indicate the presence of trisaccharides and tetrasaccharides with 2-o- and 2,6 di-o-substituted reducing end units,

but show that all the pentasaccharides have only 6-o-substituted reducing end units.

If these fragments are subjected to the same interpretations as those from components 25 and 26 (see Figures III.17. and III.20.) it would appear that component 27 could consist of a mixture of at least the six oligosaccharides shown in Fig. III.22.

Table III.14. Products from the partial acetolysis of component 27

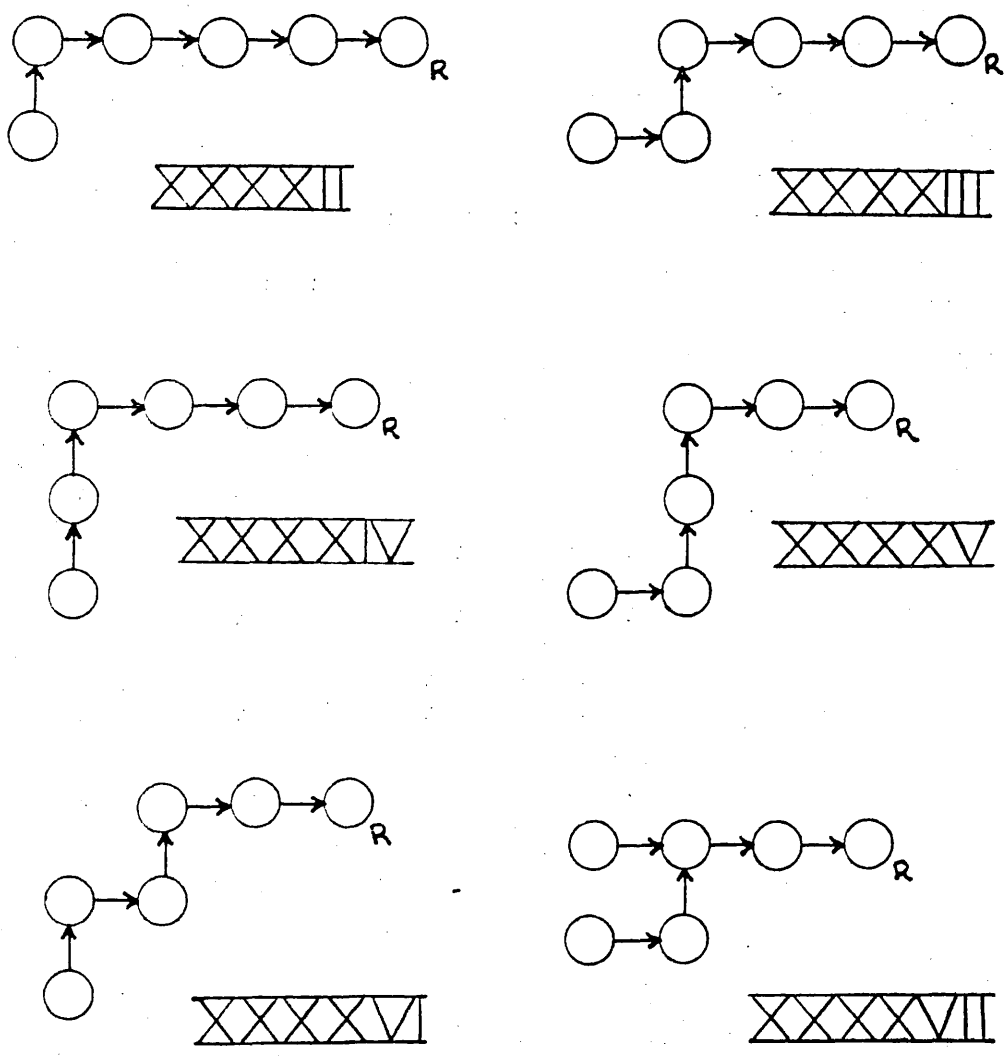
Degradation product	R_G solvent (b)	M_G borate	M_G* molybdate	Identity
27.1	1.00(3+)			Glucose
27.2	0.82(+)	0.31	0.71	Kojibiose
27.3	0.74(+)	0.72	0.71	Isomaltose
27.5	0.40(2+)	0.50(4+) 0.21(+)	0.50(9+) 0.09(+)	Mixed trisaccharides
27.6	0.28(2+)	0.42	0.42	Mixed pentasaccharides

* After reduction

The figures in parenthesis refer to the relative staining intensities of the components with spray (a).

Hexasaccharides XXXXII and XXXXIII could well be present because they have structures similar to those assigned to components 25 and 26. In addition, isomer XXXXII has an isomaltopentaose 'backbone' and could explain the results of the partial acid hydrolysis/dextranase digest experiments. Isomer XXXXIII should be stable to dextranase, although whether isomer XXXXII would also be stable is uncertain.

FIGURE III.22. —— POSSIBLE STRUCTURES FOR COMPONENT 27.



→ 1→6 LINK

↑ 1→2 LINK

O_R REDUCING GLUCOSE UNIT

The oligosaccharides XXXXIII, XXXXIV and XXXXV appear to be the only linear hexasaccharides which could contain two 1→2 links and still give the products observed in the partial acetolysate of component 27.

Partial acetolysis of component 27 indicated that the mixture contained branched structures. In the absence of a 1→2 branched pentasaccharide of the kind shown in Fig. III.7. it is unlikely that any branched hexasaccharide could be present in which there was a single 1→2 linked glucosyl unit. Hexasaccharide XXXVIImay be present for this oligosaccharide would give degradation products similar to those observed in the acetolysate of component 27.

(iv) Summary of results in Section III.D. and some general conclusions

The structures of tetrasaccharide XXIX and pentasaccharide XXXIX clearly indicate that the acid degraded AD-1299 S dextran contains intra chain 1→2 links, and provide further evidence that in the native B-1299 S dextran branches occur which are more than a single glucose unit long (see structure XXXIII, Fig. III.5.).

Although periodate oxidation of AD-1299 S dextran had indicated that 9% of the glucose units are 1→2 linked, an experiment of this kind cannot specify the site of these linkages which make it possible for the 'enzymic' oligosaccharides to have arisen from degradations at branching or non-branching sections of the molecule.

However, the absence of a 'branched' pentasaccharide in the AD-1299 S dextran hydrolysate - which had been observed in dextranase digests of B-1375[76] and B-1415[75] dextrans - is a good indication that branches consisting of a single α-(1→2) linked glucosyl unit are either not

accessible to the dextranase or not present in the acid degraded dextran.

It may therefore be assumed that the 'enzymic' oligosaccharides from AD-1299 S dextran have arisen from hydrolyses at non-branching points, or where the branches consist of more than one glucose unit.

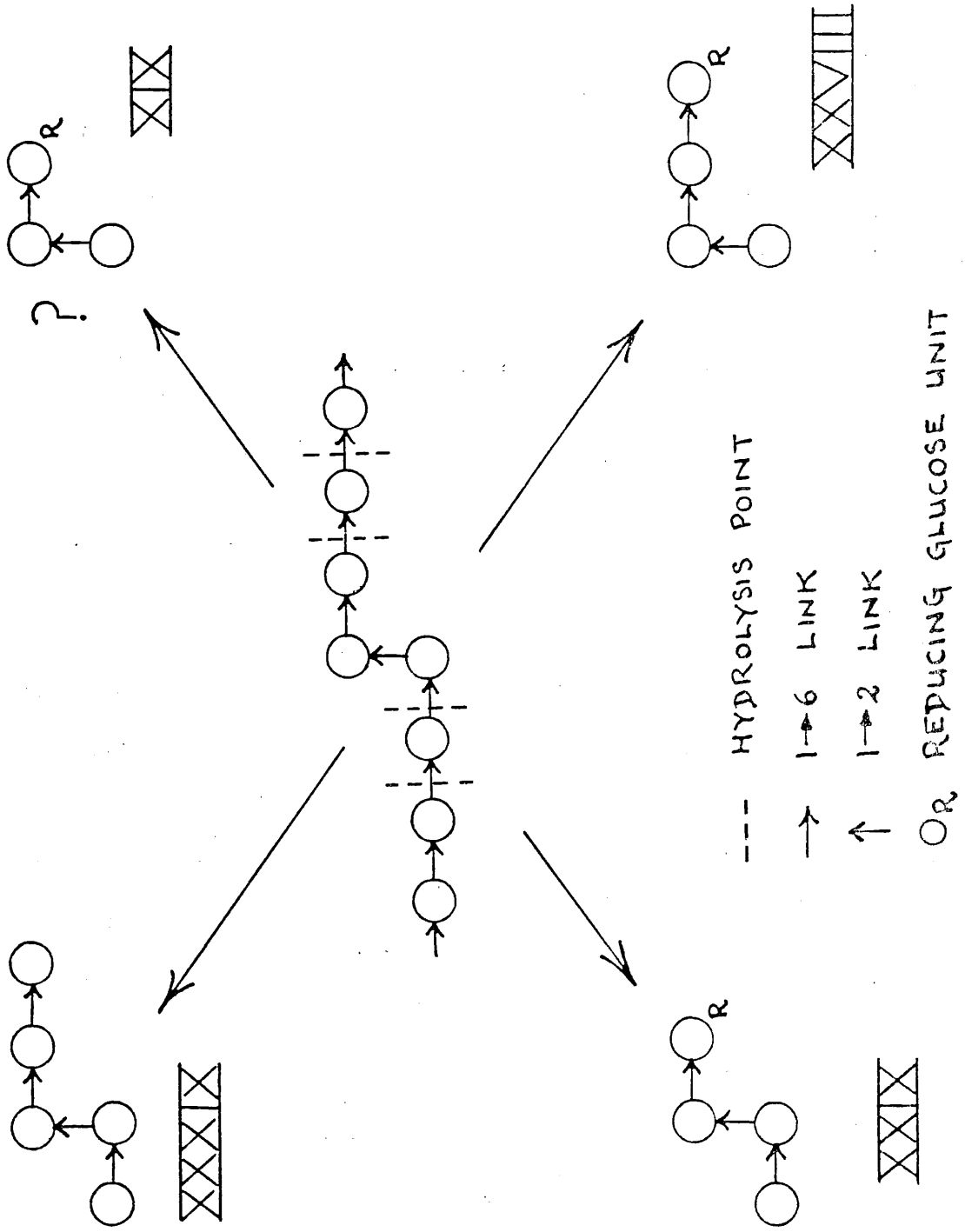
The 'action pattern' proposed by Hutson and Weigel[91] for P. lilacinum dextranase can, however, most adequately explain the structures of the tetra and pentasaccharides (and hexasaccharides XXXXII and XXXXIII if present) if it is accepted that they arise from hydrolyses at non-branching centres of the kind shown in Fig. III.23. In addition, the hexasaccharides in the mixture comprising component 27, which appear to contain two 1→2 links may well have arisen from similar hydrolyses where 1→2 linked units are adjacent or in close proximity.

It is interesting to note that if these oligosaccharides did originate from hydrolyses at non-branching centres then the 'action pattern' (Fig. III.23.) would appear to require the trisaccharide XIX to be in the hydrolysate.

The experimental evidence does not indicate that this fragment is present in the hydrolysate and it may well be that - as appears to be the case with L. bifidus dextranase[95] where a trisaccharide could also have been expected - in practice the enzyme system cannot remove a fragment of this type.

In contrast, the traces of oligosaccharides in the enzyme hydrolysate which appear to have 2,6 di-o-substituted reducing end units (see page113) are very probably fragments which the P. lilacinum dextranase has removed from the reducing ends of the AD-1299 S dextran chains, because the 'action

FIGURE III.23 ——— PROBABLE ACTION OF P. LILACINUM DEXTRANASE ON AD-1299S DEXTRAN



pattern'[91] cannot otherwise account for the presence of oligosaccharides with this arrangement at the reducing end.

Fig. III.24., for example, illustrates an arrangement at the reducing end of the AD-1299 S dextran which could have given rise to trisaccharide XXI (see page115).

The picture presented here of the probable course of the dextranase hydrolysis of AD-1299 S dextran requires many of the 1→2 links to be at (dextranase accessible) non-branching points; in contrast to the native B-1299 S dextran in which all the 1→2 links form branching points.

The reasons for these differences become apparent if the acid hydrolysis reaction is examined in greater detail.

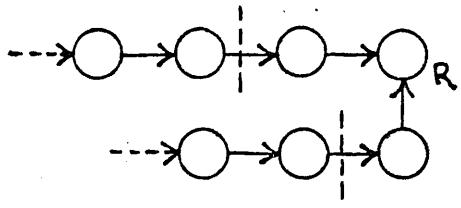
Experiments of Wolfrom[39] and Matsuda,[40] with α-D-glucopyranosyl-D-glucoses, have indicated that in these similar environments α-(1→2) glucosidic links have a far greater rate of acid hydrolysis than α-(1→6) linkages.

Where their environments are different the same α-linkages may not have equal hydrolysis rates. For example, Jones[96] has shown that the hydrolysis rate of the α-(1→6) link at the non-reducing end of isomaltotriose is 1.7 times greater than that at the reducing end of the molecule, whilst in starch hydrolysates[97] glucose is the first detectable product followed by oligosaccharides of progressively increasing molecular size.

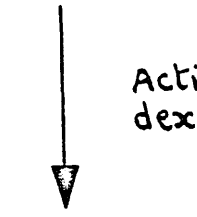
It is therefore probable that when B-1299 S dextran is acid hydrolysed:

a) an α-linkage at the non-reducing end of the chain will have a greater rate of acid hydrolysis than an α-linkage nearer the middle of

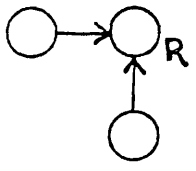
FIGURE III.24. — POSSIBLE ORIGIN OF TRISACCHARIDE XXI



'Reducing end of AD-1299 s dextran chain.'



Action of P. lilacinum dextranase.



XXI

→ 1→6 LINK

↑ 1→2 LINK

---→ α-LINKAGE

O_R REDUCING GLUCOSE UNIT

--- HYDROLYSIS POINT

the same chain.

b) α-(1→2) linkages at non-reducing ends of the chains will have a higher rate of hydrolysis than similarly positioned α-(1→6) links.

Thus hydrolysis of a 'segment' of the native B-1299 S dextran (Fig. III.25.) could be envisaged as a three stage process involving in approximate order:

a) hydrolysis of α-(1→2) linked non-reducing terminal residues

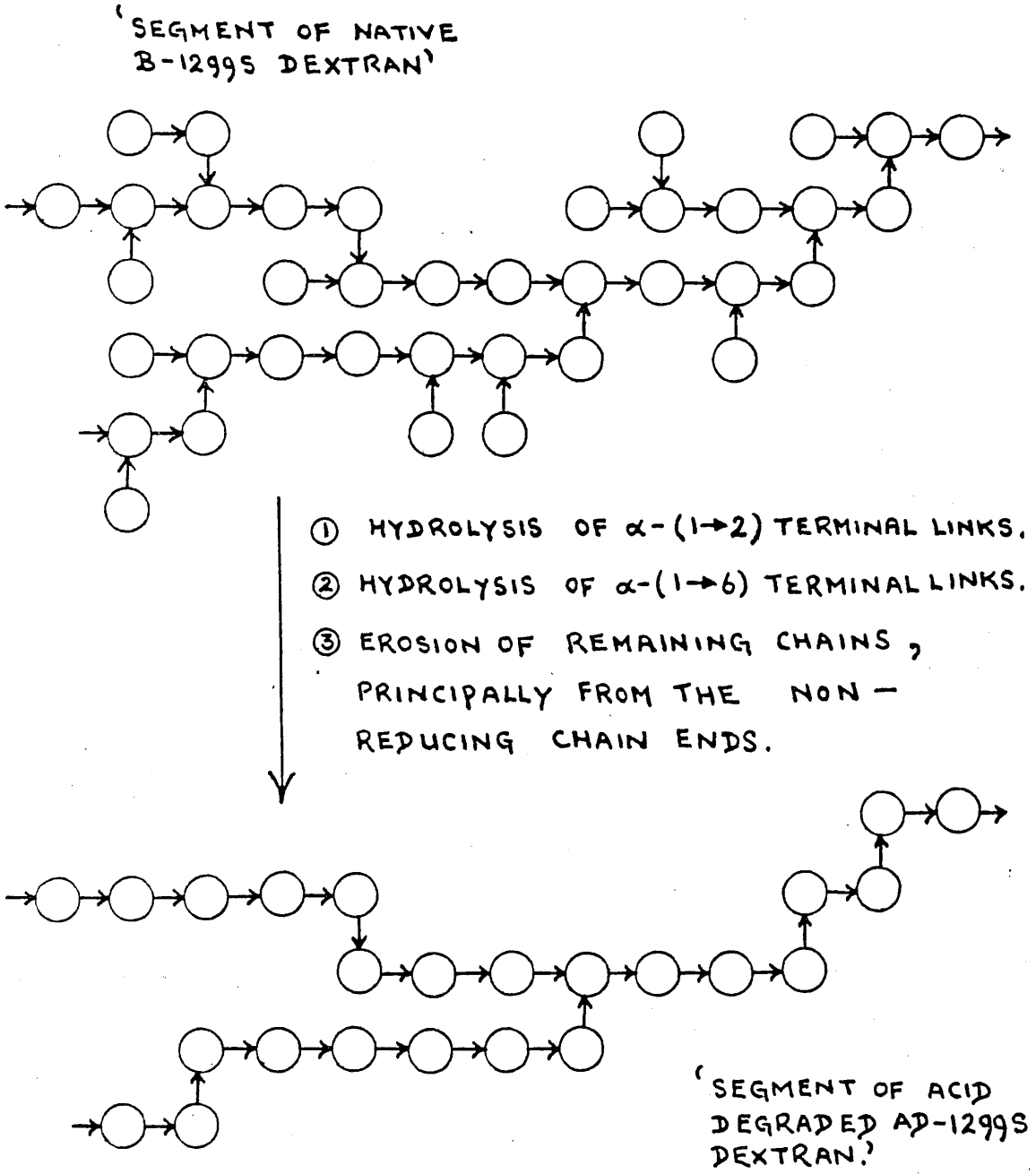
b) hydrolysis of similar α-(1→6) linked residues

c) progressive erosion of the remaining chains principally from the non-reducing ends.

Although no satisfactory reason can be advanced to explain the greater rate of acid hydrolysis of the α-(1→2) link compared with the α-(1→6) link, the greater acid lability of the non-reducing terminal linkages can be accounted for if a mechanism involving a cyclic intermediate operates in dextran hydrolysis.

This mechanism of acid hydrolysis, which was first proposed by Edward[98], has recently gained favour, and involves the steps outlined in Fig. III.26. - where it is applied to an α-glucopyranoside. Initially protonation of the glucosidic oxygen atom occurs to form the conjugated acid A, followed by heterolysis of the exocyclic oxygen to carbon atom 1 (C_1) bond to give a cyclic carbonium-oxonium ion B which probably exists in a half chair conformation C. Reaction with water then gives the protonated reducing sugar D from which the reducing sugar E is formed.

FIGURE III.25.



The rate determining step is the formation of the carbonium-oxonium ion, which involves a net counterclockwise rotation about the C_2 to C_3 and C_4 to C_5 bonds. Evidence indicates that there is a direct relationship between the hydrolysis rate and the ease of rotation about these bonds.[99] One factor which increases opposition to these changes, and so decreases the rate of glucoside hydrolysis is the presence of a bulky substituent on either positions C_2, C_3, C_4 or C_6 of the glycon ring.

The higher rate of hydrolysis of terminal glucosidic links in B-1299 S dextran could therefore be accounted for by the absence of bulky substituents on either C_2, C_3, C_4 or C_6 of the terminal non-reducing glucose residues - which would facilitate carbonium-oxonium ion formation.

The greater resistance to hydrolysis of a glucosidic link situated further from the non-reducing chain end could conversely be due to the fact that carbonium-oxonium ion formation, in this case, would require first the reorientation of an entire chain (or chains) of glucose units.

In conclusion, the more important points made in these experiments may be summarized as follows:

a) The reasons for the failure of dextranases to hydrolyse native B-1299 S have been considered:- the main factor appeared to be the high degree of branching, although experiments with an acid degraded dextran (AD-1299 S) indicated that the B-1299 S dextran had also a 'resistance' to dextranase which must have been due to some other feature of the molecule - possibly the presence of many short chains of α-(1→6) linked glucose units or of many branches more than one glucose unit long.

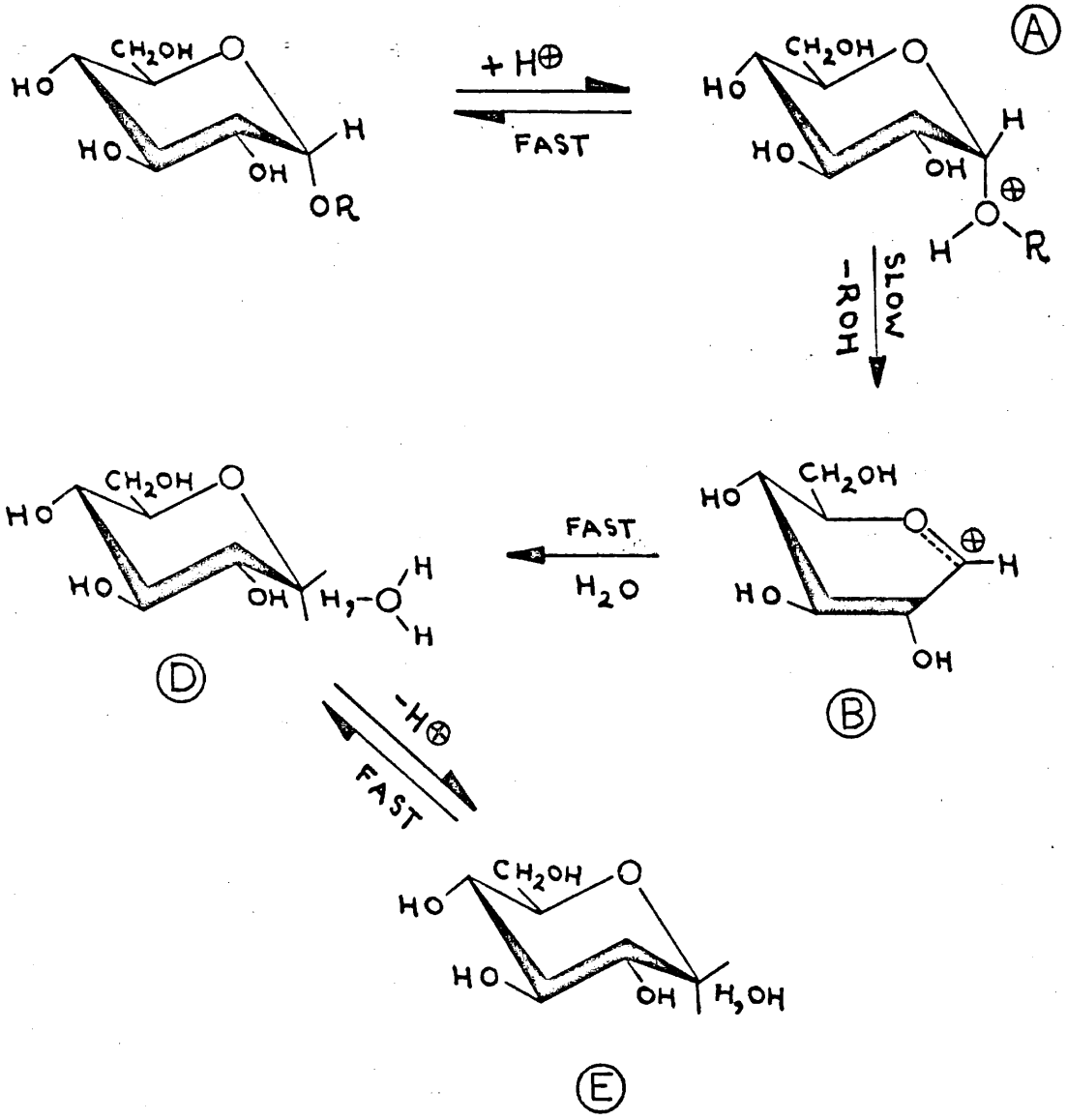
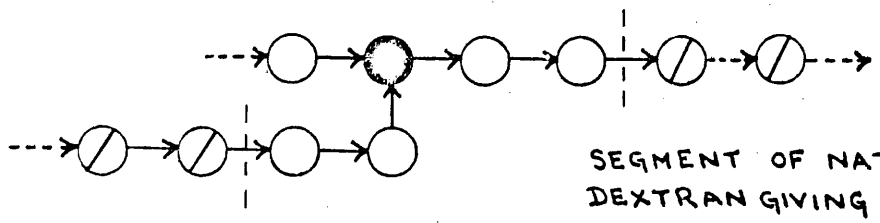


FIGURE III.26.

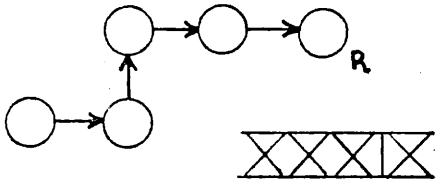
FIGURE III.27



SEGMENT OF NATIVE B-1299S DEXTRAN GIVING RISE TO PENTASACCHARIDE XXXIX

① ACID HYDROLYSIS

② ACTION OF P. LILACINUM DEXTRANASE.



XXXIX

→ 1→6 LINK, ↑ 1→2 LINK.

O_R REDUCING GLUCOSE UNIT.

---→ α — LINKAGE.

α-(1→6) LINKED GLUCOSE UNIT WHICH MUST BE PRESENT IN NATIVE B-1299S DEXTRAN. (SEE SECTION IIG)

ADDITIONAL GLUCOSE UNIT REQUIRED BY THE P. LILACINUM DEXTRANASE ACTION PATTERN.

--- POINT OF DEXTRANASE HYDROLYSIS.

b) The enzymic degradation of an acid degraded dextran (AD-1299 S) gave linear tetra and pentasaccharides whose structures were related to a previously proposed[91] 'action pattern' of P. lilacinum dextranase. Moreover, the structure of pentasaccharide XXXIX clearly indicated that in the native B-1299 S dextran some branches must consist of a minimum of two glucosyl units and these same branches are probably at least four glucosyl units long if the 'action pattern' of P. lilacinum is taken into consideration (see Fig. III.27.).

c) The partial acid hydrolysis of native B-1299 S dextran has been discussed in terms of a mechanism of acid hydrolysis.

IV. SOME GENERAL CONCLUSIONS

(i) Some general observations

Water soluble and water insoluble α-D-glucopyrans were isolated from sucrose-broth cultures of L. mesenteroides NRRL B-1299.

Determination of the optical rotations and oxidation of the two glucans with sodium metaperiodate established that they were almost certainly identical to the B-1299 S and B-1299 L dextrans isolated by Jeanes from similar cultures.[13b]

The water soluble B-1299 S dextran accounted for 95% of the total polysaccharide isolated from our cultures in contrast to the figure of 23% reported by Jeanes. The difference can, however, probably be attributed to differences of the culturing conditions employed.

Jeanes had originally reported that L. mesenteroides NRRL B-1299 belonged to an unusual class of micro organisms producing heterogeneous dextrans.[13b] The experimental results obtained in the present work do not establish whether the B-1299 dextrans are to be regarded as two distinct polysaccharides or simply as the two major fractions of the same polymer; for although they differ widely in, for example, their water solubilities they appear to have many structural features in common.

(ii) The synthesis of B-1299 dextrans

The B-1299 dextrans contain the same α-glucosidic linkages - though in different proportions - and are thus almost certainly synthesised by the same enzyme system.

Furthermore, the incidence of α-(1→6), α-(1→2) and α-(1→3) links - the latter not forming branching points - in these dextrans is an

indication that they are probably synthesised by a multi enzyme system, through a mechanism of the kind proposed by Barker and Bourne[9] rather than by the agencies of a single enzyme as has been suggested by Ebert.[120]

In particular, the close similarity which exists between the two repeating units, proposed on page 57, may reflect the fact that the water soluble B-1299 S dextran is an intermediate in the synthesis of the water insoluble B-1299 L dextran.

(iii) The α-(1→3) links in B-1299 dextrans

Methylation experiments (Section II.C) have established that both the B-1299 dextrans contain 1→3 links which do not form branching points.

Inspection of the repeating units proposed for the B-1299 dextrans (page 58) reveals that the major chemical difference between these dextrans is the number of α-(1→3) links which each contains.

It is possible that this difference could be largely responsible for the markedly different water solubilities and optical rotations of the two polymers.

These two physical properties and the results of immunochemical studies[17-21] point to the fact that the α-(1→3) links in each of these dextrans could be features at the non-reducing ends of the chains.

The immunochemical studies of Kabat and the results of Smith Degradation (Section II.F) indicate that branches consisting of 3-o-α-D-glucopyranosyl -2-o-α-D-glucopyranosyl units (see Fig. IV.1.) may be important structural features of both dextran molecules.

(iv) The branches in B-1299 dextrans

A particularly unusual feature of the B-1299 dextrans are the large

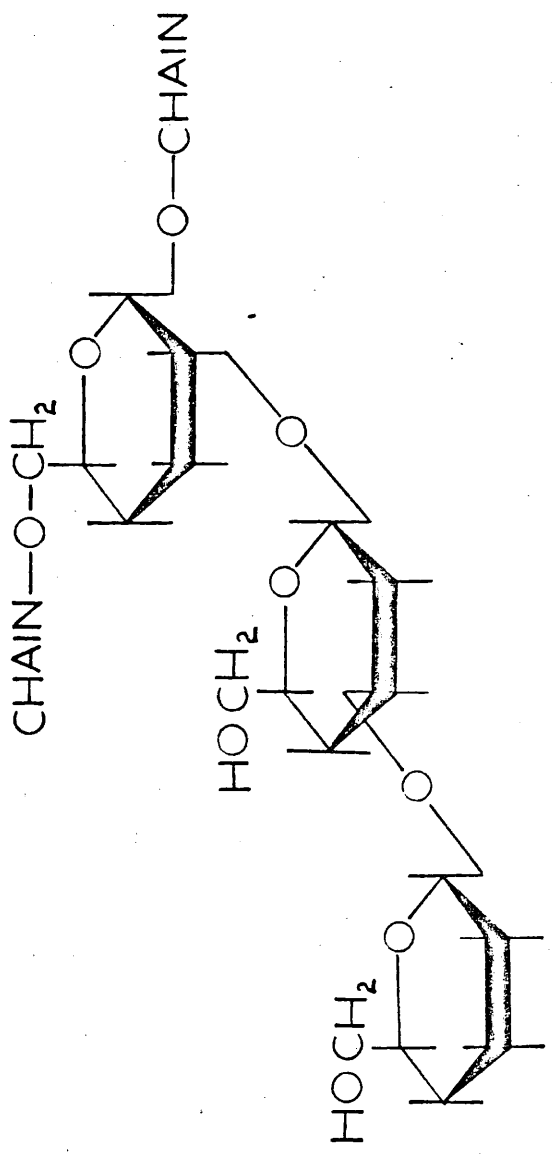


FIGURE IV.1.

percentages of secondary glucosidic linkages which each contains.

The α-(1→2,6) linked units - which form the branching points in both dextrans - appear to remain largely as isolated from each other (a possible exception is considered on page 96) within chains of α-(1→6) linked units, giving the B-1299 dextrans an essentially dextran-like structure. (See page 1).

The branches themselves may well be of three types:

(a) The products obtained from partial acetolysis (Section III.C) and enzymic degradation (Section III.D) of the B-1299 S dextran provide evidence that many branches of this dextran consist of chains of α-(1→6) linked glucose units.

(b) Branches consisting of a single α-(1→2) linked glucosyl unit must also be present in B-1299 S dextran in order to reconcile the experimental finding that this dextran contains chains of at least 6 α-(1→6) linked glucose units (Section III.B) and has a degree of branching of approximately 33%. In addition, the production of antibodies with a serological specificity directed towards the α-(1→2) glucosidic link indicates that many chains are terminated by α-(1→2) linked non-reducing glucose units.[17-21]

Attempts during the course of the present work to investigate this feature directly through catalytic oxidation experiments of the kind applied to NRRL 1375 and NRRL 1415 dextrans[27] proved unsuccessful - the degree of oxidation of available primary hydroxyl groups never exceeding 9% even after extended periods of oxidation.

Preliminary examination - by paper chromatography and electrophoresis - of an aldobiouronic acid fraction from the hydrolysed B-1299 S carboxy dextran indicated that it contained as a major product isomaltobiouronic acid - indicative of chains terminated by α-(1→6) linked non-reducing glucose units.

(c) As has been already suggested it is probable that some chains (see Fig. IV.1.) are terminated by an α-(1→3) linked non-reducing glucose unit.

If the earlier suggestion - that the water soluble B-1299 S dextran is an intermediate in the formation of the water insoluble B-1299 L dextran - is correct, then the latter may represent a limiting case where no branches remain which consist of a single α-(1→2) linked glucosyl unit - all having been terminated by an α-(1→3) linked glucose unit or extended through a chain of α-(1→6) linked glucose units.

If this assumption is correct, reference to the proposed repeating unit (page 58) reveals that 60% of all the branches could be of the kind shown in Fig. IV.1., and the remaining 40% would consist of extended chains of α-(1→6) linked glucose units. In B-1299 S dextran 40% of all the branches may likewise consist of extended chains of α-(1→6) linked glucose units, but only 20% could be of the kind shown in Fig. IV.1., and the remaining 40% must, therefore, consist of single α-(1→2) linked glucosyl units.

V. EXPERIMENTAL DETAILS

A. General Procedures

(i) Paper chromatography was carried out by descending technique on No.1 Whatman paper for general use, and on No.3 and No.17 papers for medium and large scale preparative work. The No.17 paper was washed with distilled water before use. Loadings of these papers were: No.3; 3mg.1cm. and No.17; 10mg.1cm.

The procedure used when fractionating sugars on a large scale was as follows: the sugar mixture to be separated was applied as a streak to the base line of the paper and standard compounds were spotted on the margins of the paper to aid location of the separated components. After development of the chromatogram in the chosen solvent strips were cut from the edges and centre of the paper and stained with one of the spray reagents.

The positions of the bands on these strips helped locate the components of the mixture resolved on the chromatogram.

An alternative technique,[100] used particularly with No.17 papers, was to take the chromatogram, while still wet with the solvent, lay it on a sheet of glass and place a sheet of No.1 paper upon it. An imprint was then taken by running a blunt edge up and down the No.1 paper several times, which, when sprayed up, gave a picture of the distribution of components on the chromatogram. The areas containing the separated components were cut from the chromatogram, stapled to a paper 'wick' and eluted with distilled water.

(ii) Solvent systems, used for paper chromatography were:

(a) n-butanol:pyridine:water (6:4:3, by volume).[101]

(b) the upper layer of an ethyl acetate:pyridine:water mixture (2:1:2, by volume).[102]

(c) n-butanol:ethanol:water (40:11:19, by volume).[103]

(d) n-butanol:benzene:pyridine:water (5:1:3:2, by volume).

(e) n-butanol:ethanol:water:ammonia (40:10:49:1, by volume).[105]

(f) ethyl acetate:acetic acid:formic acid:water (18:3:1:4, by volume).[89]

(iii) Paper electrophoresis was carried out in the following electrolytes:

(a) Sodium borate, pH 10.0.[63]

Standard migrating marker: glucose.

Non-migrating marker: tetra-o-methyl glucose.

(b) Sodium molybdate, pH 5.5.[80]

Standard migrating marker: glucitol.

Non-migrating marker: glucose.

(iv) Spray reagents used for location of components separated by paper chromatography or paper electrophoresis were:

(a) Silver nitrate in acetone/sodium hydroxide in ethanol.[106]

(b) p-anisidine hydrochloride.[107]

(c) Aniline/diphenylamine/phosphoric acid.[46]

(d) Diphenylamine/phosphoric acid.[34]

(v) Preparation of a carbon - celite column[108]

Equal weight of B.D.H. activated charcoal and Lights No.545 celite were mixed, treated with 4 volumes of concentrated hydrochloric acid, and

allowed to stand for 24 hours.

The mixture was then washed (by decantation) with tap water to reduce the acidity, and poured onto a large Büchner funnel. The mixture was then re-washed, under suction, with running tap water until the filtrate was neutral. After standing (24 hr) in 2 volumes of absolute ethanol the mixture was given a final washing (by decantation) with distilled water.

A 90 x 7.5 cm. glass column was packed under gravity in distilled water, which was allowed to run freely through the column throughout the packing procedure. To ensure horizontal interfaces between the layers of packing media the column was plumbed to the vertical beforehand. The packing media was supported on a hard packed layer of glass wool (5 cm.). The column was closed at the base by a layer of Whatman CF 11 cellulose (5 cm.), added as a thin aqueous slurry.

This was followed by a layer of Lights No.545 Celite (5 cm.; washed in concentrated hydrochloric acid), also added as a slurry.

The charcoal-celite slurry was then added to a depth of 70 cm. and allowed to pack down over 4-5 hours, by which time the eluent flow rate had reduced to a fast drip.

Next, a 3 cm. layer of washed Celite was added and the column closed with a layer of CF 11 cellulose (3 cm.), on top of which was placed a filter paper to hold the column surface firm. The column was eluted with distilled water for 5 days before use.

(vi) Terms used during paper chromatography and paper electrophoresis

(a) Paper chromatography

1. R_F = Distance moved by the component from the base line / Distance moved by the solvent front from the base line

2. R_X = Distance moved by the component from the base line / Distance moved by the standard component x from the base line

X, a standard marker, was usually glucose (R_G)

or 2,3,4,6 tetra-o-methyl glucose (R_{TMG})

3. R_M = $\log_{10}(\frac{1-R_X}{R_X})$

when R_X may be R_G, R_{TMG} or R_F

(b) Paper electrophoresis

M_X = Distance moved by component from the non-migrating marker / Distance moved by standard component X from the non-migrating marker

X, a standard marker, was usually glucose (M_G) in borate electrolyte, or glucitol (M_S) in molybdate electrolyte

(c) Gas liquid chromatography

R_T (the retention time) =

Time taken for the component to pass through the column / Time taken for the standard component X to pass through the column

X, the standard, was always 2,3,4,6 tetra-o-methyl, methyl glucoside ($RT_{\beta TMG}$) for o-methyl, methyl glucosides.

(vii) Borohydride reductions of sugars.[109]

Aqueous solutions of the sugars (1% w/v) were reduced with an equal volume of sodium borohydride (2% w/v) for a period of 12 hours. The solutions were then deionised by shaking with IR 120(H^+) resin and

evaporating the recidue with dry methanol.

(viii) Carbohydrate contents of the sugars were determined by the phenol-sulphuric acid method.[110] A standard graph was prepared as follows: analar D-glucose (100 mg.) was dissolved in distilled water (1 litre) and from this solution aliquots of 1.0, 0.75, 0.50, 0.25 and 0.10 ml. were removed and made to 1.0 ml. with distilled water. These solutions, and a blank (distilled water; 1 ml.) were treated with concentrated sulphuric acid (5 ml.) rapidly added from a pipette with a broken tip to generate the colour. The tubes were cooled, stoppered, mixed and the optical densities of the solutions determined at 490 mμ with a Unicam S.P.500 spectrophotometer.

(ix) Bacteriological procedures

(a) Inoculations, were made with pre-sterilized, cotton wool plugged pipettes. The tips were 'flamed', i.e. passed through a bunsen flame, before inoculations.

(b) Flasks and tubes, were sealed with sterile cotton wool plugs. During all transfers, the neck of the vessel and the plug were 'flamed' in a bunsen burner.

(c) Sterilizing. All media was sterilized at 15lb. per square inch for 10-15 minutes. The carbohydrate was sterilized separately and combined later to reduce decomposition.

(d) Media used were as follows:

1. Litmus - Milk,[111] a non-dextran producing, preservation medium consisted of:

0.3% yeast extract (Marmite Co)
1.0% glucose
1.0% calcium carbonate
Litmus indicator
(Initial pH 7.0)

The calcium carbonate neutralised acid metabolites; at 0°C organism viability could be maintained for 4 months.

2. Glucose medium,[111] a non-dextran producing medium consisted of:

Glucose	10 gm./litre
Evans peptone	10
Yeast extract	10
Microcosmic salt	5
Manganese sulphate	0.05
Magnesium sulphate	0.2
Sodium acetate	2.0
Potassium dihydrogen phosphate	1.0

(Initial pH 6.7)

This was essentially a subculturing medium in which dormant cells (from freeze dried cultures) or slow growing cells (from preservation medium) could be revitalised. Organism viability could be maintained for 48 hours at 25°C.

3. Sucrose medium,[111] a dextran producing medium consisted of:

Sucrose	100 gm./litre
Evans peptone	10
Yeast extract	10
Microcosmic salt	5
Manganese sulphate	0.05
Magnesium sulphate	0.2
Sodium acetate	2
Potassium dihydrogen phosphate	1

(Initial pH 6.7)

In this medium maximum bacterial growth occurred after an initial lag of 24 hours, with high CO_2 production. Increasing acidity usually killed the organisms after 72 hours.

4. Media used for the S. Bovis organism [92]

Long term preservation was possible on Litmus - milk, but sub-culturing took place through the following medium:

Sodium acetate	0.15 molar
Yeast extract	0.5% w/v
Bacto tryptose	1.4% w/v
Glucose	8.5% w/v
Potassium carbonate	0.004 molar

The potassium carbonate was separately sterilized. For dextran production the glucose was replaced by an equal weight of sucrose. Additions of pentothenic acid (20 mg. per litre) and biotin (0.01 mg. per litre) may be beneficial. Organism viability on both media could be maintained for 48 hours at 38°C.

(x) Determination of the degree of polymerisation of an oligosaccharide

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

(a) 10 ml. were made to 20 ml. with distilled water (active borohydride solution)

(b) 10 ml. were made to 20 ml. with 2N sulphuric acid (inactive borohydride solution)

An aqueous solution of the sugar was prepared, containing between 30 and 60 µg of carbohydrate per ml.

Aliquots (1 ml.) of this sugar solution were then treated with active borohydride solution (1 ml.) and inactive borohydride solution (1 ml.), and left (12 hours) in capped tubes.

The solutions were then treated with 4% phenol solution (1 ml.) and analar concentrated sulphuric acid (5 ml.) was added rapidly, from a pipette with a broken tip, to generate the colour.

After cooling for 0.5 hours, the optical densities of the solutions were measured at 490 mµ using a Unicam S.P.500 spectrophotometer.

The degree of polymerisation (n) was calculated from the following relationship:

$$\frac{n}{n-1} = \frac{\text{optical density of the unreduced sugar solution}}{\text{optical density of the reduced sugar solution}}$$

(xi) Determination of the optical rotation of a sugar

The sugar (approx. 15 mg.) was dissolved in distilled water (2 ml.) and an aliquot (0.3 ml.) was removed and diluted to 25 ml.

The phenol-sulphuric method (see General Procedure (viii)) was used to determine accurately the sugar concentration of this, and hence the

original, sugar solution.

The optical rotation was measured with a mercury polarimeter in a 1 dm. tube (filling on 0.7 ml.).

The specific rotation was calculated from the following expression:

$$\text{Specific rotation } [\alpha] = \frac{100.\alpha'}{L.c.} \text{ degrees}$$

when, α' - observed rotation of the sugar solution

L - optical length of the tube (1 dm.)

c - sugar concentration (gm./100 ml.)

The specific rotation (mercury 5461 Å line) $[\alpha]_{Hg}$ was converted to the sodium D line value $[\alpha]_D$ on dividing by 1.175.

(xii) Periodate oxidation of oligosaccharides

Solutions (10 ml.), each containing 15 to 20 mg. of oligosaccharide (whose concentration had been accurately determined by the phenol-sulphuric method (see General Procedure (viii)), were treated with an equal volume of standard sodium meta periodate solution and made to 100 ml. in a volumetric flask. A blank was prepared for each oligosaccharide containing standard periodate solution (10 ml.) made to 100 ml. with distilled water.

10 ml. of each standard periodate solution contained approximately twice the amount of sodium meta periodate calculated to completely oxidize an equal weight of the isomaltodextrin, whose degree of polymerisation is the same as that of the oligosaccharide to be oxidized.

The oxidation was conducted at room temperature (approx. 25°C), in the dark.

To determine the amount of periodate reduced by the oligosaccharide (as moles per mole of oligosaccharide) aliquots (5 ml.) were removed from the reaction mixture and blank solution, and treated with saturated sodium bicarbonate solution (10 ml.).

A known excess (20 ml.) of standard 0.01N sodium meta-arsenite was added, followed by 20% potassium iodide solution (2 ml.) as a catalyst. The solutions were swirled, left in the dark (15 minutes) and the unreacted arsenite was estimated by titration with standard 0.01N iodine solution.[113]

From these titres the quantity of arsenite which had reacted with the excess periodate, and hence the amount of periodate which had been reduced by the oligosaccharide, could be calculated.

The amount of formic acid liberated by the oligosaccharide (moles per mole of oligosaccharide) was determined by removing aliquots (10 ml.) from the reaction mixture and blank solution, and treating each with ethylene glycol (1 ml.) to destroy the excess sodium meta-periodate. The solutions were stoppered and left in the dark (1 hour) before the formic acid was estimated by titration with standard 0.01N sodium hydroxide, in an atmosphere of nitrogen, using phenolphthalein indicator.[114]

(xiii) Incubation of oligosaccharides with P. lilacinum dextranase

The oligosaccharide (approx. 5 mg.) was dissolved in 0.2 M sodium citrate buffer solution (2 ml.) which had been adjusted to pH 5 with citric acid.

An equal volume (2 ml.) of P. lilacinum dextranase preparation was added and the solution was mixed by inversion, sealed under a layer of toluene and incubated (48 hours) at 36°C.

After deionisation with IR 120(H^+) and IRA 400 (OH^-) resins the digests were fractionated on paper chromatograms (solvent(b)) and examined with spray reagent(a).

B. Individual Experiments

Expt. 1 Preservation of the Leuconostoc organism

The NRRL B-1299 organism was donated by the United States Department of Agriculture, Peoria, Illinois.

The bacteria were received in a dormant state, as a freeze dried suspension of cells grown on a sterile bovine serum.

The ampoule was transferred to the National Dairy Centre, Shinfield, Reading, and accepted into the collection as N.C.D.O. 1875. On request ampoules of the freeze dried organism were supplied by the Centre.

The organism was held in the Chemistry Department for limited periods on the Litmus-Milk medium at 0°C.

Expt. 2 Preparation and isolation of the dextrans

To reduce the possibility of infection most of the dextran was prepared as required (in 10 litre batches) directly from freshly opened ampoules of the freeze dried organism. Before innocculating the sucrose broth, the bacterial cells were subcultured twice through the glucose medium.

Glucose Medium (0.1ml.) was added to the dormant cells, still in the ampoule, and the suspension used to innocculate 2-3 tubes each containing

Glucose Medium (10ml.).

After 36 hours incubation, at 28°C, these cultures were used to innocculate 2-3 tubes of fresh glucose medium, which were incubated in a similar manner.

Cells from the glucose broth (1ml.) were innocculated into Sucrose Medium (1 litre).

The sucrose broth was incubated at 28°C for 4 days, by which time the pH had dropped to 3 or 4. The crude dextrans were extracted from the sucrose broth by the scheme indicated in Fig. V.1.

Expt. 3 Purification of the dextrans

The crude B-1299 S dextran, was dissolved in distilled water, reprecipitated with an equal volume of absolute ethanol, centrifuged down (3,000 r.p.m.; 15 minutes), and redissolved in distilled water.

This aqueous solution was deproteinated by shaking with an equal volume of a mixture of chloroform-amylalcohol (3.5:1 by volume).[116] The protein precipitated out at the interface of the two phases and was removed with chloroform layer. This procedure was repeated until no more protein was seen to precipitate out.

The deproteinated solution was then treated with ethanol to precipitate the dextran, which was centrifuged down, redissolved in water, and freeze dried to a white solid.

The crude B-1299 L dextran, was dissolved in warm N sodium hydroxide solution, and the pH adjusted to 4.0 with glacial acetic acid.

The dextran was precipitated from this solution by adding an equal volume of absolute ethanol. After centrifuging down (3,000 r.p.m.; 15

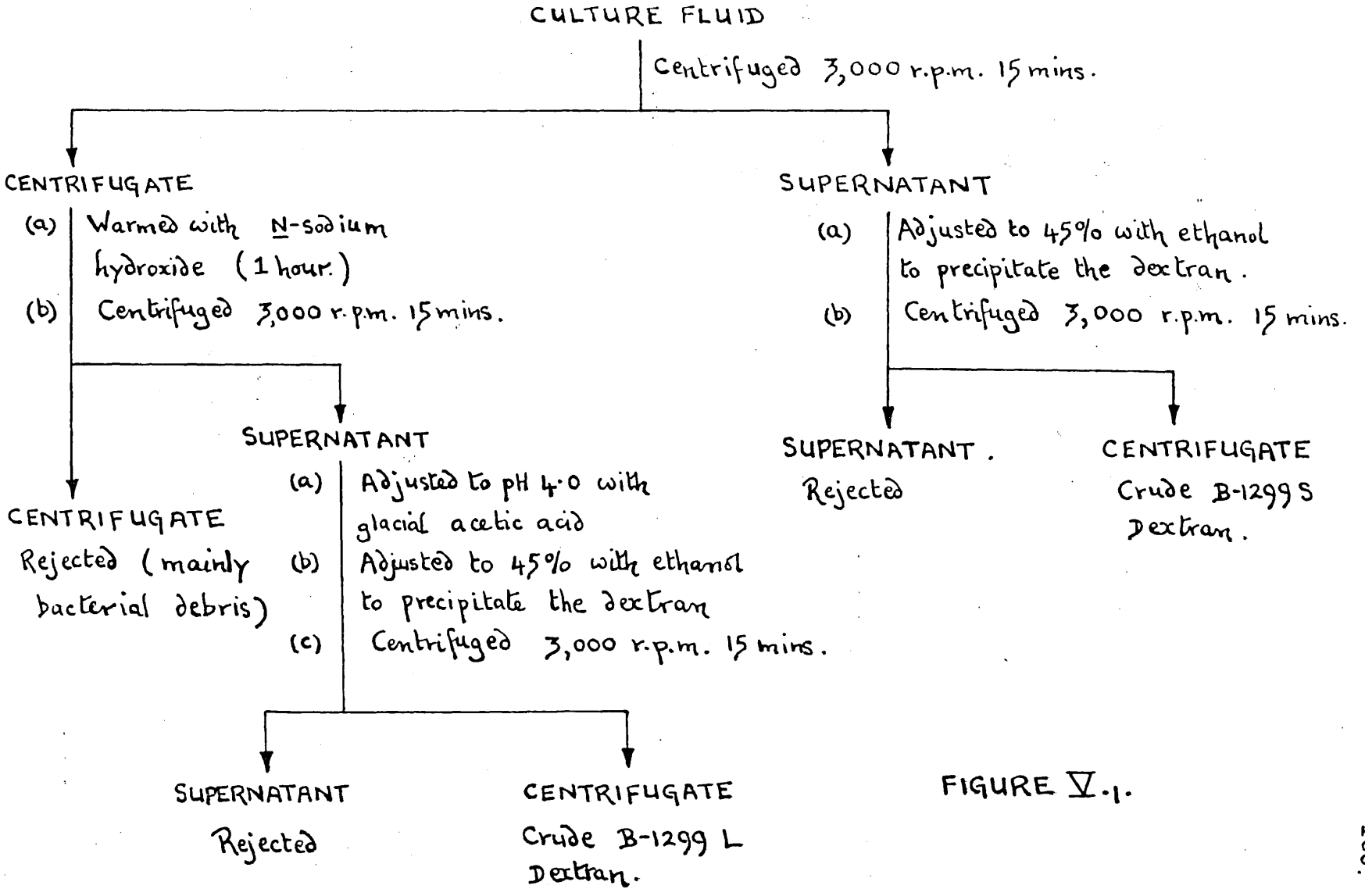

CULTURE FLUID
Centrifuged 3,000 r.p.m. 15 mins.
CENTRIFUGATE
(a) Warmed with N-sodium hydroxide (1 hour.)
(b) Centrifuged 3,000 r.p.m. 15 mins.
SUPERNATANT
(a) Adjusted to pH 4·0 with glacial acetic acid
(b) Adjusted to 45% with ethanol to precipitate the dextran
(c) Centrifuged 3,000 r.p.m. 15 mins.
CENTRIFUGATE
Rejected (mainly bacterial debris)
SUPERNATANT
Rejected
CENTRIFUGATE
Crude B-1299 L Dextran.
SUPERNATANT
(a) Adjusted to 45% with ethanol to precipitate the dextran.
(b) Centrifuged 3,000 r.p.m. 15 mins.
SUPERNATANT.
Rejected
CENTRIFUGATE
Crude B-1299 S Dextran.

FIGURE V.1.

minutes), the dextran was again dissolved in N sodium hydroxide solution and the pH adjusted with acid.

The protein was removed from this solution by shaking with chloroform-amyl alcohol mixture.

The deproteinated dextran was again precipitated by adding ethanol, centrifuged down, and finally suspended in warm water before freeze drying to a white solid.

1 litre of culture medium, containing 100gm. of sucrose produced on average the following weights of polysaccharide.

B-1299 L dextran 1 gm.

B-1299 S dextran 17.5 gm.

Expt. 4 Ash contents of the dextrans

These were determined by A. Bernhardt (Germany) on request, and are recorded in Table II.1.

Expt. 5 Optical rotations of the dextrans

Specific rotations of standard solutions of the dextrans, in N sodium hydroxide solution, were measured with a Hilger sodium polarimeter.

The dextrans had been dried over phosphorus pentoxide at 50°C, in vacuo and the weights corrected for ash content. The optical rotations are listed in Table II.1.

Expt. 6 Infrared spectra of the dextrans

These were determined upon dextran-nujol mulls held between potassium bromide plates. A Unicam S.P.100 infrared spectrophotometer was used to scan the range 650 to 1000 cm^{-1}. The main features of these spectra are shown in Table II.2.

Expt. 7 Complete acid hydrolysis experiments

(a) Optimum conditions for hydrolysis of fructosidic linkages[34]

The B-1299 polysaccharides (500mg.) were hydrolysed with 0.2N sulphuric acid (10ml.) in capped tubes placed in a water bath at 70°C for 1 hour.

The hydrolysates were neutralised with saturated barium hydroxide solution using bromothymol blue as an indicator.

The neutralised solutions were adjusted to 80% with absolute ethanol and centrifuged (10 minutes; 2,000 r.p.m.) to remove precipitated polysaccharide and barium salts.

The supernatant solutions were concentrated by rotary evaporation, in vacuo at 40°C, and subjected to chromatography in solvent (f). The chromatograms were developed with spray reagents (a) and (d).

(b) Optimum conditions for hydrolysis of glucosidic linkages[35]

The B-1299 polysaccharides (25mg.) were hydrolysed with 2N sulphuric acid (2ml.) in capped tubes placed in a water bath at 100°C for 8 hours. The hydrolysates were neutralised with an excess of barium carbonate, and the insoluble barium salts were removed by centrifugation (10 minutes, 2,000 r.p.m.).

The supernatant solutions were deionised with IR 120(H^+) and IRA 400(OH^-) resins, and concentrated by rotary evaporation, in vacuo at 40°C.

The hydrolysates were subjected to paper chromatography in solvent (f) and sodium borate electrophoresis. The reduced hydrolysates were subjected to sodium molybdate electrophoresis. The chromatograms and electrophoretograms were developed with spray reagent (a).

(c) Characterisation of the glucose from the dextrans

The B-1299 polysaccharides (1.5gm.) were hydrolysed with 2N sulphuric acid (20ml.) in sealed flasks, placed in a water bath at 100°C, for 8 hours. The hydrolysates were neutralised with excess barium carbonate, centrifuged and the supernatant solutions deionised with IR 120(H^+) and IRA 400(OH^-) resins. The solutions were then charcoaled and rotary evaporated to thin syrups, which were treated with ethanol until a slight turbidity was observed. After seeding with crystals of D-glucose the syrup crystallised out in the refrigerator.

Constants for the crystalline material are given in Table II.3.

(d) Preparation of glucose β -pentaacetate[37]

Freshly fused sodium acetate (150mg.) was mixed with glucose and refluxed with acetic anhydride (1ml.) for 40 minutes. The reaction mixture was poured onto crushed ice (5ml.) and the solution neutralised (pH 6.0) with sodium bicarbonate.

The precipitated acetate was filtered, washed with distilled water, charcoaled from ethanol and recrystallised from aqueous ethanol.

(e) Determination of the glucose content of the dextrans

The glucose was determined with glucose oxidase-peroxidase reagent obtained in a kit from Boehringer Biochemicals, Germany. The contents of the kit were used to prepare the following standard solutions.

Solution 1. (300mls) contained peroxidase (40µg/ml) and glucose oxidase (250µg/ml) in 0.10 molar phosphate buffer (pH 4.7).

Solution 2. (8mls) contained o-dianisidine hydrochloride (6.6mg/ml).

The solutions could be stored for three weeks in the refrigerator.

Fresh 'glucose reagent' was prepared by mixing solution 1 (100 parts) with solution 2 (1 part), with vigorous stirring, then equilibrating at 20°C before use.

Several standard solutions were prepared containing analar D-glucose (10 to 200 µg/ml) in distilled water. The standard glucose solutions and a distilled water blank (0.2ml.) were mixed with fresh 'glucose reagent' (5ml.) and incubated in the dark for 35 minutes.

The optical densities of the solutions were measured at 435 mµ with an S.P.500 spectrophotometer, and used to prepare a standard graph.

B-1299 L dextran (100mg.) and B-1299 S dextran (101mg.) were dried over phosphorus pentoxide, in vacuo, at 50°C, and then hydrolysed with 2N sulphuric acid (5ml.) in sealed tubes, placed in a water bath at 100°C for 9 hours.

Acid degradation of the liberated glucose was compensated for by including control solutions prepared as follows:

Analar D-glucose (500mg.) was dissolved in distilled water (20ml.), and two aliquots (4ml.) were removed, diluted by addition of 5N sulphuric acid (1ml.) and incubated alongside the two dextrans. Each of the solutions was neutralised by addition of N sodium hydroxide (5ml.) and made quantitatively to 500ml.

Aliquots (0.2ml.) were removed from these solutions, incubated with fresh 'glucose reagent' as described earlier, and the glucose content calculated from the standard graph.

The results obtained were:

B-1299 L dextran	190	µg. glucose per ml.
B-1299 S dextran	193	" " " "
Control solution	200	" " " "
Hydrolysed control	180	" " " "

These figures indicate that the loss of glucose through acid degradation was 10% and the amount of glucose liberated from each dextran, after correction, was B-1299 L (95mg.) and B-1299 S (97.5mg.).

The figures were converted into percentages of glucose (see page 16) from the fact that 162mg. of dextran would theoretically liberate 180mg. of glucose on complete hydrolysis.

Expt. 8 Partial acid hydrolysis experiments

(a) Preliminary hydrolysis. Dextran (30mg.) was hydrolysed with N sulphuric acid (3ml.) in a capped tube placed in a water bath at 100°C for 1.5 hours.

The hydrolysate was neutralised with an excess of barium carbonate and the insoluble barium salts were removed by centrifugation.

The clear supernatant was deionised with IR 120(H^+) and IRA 400(OH^-) resins and concentrated by rotary evaporation, in vacuo, at 40°C to a syrup.

The syrup was subjected to paper chromatography in solvents (a) and (f); chromatograms being developed with spray reagents (a) and (c).

(b) Large scale partial acid hydrolysis of B-1299 S dextran

B-1299 S dextran (35gm.) was treated with N sulphuric acid (700ml.) to give a 5% solution.

The solution was hydrolysed (1.25hr) in a sealed 2 litre flask, placed in a water bath at 100°C. The flask was swirled occasionally to

quicken solution of the dextran.

The hydrolysate was neutralised with excess barium carbonate and the insoluble barium salts centrifuged down. The supernatant was filtered through a No.42 Whatman paper and deionised with IR 120(H^+) and IRA 400(OH^-) resins.

The solution was concentrated to a syrup (24gm.) by rotary evaporation, in vacuo, at 40°C and checked by paper chromatography in solvent (a).

The syrup (15gm.) was taken up in a minimum quantity of distilled water and placed on a carbon-celite column (70 x 7.5 cm.)[41] - see General Procedure (V).

The column was eluted progressively with increasing percentages of aqueous ethanol, and fractions (25ml.) were collected and monitored polarimetrically (2Dm. tube) and chromatographically in solvent (a).

Details of the separation are recorded in Table V.i. and histogram V.2B.

All syrups were subsequently refractionated by paper chromatography in solvent (a) on No.17 Whatman papers. The separated sugars were charcoaled from aqueous ethanol, filtered and freeze dried to white powders.

Details are given in Table III.3.

Table V.1. Separation of oligosaccharides from partially acid hydrolysed B-1299 S dextran

Eluant	Fractions (each 25ml.)	Component	Chromatographing as: (in solvent (a))	Weight (gm.)
Distilled water	145-260	1	Glucose	4.8
6% aqueous ethanol	490-590	2 8	Isomaltose Kojibiose	0.75
16% aqueous ethanol	770-810	3	Isomaltotriose	0.65
↓	810-880	3 4	Isomaltotriose Isomaltotetraose	0.73
↓	880-950	4 5	Isomaltotetraose Isomaltopentaose	0.68
25% aqueous ethanol	950-1080	5 6 7	Isomaltopentaose Isomaltohexaose Isomaltoheptaose	1.00

(c) Determination of the degrees of polymerisation of the oligosaccharides from the partially hydrolysed B-1299 S dextran

The experimental procedure is outlined in General Procedure (X). The results are summarised in Table V.2.

Table V.2.

Component	Optical density (490mμ)		
	Unreduced	Reduced	
2	0.305	0.146	1.9
3	0.301	0.137	2.7
4	0.297	0.223	4.0
5	0.262	0.207	4.8
6	0.295	0.244	5.8
8	0.425	0.225	2.1

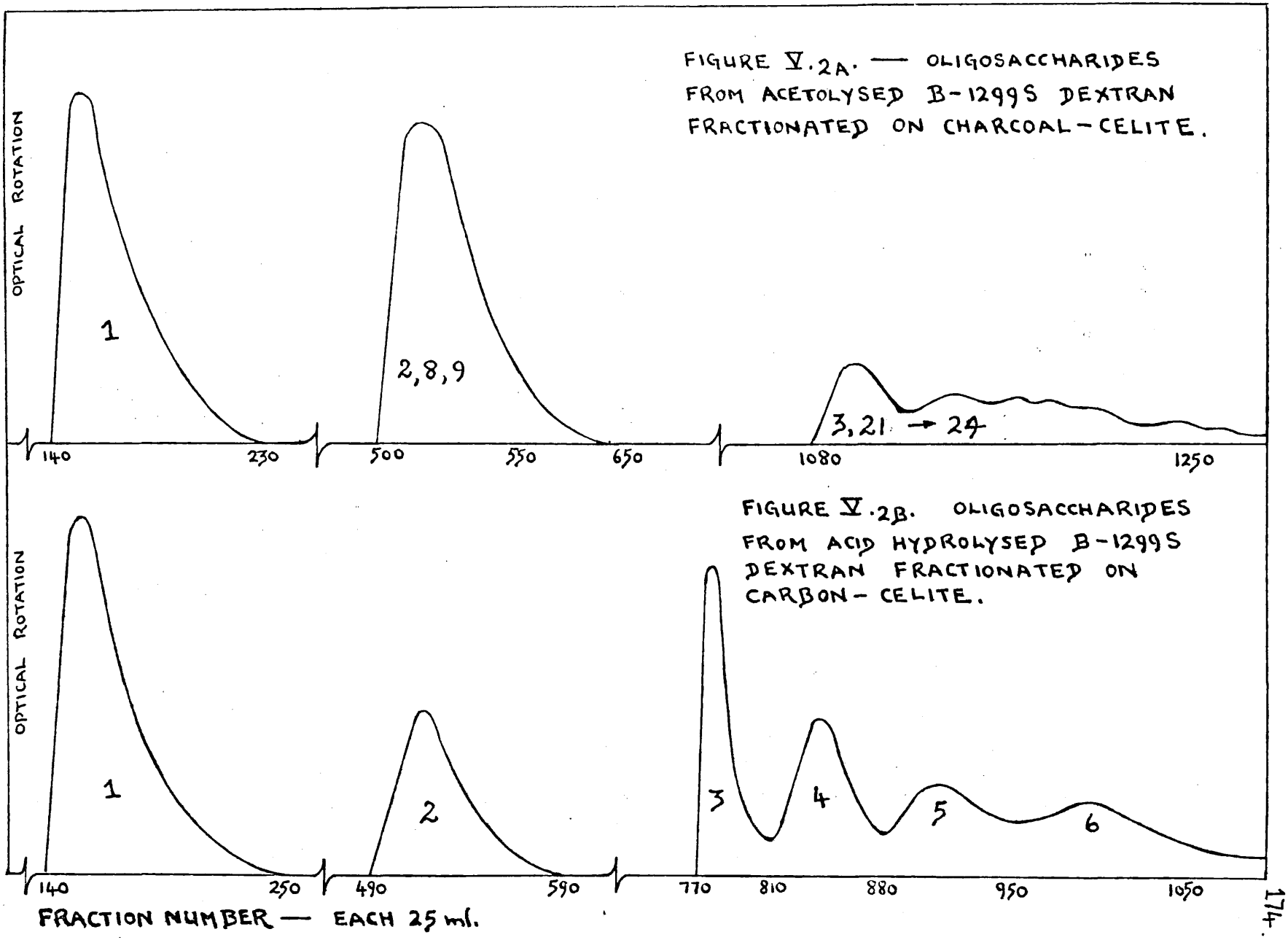


FIGURE V.2A. — OLIGOSACCHARIDES FROM ACETOLYSED B-1299S DEXTRAN FRACTIONATED ON CHARCOAL-CELITE.

FIGURE V.2B. OLIGOSACCHARIDES FROM ACID HYDROLYSED B-1299S DEXTRAN FRACTIONATED ON CARBON-CELITE.

(d) Determination of the optical rotations of the oligosaccharides from the partially hydrolysed B-1299 S dextran[41]

The experimental procedure is outlined in General Procedure (xi). The results are summarized in Table V.3.

Table V.3.

Component	Concentration gm/100 ml.	Observed rotation	Specific rotation	
			Hg light	Na light
Monosaccharide 1	0.571	+ 0.700°	+ 61°	+ 51.9
Disaccharide 2	1.115	+ 1.575°	+ 141°	+ 120
Trisaccharide 3	0.550	+ 0.910°	+ 165°	+ 141
Tetrasaccharide 4	0.633	+ 1.080°	+ 171°	+ 150
Pentasaccharide 5	0.658	+ 1.220°	+ 186°	+ 158
Hexasaccharide 6	0.866	+ 1.640°	+ 190°	+ 162

(e) Preparation of Isomaltose β -octaacetate[41]

The sugar (50mg.) was mixed with freshly fused sodium acetate (75mg.) and gently heated under reflux with acetic anhydride (1ml.) for 1 hour. The reaction mixture was poured onto crushed ice (5ml.) and the solution neutralised to pH 6.0 with solid sodium bicarbonate. The precipitated acetate was filtered, washed with distilled water charcoaled from ethanol and crystallised from aqueous ethanol. Mpt. 139-141°C undepressed by the authentic derivative. Yield 15mg.

(f) Incubation of the oligosaccharides from partially hydrolysed B-1299 S dextran with P. lilacinum dextranase

The experimental procedure is outlined in General Procedure (xiii). Results are listed in Table III.4.

(g) Periodate oxidations of the oligosaccharides from partially hydrolysed B-1299 S dextran

The experimental procedure is outlined in General Procedure (xii). The

results are summarized in Table V.5., and illustrated by the curves in Fig. III.3. The calculated amounts of periodate reduced and formic acid liberated per mole of each member of the series of isomaltose homologues are summarized in Table V.4.

Table V.4.

Isomaltose homologue	Periodate reduced Moles/mole of sugar	Formic acid liberated Moles/mole of sugar
Isomaltose	6	5
Isomaltotriose	8	6
Isomaltotetraose	10	7
Isomaltopentaose	12	8
Isomaltohexaose	14	9

Expt. 9 Partial acetolysis experiments[42]

(a) Preliminary acetolysis. Dextran (150mg.) was treated with acetic anhydride-concentrated sulphuric acid 100:9 volume/volume (1.2ml.). The acetolysis mixture, in a capped tube, was held at 35-37°C for 30 hours with occasional shaking to quicken solution.

The reaction mixture was poured onto crushed ice (20ml.) and neutralised (pH 6.0) with sodium bicarbonate. The precipitated acetates were extracted with chloroform (3x50 ml.) and the chloroform extracts washed with dilute sodium bicarbonate solution and distilled water. The extracts were dried over anhydrous sodium sulphate (24hr), filtered and rotary evaporated, in vacuo, at 40°C to a syrup. The syrup was taken up in dry methanol (10ml.), and deacetylated by addition of a small piece of sodium metal.[115].

The deacetylating solution was left in the refrigerator (24 hours),

Table V.5. The periodate oxidations of oligosaccharides from acid hydrolysed B-1299 S dextran

Component	Periodate reduced (mole/mole sugar) in time (hour)						Formic acid liberated (mole/mole sugar in time (hour)					
	3	12	25	50	75	100	3	12	25	50	75	100
Disaccharide 2	4.54	5.49	5.85	6.09	6.09	-	3.22	4.34	4.57	4.96	5.00	-
Trisaccharide 3	5.61	6.81	7.75	8.02	8.02	-	3.47	4.89	5.50	5.90	6.01	-
Tetrasaccharide 4	5.94	8.92	9.10	9.66	10.03	10.03	3.75	5.65	6.20	6.46	6.91	6.91
Pentasaccharide 5	7.32	9.91	11.42	12.06	12.06	-	4.31	6.20	7.15	7.76	7.89	-
Hexasaccharide 6	7.30	12.36	12.92	13.50	14.04	14.04	3.93	7.00	8.14	8.65	9.00	8.90

treated with distilled water (2ml.) and deionised with IR 120(H^+) and IRA 400(OH^-) resins.

The deionised solution was concentrated in vacuo to a syrup and subjected to paper chromatography in solvent (a). Chromatograms were developed with spray reagents (a) and (c).

The remaining syrup was fractionated on a Whatman No.3 paper (solvent (a)), and the separated sugars were eluted from the paper and subjected to electrophoresis in borate and (after reduction) molybdate electrolytes.

The results are summarized in Table II.5.

(b) Large scale acetolysis of B-1299 S dextran

B-1299 S dextran (30gm.) was treated with acetic anhydride-concentrated sulphuric acid 100:9 volume/volume (120ml.). The reaction mixture, in a 250ml. flask, was placed in a water bath at 35°C for 32 hours.

For the first 12 hours the solution was stirred, under a mercury seal, to quicken solution. The reaction mixture was poured onto crushed ice (1 litre) and neutralised (pH 6.0) carefully with sodium bicarbonate. The precipitated acetates were extracted with chloroform (4x500ml.) and the chloroform extracts washed with saturated sodium bicarbonate solution (3x750ml.) and distilled water.

The extracts were dried over anhydrous sodium sulphate (48 hrs), filtered and evaporated in vacuo to a syrup (50gm.).

Deacetylation.[115] The syrupy acetates were dissolved in methanol (700ml.) and deacetylated by adding clean dry sodium metal (1gm.). The deacetylating solution was left in the refrigerator (24hr), treated with distilled water (150ml.) and deionised with IR 120 (H^+) and IRA 400(OH^-) resins.

The deionised solution was concentrated, in vacuo, to a syrup (19.5gm.) which was examined by paper chromatography in solvent (c), to ensure complete deacetylation.

The syrup (13.5gm.) was taken up in a minimum quantity of distilled water and placed on a carbon-celite column (70x7.5cm.) - see General Procedure (V).

The column was eluted progressively with increasing percentages of aqueous ethanol. Fractions (25ml.) were collected and monitored both polarimetrically (2 Dm. tube) and by paper chromatography in solvent (a).

Details of the separation are recorded in Table V.6. and histogram V.2A.

Table V.6. Separation of oligosaccharides from acetolysed B-1299 S dextran

Eluant	Fractions (each 25ml.)	Component	Chromatographing as (solvent (a))	Weight (gm.)
Distilled water	140-230	1	Glucose	5.3
6% aqueous ethanol	500-550	8	Kojibiose	3.3
6% aqueous ethanol	550-650	2 8 9	Isomaltose Kojibiose Nigerose	1.5
16% aqueous ethanol ↓ 25% aqueous ethanol	1080-1250	3 21 22 23 24	Isomaltotriose - - - -	1.0

The syrups from disaccharide fraction 550-650 and the higher oligosaccharides from fractions 1080 to 1250 were reseparated on No.3 Whatman papers (solvent (a)). Details are given in Table III.6.

(c) Fractionation of the higher oligosaccharides from acetolysed B-1299 S dextran

The mixed oligosaccharide syrup was streaked on a series of No.3 Whatman papers (approximately 30mg. per paper) and electrophoretised in sodium borate electrolyte (pH 10) for 1.5 hours at 2,000 volts.

The separated components were located by laying two thin strips of No.1 Whatman paper along the wet electrophoretogram and taking an impression which was developed with spray reagent (a). The areas containing the components were eluted with distilled water, and deionised by shaking with IR 120(H^+) resin and then evaporating with dry methanol. As some decomposition had occurred, the sugars were refractionated on No.3 Whatman papers in solvent (a), eluted, and concentrated to syrups.

(d) Preparation of kojibiose β-octaacetate

The sugar (200mg.) was mixed with freshly fused sodium acetate (100mg.) and heated under reflux on a water bath with acetic anhydride (2ml.), for 2 hours.

The mixture was brought to reflux over a bunsen for 1 minute and poured onto crushed ice (5ml.). The solution was neutralised to pH 6.0 with solid sodium bicarbonate. The precipitated acetate was filtered, washed with distilled water, charcoaled from ethanol, and recrystallised from aqueous ethanol (twice). Yield 50mg. Details of the physical constants are given in Table III.5.

(e) Determination of the degrees of polymerisation of the oligosaccharides from acetolysed B-1299 S dextran

The experimental procedure is outlined in General Procedure (x). The results are summarized in Table V.7.

Table V.7.

Component	Optical density (490mμ)		
	Unreduced	Reduced	
2	0.400	0.195	2.0
3	0.380	0.250	2.9
8	0.380	0.182	1.9
9	0.375	0.175	1.9
21	0.220	0.155	3.0
22	0.410	0.310	4.1
23	0.710	0.530	3.7
24	1.100	0.820	3.9

(f) Determination of the optical rotations of the oligosaccharides from acetolysed B-1299 S dextran

The experimental procedure is outlined in General Procedure (xi). The results are summarized in Table V.8.

Table V.8.

Component	Concentration gm/100ml.	Observed rotation	Specific rotation	
			Hg light	Na light
Monosaccharide 1	0.501	+ 0.618°	+ 60.4°	+ 51.3°
Disaccharide 8	0.720	+ 1.16°	+ 161°	+ 137°
Disaccharide 9	0.630	+ 0.80°	+ 127°	+ 108°

Expt. 10 Nuclear Magnetic Resonance (NMR) spectra of the dextrans[50]

Before introducing the dextran sample into the spectrometer it was treated with deuterium oxide to exchange the labile protons of the hydroxyl groups with deuterons. The procedure used was as follows:

Dextran (approximately 80mg.) was dried over phosphorus pentaoxide (50°C, in vacuo for 24 hours) and dissolved (or suspended) in deuterium oxide (approximately 3ml.) - warming if necessary.

The dextran solution was allowed to stand in a stoppered flask for 1 hour before freeze drying.

The procedure was repeated thrice more. Finally the deuterated dextran was redissolved in deuterium oxide, introduced into a sample tube and the spectrum recorded on a Varian A60 IL machine.

TMS Tetramethylsilane) was used as an external standard for all peak assignments.

In this experiment the NMR spectra of the following dextrans were recorded: NRRL B-512 (see Fig. II.3.), NRRL B-1299 L and NRRL B-1299 S (see Fig. II.4.) and AD-1299 S (an acid degraded dextran, see Fig. III.11.).

Results are discussed in Sections II.E. and III.D(ii).

Expt. 11 Periodate oxidation of the dextrans[13]

The dextran - B-1299 S (312mg.) and B-1299 L (279mg.) - was dried over phosphorus pentaoxide in vacuo at 50°C for 48 hours, and dissolved (or suspended) in distilled water (300ml.) in a 500ml. volumetric flask.

A standard solution (100ml.) containing sodium metaperiodate (950mg.) - sufficient to allow approximately 2.2 moles per anhydro glucose unit (AGU) of the dextran - was added to the dextran solution, and the total volume was made to 500ml.

A blank solution (250ml.), containing sodium metaperiodate (475mg.) was also prepared.

The dextran was oxidized in the dark at room temperature, and the

periodate reduced and formic acid released per AGU of dextran were measured at regular intervals by the same method as that outlined in General Procedure (xii) for the oligosaccharides.

The experimental results - corrected for ash contents of the dextrans - are summarised in Table V.9. and by Figure II.7.

Table V.9. The results from oxidations of B-1299 dextrans with sodium metaperiodate

Time (hours)	Periodate reduced. moles per mole AGU		Formic acid released. moles per mole AGU	
	B-1299 S	B-1299 L	B-1299 S	B-1299 L
2.50	-	0.46	-	0.10
3.00	0.76	-	0.16	-
8.00	1.13	-	0.26	-
10.00	-	0.78	-	0.27
14.00	1.26	-	0.34	-
21.00	1.36	1.26	0.39	0.51
32.0	1.43		0.42	
48.0	1.46	1.51	0.47	0.55
72.0	1.53	1.55	0.51	0.60
102.0	1.55	1.59	0.52	0.62
150.0	1.61	1.60	0.54	0.65
199.0	1.62	1.61	0.55	0.69

Expt. 12 Fragmentation of the reduced, periodate oxidized dextrans

(a) Preparation of the polyalcohols of B-1299 dextrans

The dextran (8gm., dried over phosphorus pentaoxide in vacuo at 50°C for 48 hours) was oxidized in 0.4% aqueous solution with sodium metaperiodate in the dark at room temperature.

The course of the oxidation reaction was followed by oxidizing separately a smaller quantity (1gm.) of the dextran under identical conditions.

Aliquots (1ml. and 2ml. respectively) were removed from this solution

and used to estimate the periodate reduced and formic acid liberated - see General Procedure (xii).

The oxidation of B-1299 S dextran was stopped after 7 days and B-1299 L dextran after 16 days; when each dextran had reduced the amounts of periodate shown in Table V.10.

Table V.10.

Time (days)	Periodate reduced. moles per mole AGU		Formic acid released. moles per mole AGU	
	B-1299 S	B-1299 L	B-1299 S	B-1299 L
1.50	1.44	1.31	0.47	0.40
5.50	1.49	1.38	0.50	0.49
7.00	1.54	-	0.53	-
8.00	-	1.48	-	0.51
10.25	-	1.51	-	0.52
13.25	-	1.54	-	0.53
16.00	-	1.56	-	0.54

The solution of periodate oxidized dextran (dextran polyaldehyde) was treated with ethylene glycol (20ml.) to destroy unreacted sodium metaperiodate and then dialysed (3 days) against running tap water.

Reduction of the dextran polyaldehyde. Sodium borohydride (4gm.) was then added to the dialysed solution, followed by boric acid solution (9.6gm. of the acid in 100ml.) to buffer the reduction.

The solution was left (24 hours) at room temperature.

A further addition (2gm.) of borohydride was made and the solution left a further 12 hours to ensure complete reduction.

The dextran polyalcohol was then dialysed (5 days) against running tap water, concentrated to approximately 200ml. on a rotary evaporator in vacuo at 30°C and freeze dried to a white solid.

Yields were:

B-1299 S dextran polyalcohol 3.5gm.

B-1299 L dextran polyalcohol 7.7gm.

(Ash contents were negligible)

(b) Complete acid hydrolysis of the dextran polyalcohols

The dextran polyalcohol (50mg.) was hydrolysed with N sulphuric acid (3ml.) in a sealed tube placed in a water bath at 100°C for 7 hours. The hydrolysate was neutralised with excess barium carbonate and the insoluble barium salts centrifuged down. The supernatant, with ethanolic washings of the barium salts, was deionised with IR 120(H^+) and IRA 400(OH^-) resins.

The solution was concentrated to a syrup by rotary evaporation, in vacuo, at 30°C and subjected to paper chromatography in solvents (c) and (d) and electrophoresis in borate electrolyte.

The results of these experiments are shown in Table II.9.

(c) Determination of the ratio of glucose: glycollic aldehyde: glyceraldehyde

(i) Glucose was estimated with the glucose oxidase reagent.

A standard graph was prepared with anular D-glucose as outlined in expt. 7(e).

(ii) Glycollic aldehyde and glyceraldehyde were determined by the colorimetric method of Dische and Borenfreund.[117]

Glycollic aldehyde reacts with diphenylamine to produce a deep green colour, having an absorption maximum at 660 mμ. At 660 mμ, the presence of equal quantities of glucose, glycerol and glyceraldehyde, cause a combined error of not more than 2-3%, in colorimetric estimations of glycollic aldehyde.

Glyceraldehyde also reacts with diphenylamine to produce a brown colour with an absorption maximum at 510 mμ. At this wavelength the error in quantitative estimations of glyceraldehyde due to the presence of equal quantities of glucose and glycerol is not more than 2-3%. However, the error due to glycollic aldehyde, which also has a large absorption at 510 mμ, is considerable.

In this experiment the error due to glycollic aldehyde absorption at 510 mμ was corrected as follows:

The optical densities of standard solutions of glycollic aldehyde, after reaction with diphenylamine reagent, were determined at 660 and 510 mμ and that of glyceraldehyde, after reaction with diphenylamine reagent, at 510 mμ.

Reference to the glycollic aldehyde standard graph at 660 mμ enabled this component to be estimated in a mixture of dextran polyalcohol hydrolysis products, and also determined the glycollic aldehyde absorption at 510 mμ.

Subtraction of the latter from the observed absorption at 510 mμ fixed the absorption due to the glyceraldehyde which was then estimated from the glyceraldehyde standard graph.

Preparation of standard graphs. Standard solutions (1.5ml.) containing glycollic aldehyde (5 to 40 mg.) and a blank (distilled water) were treated with a solution (0.3ml.) of trichloroacetic acid (1gm./ml. of total solution) and freshly prepared 1% recrystallised diphenylamine in glacial acetic acid (3.6ml.).

The tubes were well shaken and placed in a boiling water bath (30

minutes), cooled, and the optical densities measured at 660 and 510 mμ with an S.P.500 spectrophotometer. The results were used to plot two standard graphs.

Standard solutions (1.5ml.) containing glyceraldehyde (50 to 300 mg.) and a blank (distilled water) were treated with trichloroacetic acid solution (0.3ml.) and diphenylamine reagent (3.6ml.). The tubes were shaken, placed in a boiling water bath (30 minutes), cooled, and the optical densities measured at 510 mμ. A standard graph was then plotted.

(iii) Hydrolysis of dextran polyalcohols and control solutions

The dextran polyalcohols (100mg.) were hydrolysed with N-sulphuric acid (5ml.) in sealed tubes placed in a water bath at 100°C for 7 hours. The hydrolysed materials were diluted quantitatively to 25ml. (solution A).

Control solutions of glucose, glycollic aldehyde and glyceraldehyde in N-sulphuric acid (5ml.) were incubated alongside the dextran polyalcohols, and later used to apply corrections for losses, resulting from acid degradation, during the hydrolysis reaction.

(iv) Determination of the percentage degradation of glycollic aldehyde, glyceraldehyde and glucose

The acid degraded glycollic aldehyde control was neutralised (pH 7.0) with barium carbonate and the insoluble barium salts centrifuged down. Portions of the supernatant (1.5ml.) were treated as described earlier with diphenylamine reagent. Undegraded control solutions, diluted to the same extent, were similarly treated.

Glycollic aldehyde content of the control	29 μg/ml.
Glycollic aldehyde content of the acid degraded control	23 μg/ml.
Acid degradation	17.8%

The acid degraded glyceraldehyde control was neutralised (pH 7.0) with barium carbonate and the insoluble barium salts were centrifuged down. Portions of the supernatant (1.5ml.) were treated, as described earlier, with diphenylamine reagent.

Undegraded control solutions, diluted to the same extent, were similarly treated.

Glyceraldehyde content of the control	285 µg/ml.
Glyceraldehyde content of the acid degraded control	263 µg/ml.

Acid degradation 7.7%

The acid degraded glucose control was treated with N-sodium hydroxide (5ml.). Aliquots (1ml.) were treated with glucose oxidase reagents as described in expt. 7(e).

Undegraded control solutions, diluted to the same extent, were similarly treated.

Glucose content of the control	77.5 µg/ml.
Glucose content of the acid degraded control	72.5 µg/ml.

Acid degradation 6.5%

(v) Estimation of the glycollic aldehyde and glyceraldehyde from the dextran polyalcohols

Aliquots (approximately 4ml.) were removed from the hydrolysed dextran polyalcohol solution A, neutralised with barium carbonate, centrifuged, and the clear supernatants (2.5ml.) diluted to 15ml. with distilled water.

After reacting with diphenylamine reagent the optical densities of the solutions were determined at 660 and 510 mµ as described earlier, and the glycollic aldehyde and glyceraldehyde estimated.

The results are shown in Tables V.11. and V.12.

(vi.) Estimation of the glucose from the dextran polyalcohols

Aliquots (5ml.) were removed from the hydrolysed dextran polyalcohol solution A, and neutralised with 0.2N-sodium hydroxide solution (5ml.). Glucose was estimated as described in expt. 7(e).

The results are shown in Tables V.11. and V.12.

Table V.11. Ratio of the fragments from B-1299 L polyalcohol.

Fragment	Concentration* (µg/ 1.5ml.)in solution A	Molecular weight	Molecular ratio
Glycollic aldehyde	234.0	60	0.49
Glyceraldehyde	226.2	90	0.32
Glucose	89.2	180	0.19

Table V.12. Ratio of the fragments from B-1299 S polyalcohol

Fragment	Concentration* (µg/ 1.5ml.)in solution A	Molecular weight	Molecular ratio
Glycollic aldehyde	234.0	60	0.56
Glyceraldehyde	225.0	90	0.36
Glucose	271.5	180	0.07

* corrected for acid degradation

A duplicate estimation, carried out under similar conditions, gave the following molar ratios.

	Glycollic aldehyde	Glyceraldehyde	Glucose
B-1299 L polyalcohol	0.49	0.32	0.19
B-1299 S polyalcohol	0.56	0.36	0.08

(d) Partial acid hydrolysis of the dextran polyalcohols

The dextran polyalcohol (50mg.) was hydrolysed with 0.2N sulphuric acid (5ml.) in a capped tube at room temperature for 24 hours. The hydrolysate was neutralised with excess barium carbonate and the insoluble barium salts centrifuged down.

The supernatant solution was deionised with IR 120(H^+) and IRA 400(OH^-) resins and was then concentrated to a syrup by rotary evaporation at 30°C.

The syrup was subjected to paper chromatography in solvent (c) and, after elution from the chromatogram, to electrophoresis in sodium borate electrolyte.

The results are summarised in Table II.11.

Expt. 13 Methylation of the dextrans[59]

(a) Methylation procedure. The methylations were conducted in the apparatus illustrated in Fig. V.3.. Liquid ammonia from a storage cylinder was allowed to evaporate into container A, where it was reliquified, by external cooling to -78°C (acetone-card ice bath), and dried by addition of small pieces of sodium metal (indicated by a faint blue colour).

The dry liquid ammonia, under pressure from the ammonia cylinder, was passed into the unsilvered Dewar vessel B, in which the methylation reaction took place at -34°C. (the boiling point of liquid ammonia)

Except during addition of reagents the system was closed to the atmosphere. The boiling liquid ammonia was conducted away to be reliquified in the card ice-acetone trap C.

A weighed amount of dextran (dried over phosphorus pentoxide, in vacuo, at 50°C) was suspended in liquid ammonia (100-150ml.) and stirred

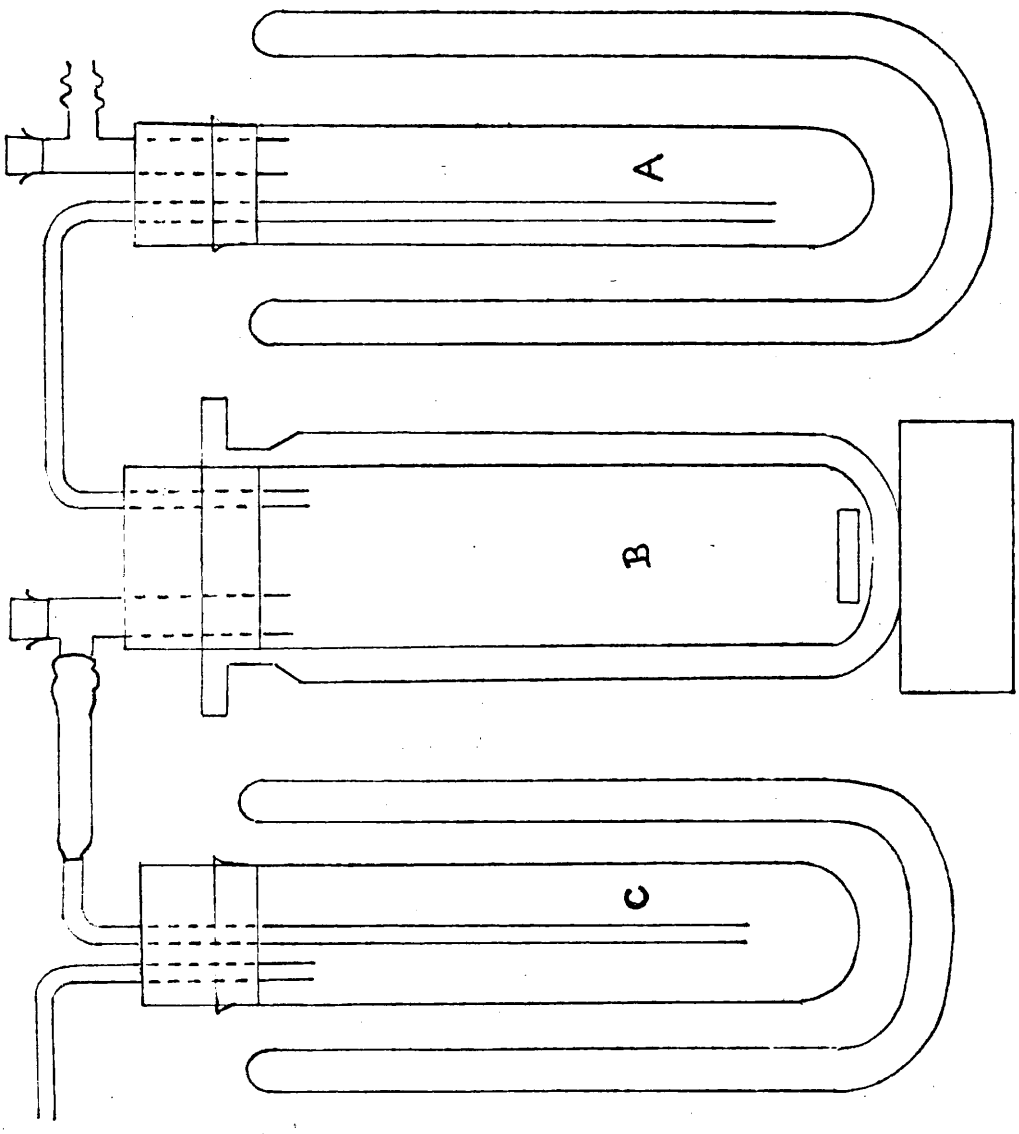


FIGURE V.3.

magnetically. A calculated amount of sodium metal was added to convert the dextran to its sodium derivative. To reduce the possibility of degradation reactions caused by large excess of sodium metal or sodamide the following precautions were observed during these additions:

I. The presence of a large excess of sodium metal in the system was avoided by adding the total (calculated) amount of sodium in the form of small pellets over a period of time.

II. Free sodium (indicated by a blue colour) was not allowed to remain unreacted in the system for more than 15 minutes, so reducing the risk of sodamide formation.

When the calculated amount of sodium had reacted, or uptake of sodium by the dextran had ceased, the reaction was terminated by rapid addition of acalculated volume of redistilled methyl iodide (from a plunger type pipette). The addition of reagents took place over approximately 1 to 2 hours, at the end of which the reaction mixture was stirred for 6 hours, before the ammonia was allowed to evaporate away.

The residue was suspended in distilled water and dialysed, initially against distilled water, (5 litres; 24 hours) then against running tap water (48 hours), before freeze drying to a white solid. Methoxyl contents were determined by the method of Zeizel.[119]

The distilled water dialysate was deionised with IR 120(H^+) and IRA 400(OH^-) resins concentrated, in vacuo, at 40°C, and examined by paper chromatography (solvent (e)) for small degradation fragments.

(In the absence of these fragments, this step was omitted in later methylations.)

The results of the methylations are shown in Table V.13.

Chloroform extraction. Each methylated dextran was extracted, by shaking at room temperature with chloroform (200ml.) and, after evaporation of the chloroform, suspended in distilled water and freeze dried to a white solid.

Details of the fractionation are summarized in Table V.14.

Table V.13.

Dextran	Number of Treatments	Weight of dextran methylated (gm.)	Sodium gm. atoms per AGU*	Methyl iodide gm. moles per AGU*	Product weight (gm.)	% OCH_3
B-1299 S	1	13.00	2.0	2.2	-	-
	2	11.79	2.0	2.2	-	-
	3	11.05	2.0	2.2	-	-
	4	9.60	1.0	1.1	9.10	37.8
	5	8.55	0.5	0.55	8.15	38.0
B-1299 L	1	8.21	2.0	2.2	-	-
	2	7.65	2.0	2.2	-	-
	3	7.43	1.0	1.1	7.35	33.7
	4	6.60	0.5	0.55	6.50	33.8

* AGU anhydro glucose unit

Table V.14.

	Methylated B-1299 S dextran		Methylated B-1299 L dextran	
	Weight (gm.)	% OCH_3	Weight (gm.)	% OCH_3
Material extracted	8.15	38.0	6.50	33.8
Chloroform soluble	7.25	41.2*	5.45	40.9*
Chloroform insoluble	0.45	-	0.60	-

* Ash contents: methylated B-1299 S dextran (1.2%), methylated B-1299 L dextran (1.60%).

(b) Complete acid hydrolysis of the methylated dextrans[64]

The methylated dextran (30mg.) was treated with 72% vol./vol. sulphuric acid (0.5ml.) and left for 1 hour at room temperature in a sealed tube. Distilled water (4.0ml.) was then added (giving an 8% vol./vol. sulphuric acid solution) and hydrolysis was completed by placing the tube in a water bath at 100°C for 4 hours. The hydrolysate was neutralised with an excess of barium carbonate, and the insoluble barium salts centrifuged down.

The supernatant solution was deionised with IR 120(H^+) and IRA 400(OH^-) resins and rotary evaporated, in vacuo, at 35°C to a syrup.

The syrup was examined by paper chromatography in solvent (e) using spray reagent (b) and, after separation in the same solvent, by electrophoresis in borate buffer.

The results are summarised in Table II.12.

(c) Methanolysis of the methylated dextrans

The methylated dextran (30mg.) was refluxed for 24 hours in 7.2% methanolic hydrogen chloride (10ml.), prepared by adding acetyl chloride (7ml.) to cooled dry methanol (27ml.).

$$CH_3.CO.Cl + CH_3OH \longrightarrow CH_3.CO.OCH_3 + HCl$$

(excess)

The solution was then neutralised with excess silver carbonate, filtered, and the filtrates evaporated to dryness at 30°C. The residue was extracted with chloroform (10ml.) and the extract rotary evaporated to a syrup at 30°C.

The syrup was dissolved in sodium dried ether (1ml.) and examined by

vapour phase chromatography. Samples were injected onto the following columns:

I. Butan 1-4 diol succinate polyester (175°C).[61]

II. Polyphenyl ether (m-bis [m-phenoxyphenoxy]) benzene (200°C).[61]

The results are shown in Table II.14.

(d) Large scale acid hydrolysis of the methylated dextrans

Methylated B-1299 L dextran (4gm.) and B-1299 S dextran (5.4gm.) were hydrolysed as described in expt. 13(b). Yields of sugar syrups were:

3.9 gm., from B-1299 L dextran

4.5 gm., from B-1299 S dextran

(e) Determination of the ratio of the methyl sugars

(i) Hypoiodite oxidation method[65]

Preparation of a standard graph. 0.1N iodine solution (1ml.) was added to standard sugar solutions (5ml.) each containing between 0.25 and 2.5mg. of tetra-o-methyl glucose, and a distilled water blank, contained in stoppered tubes.

Solutions (2ml.) 0.2M with respect to both sodium hydrogen carbonate and sodium carbonate (pH 10.6) were added and, after moistening the stoppers with 20% potassium iodide, the oxidations allowed to proceed in the dark at room temperature for 2.5 hours.

The potassium iodide on the stoppers was washed into the reaction mixtures which were diluted to 15ml. with distilled water and acidified with 2N sulphuric acid. The contents of the tubes were mixed and titrated against 0.01N sodium thiosulphate, using sodium starch glycollate as indicator.

These results were used to plot the standard graph illustrated in Fig. V.4.

Estimation of the methyl sugars. The methyl sugar syrup from expt. 13(d) (100mg.) was separated on a No.3 Whatman paper in solvent (e). Vertical parallel sided strips (2cm. across) were cut from the chromatogram and the areas containing the methyl glucoses each separately eluted with distilled water (5ml.). Blank solutions (5ml.) were prepared for each methyl glucose by eluting an identical area of No.3 paper.

The sugars were determined (as tetra-o-methyl glucose equivalent) by hypoiodite oxidation. The results are summarized in Table V.15. and V.16.

Table V.15. Ratio of the methyl glucoses from B-1299 S dextran

Methyl glucose	Tetra	Tri	Di	Mono	Glucose
Experimental titre 0.01N thiosulphate (ml.)	1.43	1.46	1.27	0.26	-
Tetra-o-methyl glucose equivalent (mg.)	1.79	1.83	1.59	0.33	-
Molecular weights of the methyl sugars	236	222	208	194	-
Molar ratio	1.00	1.08	1.01	0.22	-
Molar percentages	30	33	30	7	-

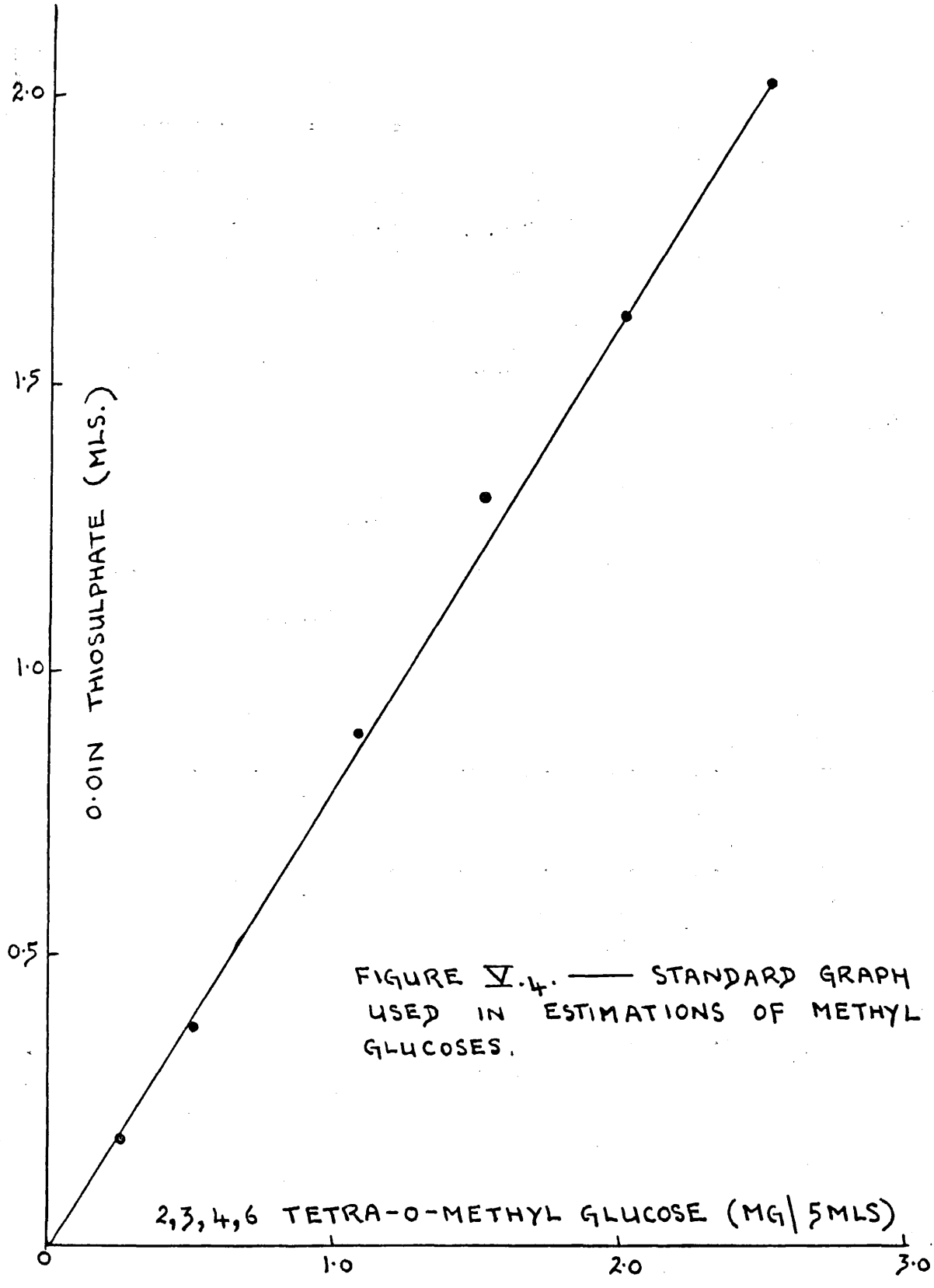


FIGURE V.4. —— STANDARD GRAPH USED IN ESTIMATIONS OF METHYL GLUCOSES.

Table V.16. Ratio of the methyl glucoses from B-1299 L dextran

Methyl glucose	Tetra	Tri	Di	Mono	Glucose
Experimental titre 0.01N thiosulphate (ml.)	1.10	1.36	1.06	0.21	-
Tetra-o-methyl glucose equivalent (mg.)	1.38	1.70	1.33	0.26	-
Molar ratio	1.00	1.30	1.09	0.22	-
Molar percentages	28	36	30	6	-

(ii) Measurement of peak areas

The separations of the methyl glucoses (expt. 13(f)) were followed polarimetrically and are represented by the optical rotational curves in Figure V.5. The individual equilibrium optical rotations of the separated methyl sugars varied by less than 10%, which enabled a ratio for the methyl glucoses to be calculated from determinations of the areas under the peaks in Figure V.5.

The results are summarized in Table V.17. and V.18.

Table V.17. Ratio of the methyl glucoses from B-1299 S dextran

Methyl glucose	Tetra	2,3,4 Tri	2,4,6 Tri	Di
Area under curve ($cm.^3$)	44.4	32.4	9.0	37.2
Molecular weights of the methyl glucoses	236	222	222	208
Molar ratio	1.00	0.76	0.20	0.94
Molar percentages	34.0	26.4	7.2	32.3

Table V.18. Ratio of the methyl glucoses from B-1299 L dextran

Methyl glucose	Tetra	2,3,4 Tri	2,4,6 Tri	Di
Area under curve ($cm.^3$)	50.4	40.7	25.0	53.0
Molar ratio	1.00	0.86	0.53	1.17
Molar percentages	27.9	24.0	14.6	31.0

(f) Fractionation of the sugars from the methylated dextrans[104,73].

Preparation of a cellulose column. No.11 Whatman cellulose was slurried in acetone, and packed under pressure on a bed of glass wool (3cm.) to give a 80 x 5cm. column.

The column was washed with a mixture of redistilled n-butanol:petroleum ether (bpt. 100-120°C) 3:7 volume/volume saturated with water.

The methyl glucoses from hydrolysed methylated B-1299 L dextran (3.8gm.) were applied to the cellulose column as a thick syrup.

The separation was followed polarimetrically (2dm. tube) and paper chromatographically in solvent (e).

Components 16 (tetra-o-methyl glucose), 17 (tri-o-methyl glucose) and 18 (tri-o-methyl glucose) were eluted from the column with n-butanol: petroleum ether (bpt. 100-120°C) 3:7 volume/volume.

Component 19 (di-o-methyl glucose) was eluted by changing the eluant proportions to 4:6 volume/volume.

The syrupy hydrolysate from methylated B-1299 S dextran (4.4gm.) was separated on a cellulose column into components 16, 17, 18 and 19 by an identical procedure.

Details of these separations are given in Table II.17. and illustrated in Fig. V.5.

(g) Characterisation of component 16 from each methylated dextran

Fractions containing component 16 were combined and evaporated to a semi crystalline solid which chromatographed (solvent (e)) as tetra-o-methyl glucose.

The crude material was washed with a little petroleum ether (bpt. 60-80°C).

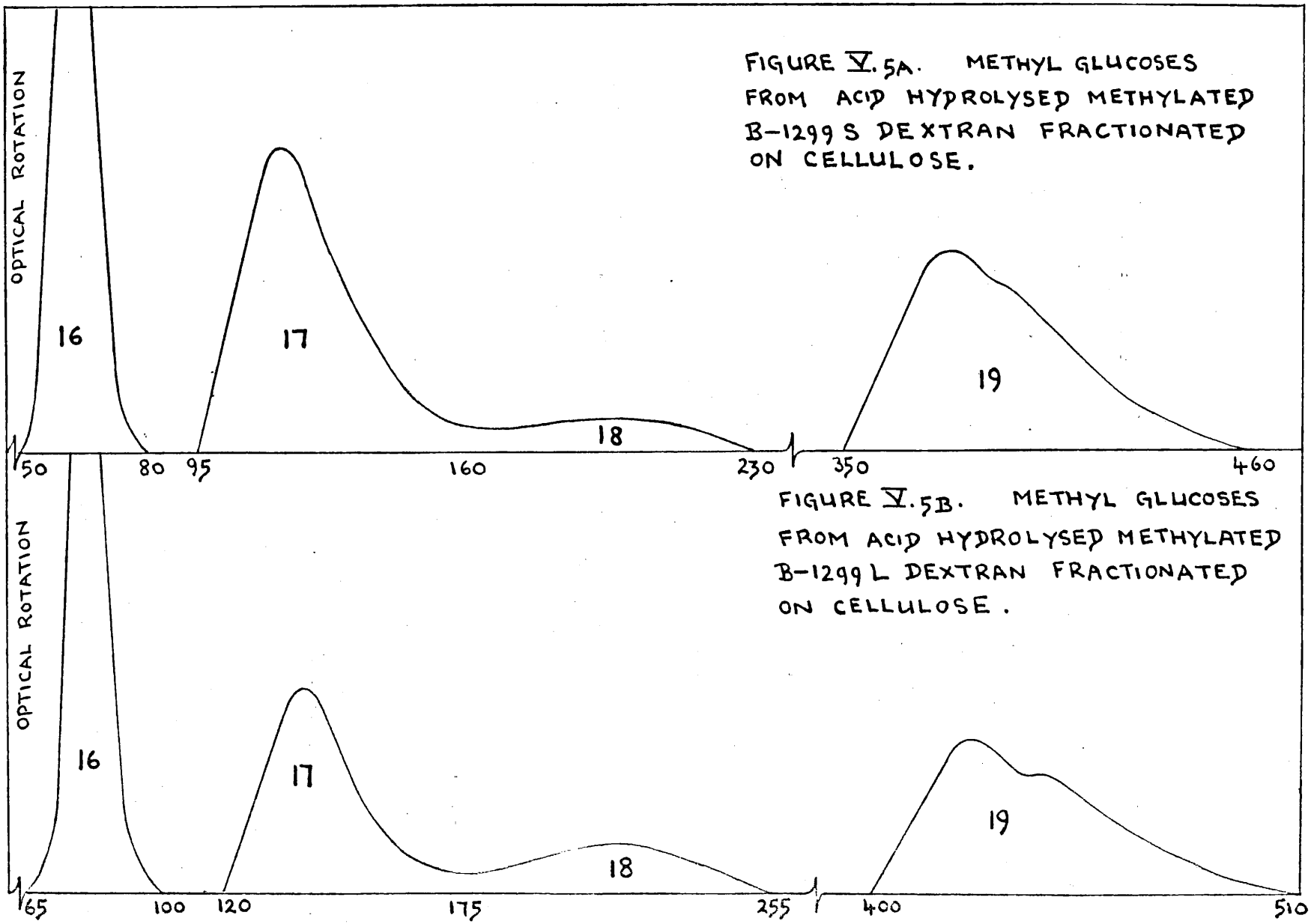


FIGURE V.5A. METHYL GLUCOSES FROM ACID HYDROLYSED METHYLATED B-1299 S DEXTRAN FRACTIONATED ON CELLULOSE.

FIGURE V.5B. METHYL GLUCOSES FROM ACID HYDROLYSED METHYLATED B-1299 L DEXTRAN FRACTIONATED ON CELLULOSE.

weighed, charcoaled from ethanol and recrystallised from ethanol-petroleum ether (bpt. 40-60°C). The recrystallised material was examined as follows:

(i) with spray reagent (b) after paper chromatography in solvent (e)

(ii) the sugar (5mg.) was methanolysed by refluxing in 4% methanolic hydrogen chloride (10 hours) and the methyl glucoside was subjected to vapour phase chromatography on a butan-1-4-diol succinate polyester (175°C)[61] - column I

(iii) the optical rotation of the sugar was determined in distilled water with a sodium polarimeter (1dm. tube, filling on approximately 0.7ml.)

The results were:

Component 16	Concentration gm./100ml.	Observed rotation	Specific rotation
From B-1299 L dextran	0.204	0.201° → 0.171°	+99° → +83°
From B-1299 S dextran	0.212	0.208° → 0.175°	+98° → +83°

(iv) the melting point, and mixed melting point with authentic 2,3,4,6 tri-o-methyl glucose, was determined

(v) a sample (5mg.) was sent for independent methoxyl content determination.

The yield and physical constants of component 16 from each methylated dextran are shown in Table II.17.

(h) Characterisation of component 17 from each methylated dextran

Fractions containing component 17 were combined and evaporated to a syrup, which chromatographed (solvent (e)) as 2,3,4-tri-o-methyl glucose. The crude material was taken up in ethanol charcoaled, re-evaporated and weighed as a syrup. The syrup was examined as follows:

(i) with spray reagent (b) after paper chromatography in solvent (e)

(ii) the sugar (5mg.) was methanolysed and subjected to vapour phase chromatography (see expt. 13(g))

(iii) the syrup from methylated B-1299 S dextran was converted into the N-phenyl glucosylamine ('anilide')[74] derivative.

Syrup 17 (80mg.) was treated with redistilled aniline (55mg.) and refluxed in absolute ethanol (1ml.) for 4.5 hours on a water bath. The reaction mixture was cooled and concentrated by rotary evaporation, in vacuo, at 30°C to an off white solid. The crude 'anilide' was charcoaled from a large excess of ether and recrystallised (twice) from ether:petroleum ether (bpt. 40°C). Yield 12mg. The physical properties of the 'anilide' are shown in Table II.17.

The yield and physical constants of component 17 from each methylated dextran are shown in Table II.17.

(i) Characterisation of component 18 from each methylated dextran

Fractions containing component 18 were combined and evaporated to a semi crystalline solid which chromatographed (solvent (e)) as 2,4,6 tri-o-methyl glucose. The crude material was washed with a little petroleum ether (bpt. 60-80°C), weighed, charcoaled from ethanol, and recrystallised from ethanol:petroleum ether (bpt. 40-60°C). The recrystallised material

was examined as follows:

(i) with spray reagent (b) after paper chromatography in solvent (e)

(ii) the sugar (5mg.) was methanolysed and subjected to vapour phase chromatography (see expt. 13(g))

(iii) the optical rotation of the sugar was determined in methanol (see expt. 13(g)).

The results were:

Component 18	Concentration gm./100ml.	Observed rotation	Specific rotation
From B-1299 L dextran	1.025	1.01° → 0.745°	+98° → +73°
From B-1299 S dextran	0.816	0.775° → 0.603°	+95° → +74°

(iv) the melting point, and mixed melting point with authentic 2,4,6 tri-o-methyl glucose, was determined.

(v) a sample (5mg.) was sent for independent methoxyl content determination.

The yield and physical constants of component 18 from each methylated dextran are shown in Table II.17.

(j) Characterisation of component 19 from each methylated dextran

Fractions containing component 19 were combined and evaporated to a semi crystalline solid which chromatographed (solvent (e)) as a di-o-methyl glucose and in borate electrophoresis migrated as 3,4 di-o-methyl glucose although traces of both 2,3 di-o-methyl and 2,4 di-o-methyl glucose appeared to be present.

The crude material was washed with a little petroleum ether (bpt. 60-80°C), weighed, charcoaled from n-propanol and recrystallised from ethyl acetate. The recrystallised material was examined as follows:

(i) with spray reagent (b) after paper chromatography in solvent (e)

(ii) the sugar (5mg.) was methanolysed and subjected to vapour phase chromatography (see expt. 13(g))

(iii) the optical rotation of the sugar was determined in distilled water (see expt. 13(g)).

The results were:

Component 19	Concentration gm./100ml.	Observed rotation	Specific rotation
From B-1299 L dextran	0.991	1.076° 0.735°	+108° +74°
From B-1299 S dextran	0.999	0.735°	+74°

(iv) a sample (5mg.) was sent for independent determination of the methoxyl content

(v) the melting point was determined

(vi) the sugar from methylated B-1299 S dextran was converted into the N-phenyl glycosylamine ('anilide') derivative.[72]

Component 19 (50mg.) was treated with redistilled aniline (32mg.) and refluxed in absolute ethanol (1ml.) on a water bath for 4 hours. Ethanol (10ml.) was added to the reaction mixture which was charcoaled, filtered and concentrated by rotary evaporation in vacuo at 30°C.

The crude material was recrystallised (twice) from a mixture of n-butanol:n-heptane. Yield 10mg. The physical constants of the 'anilide' appear in Table II.17.

The yield and physical constants of component 19 from each methylated dextran are shown in Table II.17.

Expt. 14 Enzymic degradation of the B-1299 S dextran

(a) Preservation of the Penicillium lilacinum mould and preparation of the dextranase

(i) Preservation of moulds

The mould Penicillium lilacinum was obtained from the Commonwealth Mycological Institute, Kew. The mould was preserved on agar slopes of the following composition:

Sodium nitrate	2.0	gm./litre
Potassium dihydrogen phosphate	1.0	
Magnesium sulphate	0.5	
Potassium chloride	0.5	
Ferrous sulphate, hepta hydrate	0.01	
Sucrose	30.0	
Agar Powder	20.0	

At 0°C, viability of the moulds could be maintained for 4 months, before subculturing, on this medium.

(ii) Preparation of the Penicillium lilacinum dextranase[90]

Agar stabs of the P. lilacirum mould were innocculated into a sterile medium (10ml.) containing

1% S. Bovis dextran
1.5% Yeast extract (Marmite C.o)

and swirled at 25-29°C.

After subculturing 5 times at 3 day intervals through 10ml. of the medium the mycelia were introduced into 6 conical flasks each containing 200ml. of the medium. These flasks were swirled for 7 days at 25-29°C. The culture medium (cooled to 0°C) was filtered under reduced pressure through glass wool to remove most of the mycelia, and the filtrate centrifuged (3,000 r.p.m., 15 minutes) at 0°C. The supernatant liquor (1.1 litres) was made 5 m.molar with respect to sodium citrate (adjusted to pH 6.0 with citric acid), dialysed against 4 x 6 litres of citrate buffer (5 m.molar, pH 6.0) at 0°C and stored in the refrigerator at 0°C for use.

(b) Preparation of S. Bovis dextran[92]

The dextran producing organism Streptococcus Bovis strain 1 was obtained from the National Dairy Centre, Shinfield, Reading. The organism, from a freeze dried state, was subcultured (twice) through glucose media, before innoculating a sucrose broth. The compositions of these media are given under General Procedure (ix)d.

After incubation for 3 days at 38°C the S. Bovis dextran was extracted from the culture fluid and purified, by the methods described for the water soluble B-1299 S dextran (expt. 2 and 3). The average yield from 1 litre of medium was 4.2gm. of dextran; ash content 0.19%.

(c) Periodate oxidation of the S. Bovis dextran[13]

S. Bovis dextran (310mg.), dried over phosphorus pentaoxide, in vacuo, for 48 hours at 50°C, was dissolved in distilled water (300ml.) in a 500ml. volumetric flask.

A standard solution (100ml.), containing sodium metaperiodate (950mg.), was added to the dextran solution, and the total volume was made to 500ml.

A blank solution (250ml.) containing sodium metaperiodate (475mg.) was also prepared. The dextran was oxidized in the dark at room temperature, and the periodate reduced and formic acid liberated per AGU were measured at regular intervals - see General Procedure (xii).

The experimental results - corrected for ash content of the dextran - are summarized in Table V.19. and Figure V.7.

Table V.19. The results from oxidations of S. Bovis dextran with sodium metaperiodate

Time (hours)	Periodate reduced: moles per mole AGU	Formic acid released: moles per mole AGU
4.00	1.17	0.36
10.00	1.41	0.51
19.50	1.61	0.69
49.50	1.86	0.90
75.50	1.92	0.96
98.50	1.94	0.97
154.50	1.95	0.97

After extrapolating the curves in Figure V.7. back to zero time (see page 32) the corrected figures were:

periodate reduced 1.90 moles per AGU

formic acid liberated 0.95 moles per AGU

Applying the equations in Table II.7. this indicated that the S. Bovis dextran contained 95% 1→6 and 5% 1→3 links.

(d) The standard dextran/dextranase digest contained dextran (5mg.) in 0.2M sodium citrate (pH 5.0, 2ml.) and dextranase solution (2ml.) which were incubated under toluene (1ml.) for 48 hours at 37°C.

(e) Determination of the activity of P. lilacinum dextranase

Tsuchiya,[89] defined dextranase activity in terms of the 'dextranase unit'. One 'dextranase unit' is the amount of enzyme which will liberate 1mg. equivalent of isomaltose monohydrate in 1 hour, at 40°C from a suitable substrate.

S. Bovis dextran (50mg.) in distilled water (1ml.) was buffered with 0.2M sodium citrate, pH 5.0 (2ml.) and treated with 0.05ml. of dextranase preparation diluted to 1ml. with distilled water. An identical solution was also prepared in which the dextranase had been inactivated by boiling for 1 minute.

The solutions were incubated in capped tubes for 2 hours at 39°C. An aliquot (1ml.) was removed from each solution and the reducing sugars estimated (as isomaltose) by the Nelson-Somogyi method.[118]

A standard graph for this determination was prepared as follows:

Three standard reagents were first made up.

I. Arsenomolybdate reagent. Ammonium molybdate (25gm.) was dissolved in water (450ml.) and concentrated sulphuric acid (21ml.) added with stirring. Sodium arsenate heptahydrate (3gm.) was dissolved in distilled water (25ml.) and added to the molybdate solution. The resulting solution was left for 2 days at 37°C and then stored at 0°C in a brown bottle.

Copper reagents

II. Rochelle salt (15gm.) and anhydrous sodium carbonate (30gm.) were dissolved in distilled water (300ml.), and sodium bicarbonate (20gm.) then added. The solution was cooled and combined with a solution (500ml.) containing anhydrous sodium sulphate (180gm.). The resulting solution was diluted to 1 litre with distilled water.

III. Copper sulphate pentahydrate (5gm.) and anhydrous sodium sulphate (45gm.) were dissolved in distilled water (250ml.).

Just before use solution II (4 parts) was combined with solution III (1 part).

Standard sugar solutions (1ml.) containing isomaltose (20 to 200 µg.) and a distilled water blank were pipetted into pyrex tubes (1.3 x 10cm.) and treated with copper reagent (1ml.). The tubes were capped with glass bulbs and heated on a boiling water bath (30 min.) cooled and arseno-molybdate reagent (1ml.) added.

The solutions were mixed (by inversion) diluted to 6ml. with distilled water and the optical density measured at 520 mµ. with an S.P.500 spectrophotometer.

Results: the optical density of the solution from the dextranase digest was equivalent to that from a solution containing 105 µg. of isomaltose per ml. This was equivalent to

$$\frac{105 \times 10^{-3} \times 4 \times 20}{2 \times 360}$$ mg. equivalent of

isomaltose per ml. of dextranase solution or 0.12 'dextranase units' per ml. of dextranase solution.

(f) Acid degradation of the native B-1299 S dextran

Matsuda[40] determined that, when hydrolysed with N-sulphuric acid at 90°C, the percentage degradation of the α-(1→2) glucosidic link of kojibiose was as follows.

TIME (min.)	Degradation (%)
30	50
60	66
90	78
120	88
150	93
180	98
210	(100)

These figures were taken as an approximate guide for the acid hydrolysis of the α-(1→2) links in B-1299 S dextran.

(i) Preliminary hydrolyses

B-1299 S dextran (15gm.) was dissolved in warm distilled water (300ml.) and incubated (in a sealed flask) in a thermostated bath at 90°C (± 3°C). 5N-sulphuric acid (75ml.) was incubated in a similar manner. When the two solutions had attained 90°C they were quickly combined, mixed and returned to the bath.

Aliquots (25ml.) were removed from the hydrolysis reaction after 30, 60, 90, 120, 150, 180, 210, 240 and 270 minutes. The aliquots were neutralised with barium carbonate, and the insoluble barium salts centrifuged down. The supernatant solution was passed through a No.42 Whatmans' filter, and dialysed (48 hrs) against running tap water. The

dialysed solution was concentrated, by rotary evaporation, in vacuo, at 40°C and freeze dried to a white solid. The weights of the freeze dried degraded dextran appear in Fig. V.6. Each degraded dextran (5mg.) was incubated with dextranase (expt. 14(d)) and, after deionisation, was subjected to paper chromatography in solvent (b). The results are discussed on page 106 .

(ii) Large scale acid hydrolysis. B-1299 S dextran (200gm.) was dissolved in warm distilled water (4 litres) and incubated at 90°C, in a thermostated bath. 5N-sulphuric acid (1 litre) was incubated in a similar manner. The two solutions were then combined, to give a 4% solution of dextran in N sulphuric acid, mixed and reincubated at 90°C for 1.75 hours. The hydrolysate was neutralised with excess barium carbonate, centrifuged and filtered. The filtrate was dialysed against distilled water (10 litres), which was concentrated down to yield glucose plus isomaltodextrins (35gm.), then dialysed (3 days) against running tap water. The dialysed solution was concentrated in vacuo at 40°C, and the acid degraded dextran/freeze dried to a white solid (55gm.). Ash content 0.00%.

(g) Periodate oxidation of the acid degraded AD-1299 S dextran[13]

AD-1299 S dextran (308mg.) dried over phosphorus pentaoxide, in vacuo, at 50°C for 48 hours, was dissolved in distilled water (300ml.) in a 500ml. volumetric flask.

A standard solution (100ml.), containing sodium metaperiodate (940mg.), was added to the dextran solution and the total volume was made to 500ml.

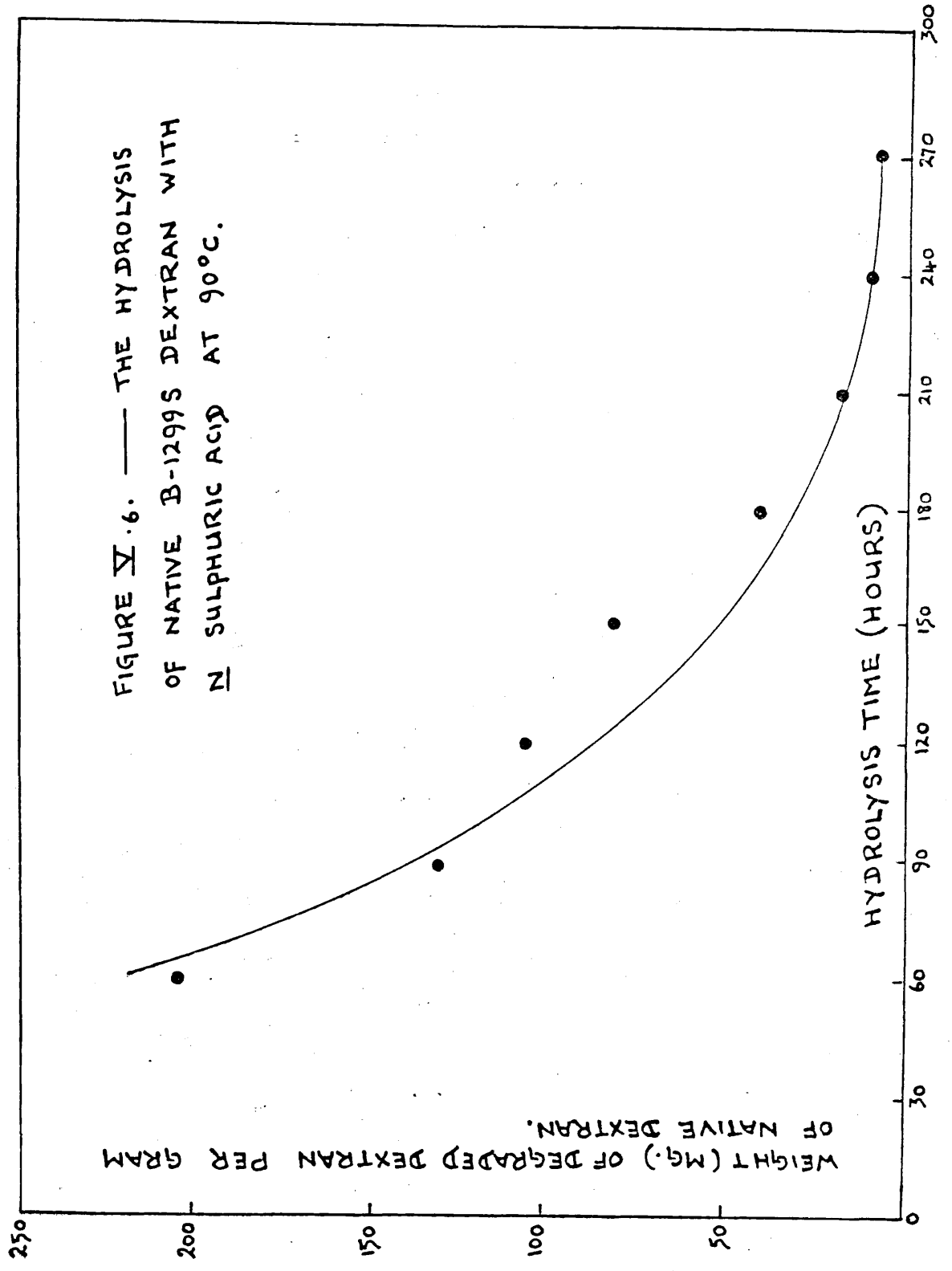


FIGURE V.6. —— THE HYDROLYSIS OF NATIVE B-1299S DEXTRAN WITH N SULPHURIC ACID AT 90°C.

A blank solution (250ml.) containing sodium metaperiodate (470mg.) was also prepared. The dextran was oxidized in the dark at room temperature, and the periodate reduced and formic acid liberated per AGU were measured at regular intervals - see General Procedure (xii).

The experimental results are summarized in Table V.20. and Figure V.7.

Table V.20. The results of oxidations of AD-1299 S dextran with sodium metaperiodate

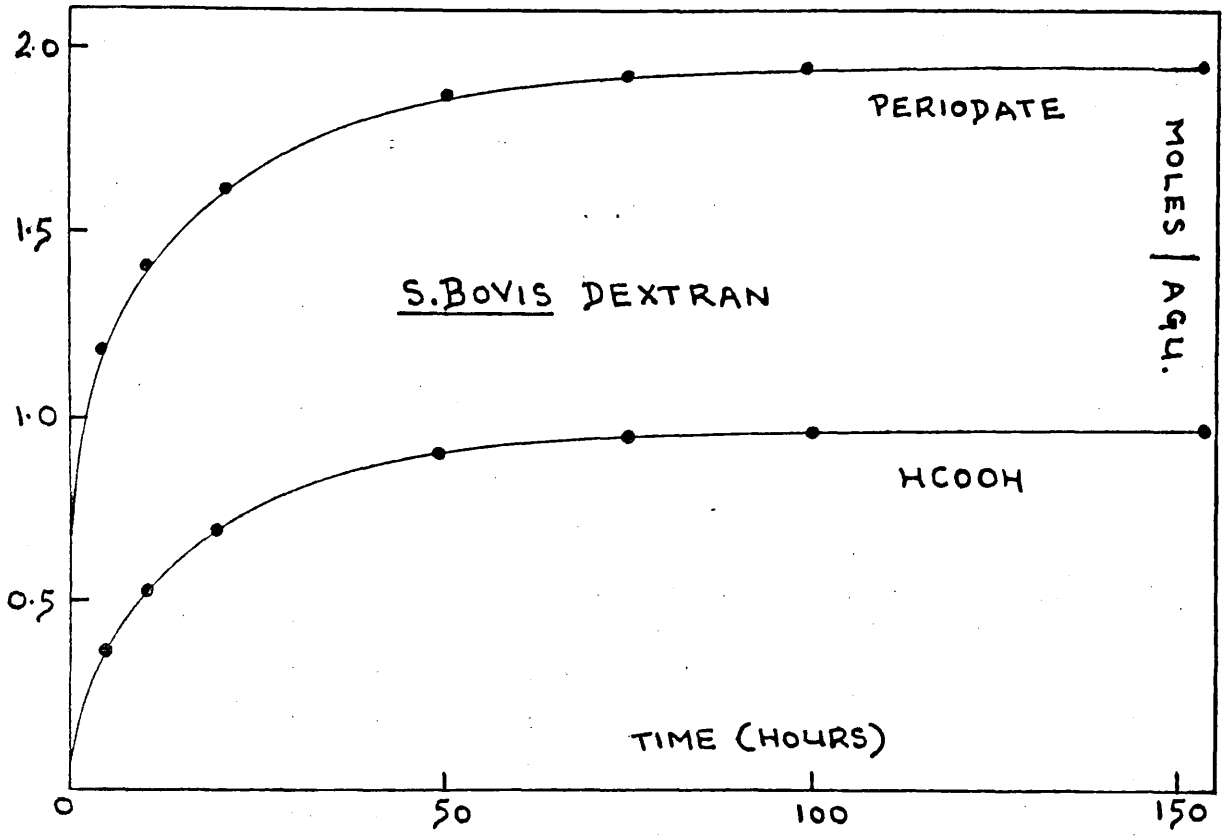
Time (hours)	Periodate reduced: moles per mole AGU	Formic acid released: moles per mole AGU
3.00	1.17	0.46
9.00	1.55	0.65
19.50	1.70	0.79
48.50	1.86	0.92
73.50	1.94	0.94
100.00	1.95	0.97
133.0	1.98	0.98

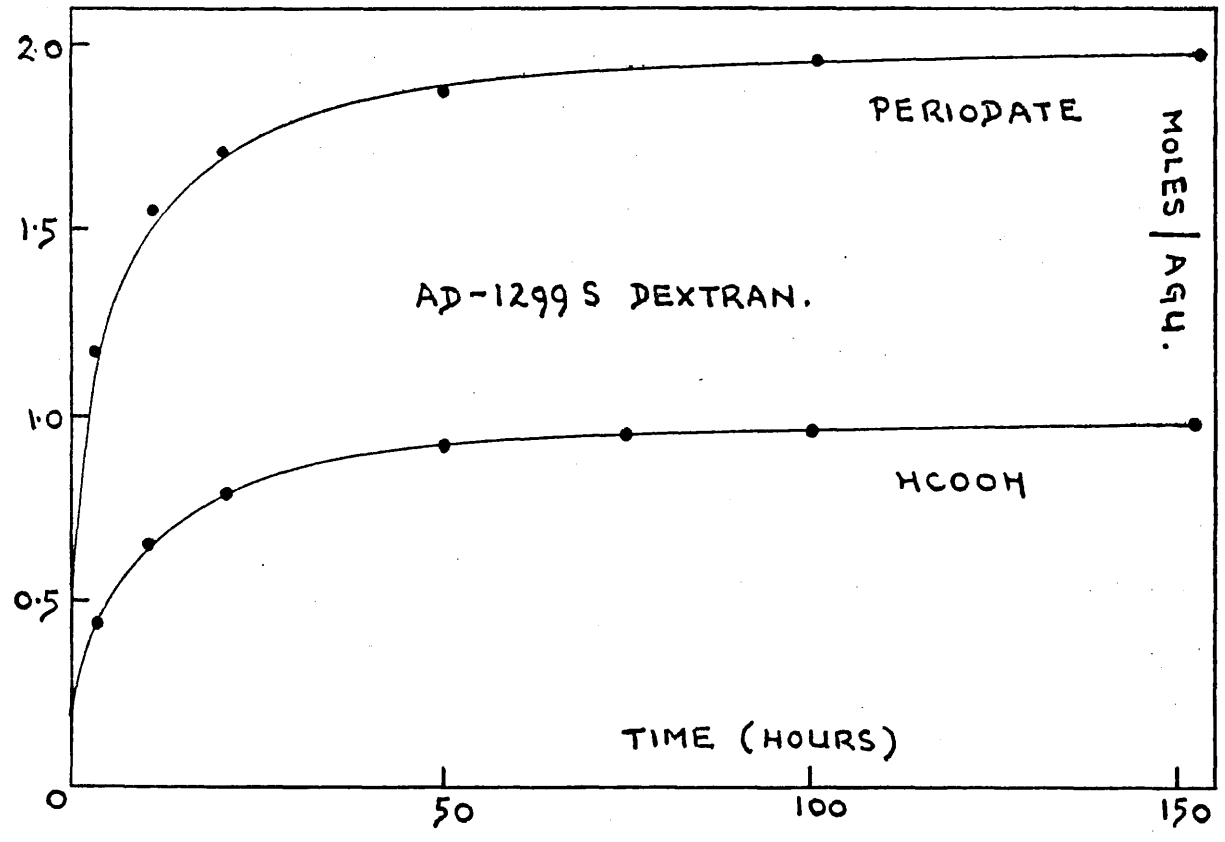
(h) Liberation of reducing sugars from the acid degraded AD-1299 S dextran by the action of P. lilacinum dextranase

(i) Qualitative experiment. The AD-1299 S dextran (5mg.) was incubated in a standard digest (expt. 14(d)). The products were subjected to chromatography in solvent (b).

(ii) Quantitative experiment. The AD-1299 S dextran (50mg.) - dried over phosphorus pentaoxide at 50°C, in vacuo, for 3 days - was dissolved in distilled water (1ml.), buffered with 0.2M citrate buffer (pH 5.0, 2ml.) and treated with P. lilacinum dextranase extract (0.05ml. - made to 1ml. with distilled water). An identical blank solution was prepared in which

FIGURE V.7. THE PERIODATE OXIDATIONS OF TWO DEXTRANS.





the enzyme was inactivated by boiling for 1 minute.

The digestw were incubated in capped tubes, at 39°C.

At intervals aliquots (0.02ml.) were removed and the reducing sugars estimated, as isomaltose equivalent, by the method of Nelson-Somogyi[118] (see experiment 14(e)).

The results are shown in Table V.21. and illustrated by Figure III.11.

Table V.21.

Time (hours)	Isomaltose equivalent (µg./ml.)	Isomaltose liberated mg./100 mg. dextran
0.00	43.3	17.3
1.50	50.5	20.2
5.00	53.5	21.4
11.50	60.0	24.0
27.00	71.0	28.4
52.50	78.0	31.2
93.00	82.5	33.0
120.00	85.0	34.0

(i) Large scale dextranase digest of the AD-1299 S dextran

AD-1299 S dextran (50gm.) was dissolved in sodium citrate buffer 0·2M (pH 5.0; 500ml.) and treated with P. lilacinum dextranase solution (500ml.). The digest was incubated, under toluene, at 37°C for 6 days.

The solution was deionised with IR 120(H^+) and IRA 400(OH^-) resins, concentrated to 50ml. and placed on a carbon-celite column (70x7.5cm.). The column was eluted with increasing percentages of aqueous ethanol. Due

to overloading, sugar separations were poor. The column was therefore washed with 35% aqueous ethanol (to remove sugars of D.P. 2 to 9) and the washings combined and concentrated to a syrup. This syrup was placed on a second carbon column (70x7.5cm.) which was eluted with aqueous ethanolic solutions. The separation was followed polarimetrically (2Dm. tube) and by paper chromatography in solvent (b). Details of the separation are recorded in Table V.22.

Note. In retrospect a better work up of the digest at this stage would probably have been initial deionisation, dialysis (to remove sugars of D.P. < 9) and carbon column separation of the sugars in the dialysate.

The syrups from fractions 510-565 (each 25ml.) were streaked on washed No.17 Whatman papers and refractionated in solvent (b). The oligosaccharides were charcoaled from aqueous ethanol, filtered, and freeze dried to white solids. Yields were: component 25 (1.1gm.), component 26 (1.6gm.) and component 27 (0.60gm.).

Table V.22. Separation of oligosaccharides from enzymically degraded AD-1299 S dextran

Eluant	Fractions (each 25ml.)	Component	Chromatographing as:(in solvent (b))	Weight (gm.)
Distilled water	160-220	1	Glucose	0.6
6% aqueous ethanol	255-285	2	Isomaltose	4.7
6% aqueous ethanol	285-380	2 3	Isomaltose Isomaltotriose	5.5
6% aqueous ethanol ↓ 16% aqueous ethanol	380 -510	2 3	Isomaltose Isomaltotriose	7.1
16% aqueous ethanol ↓	510- 565	3 25 26 27	Isomaltotriose - - -	4.2
20% aqueous ethanol	565- 645	26 27 28	- - -	3.5
20% aqueous ethanol ↓ 25% aqueous ethanol	A further 10 litres of eluant	27 28 29	- - -	8.0

Expt. 15. Characterisation of the oligosaccharides from enzymically degraded AD-1299 S dextran

(a) Determination of the degrees of polymerisation of the oligosaccharides from AD-1299 S dextran

The experimental procedure is outlined in General Procedure (x). The results are summarized in Table V.23.

Table V.23

Component	Optical density (490 mμ.)		
	Unreduced	Reduced	
2	0.800	0.400	2.0
3/21	0.580	0.380	2.9
25	0.575	0.430	4.0
26	0.600	0.400	5.0
27	0.590	0.490	5.9

(b) Determination of the optical rotation of the oligosaccharides from AD-1299 S dextran

The experimental procedure is outlined in General Procedure (xi). The results are shown in Table V.24.

Table V.24.

Component	Concentration gm./100 ml.	Observed rotation	Specific rotation	
			Hg light	Na light
Disaccharide 2	0.744	+1.05°	+141°	+120°
Tetrasaccharide 25	0.620	+1.14°	+184°	+157°
Pentasaccharide 26	0.700	+1.39°	+198°	+169°
Hexasaccharide 27	0.687	+1.38°	+201°	+171°

(c) Periodate oxidation of the oligosaccharides from AD-1299 S dextran

The experimental procedure is outlined in General Procedure (xii). The results are summarized in Table V.25. and illustrated by the curves in Fig. III.14.

(d) Partial acetolysis of the oligosaccharides from AD-1299 S dextran

The oligosaccharide (approximately 90mg.) was treated with an acetolysis

Table V.25. The periodate oxidations of oligosaccharides from enzymically degraded AD-1299 S dextran

Component	Periodate reduced (Moles/mole AGU) - in time (hrs)						HCOOH released (Moles/mole AGU) - in time (hrs)					
	3.0	12.0	25.0	50.0	76.0	100.0	3.0	12.0	25.0	50.0	76.0	100.0
Disaccharide 2	4.5	5.4	5.8	6.1	6.1	-	3.4	4.3	4.6	4.9	5.0	-
Trisaccharide 3/21	5.4	(16 hours) 7.10		7.7	7.7	-	3.2	(16 hours) 4.9		5.6	5.6	-
Tetrasaccharide 25	6.6	7.6	8.6	9.0	9.0	-	4.1	5.0	5.4	5.85	5.9	-
Pentasaccharide 26	7.6	9.4	10.3	10.8	10.82	10.82	4.3	5.8	6.3	6.75	6.85	6.85
Hexasaccharide 27	8.1	10.6	11.6	12.4	12.6	12.6	4.4	6.0	6.7	7.3	7.6	7.6

mixture (3ml.) consisting of acetic anhydride: concentrated sulphuric acid, 100:9 volume/volume.

After warming to complete solution, the sugar solution was cooled and incubated at 35°C for 2 hours in a water bath.

The acetolysis reaction mixture was then poured onto crushed ice (10ml.) and neutralised with sodium bicarbonate (pH 6.0). The precipitated acetates were extracted with chloroform (4x10ml.), and the extracts washed with dilute sodium bicarbonate solution and then distilled water.

The chloroform extracts were dried over anhydrous sodium sulphate (24 hours), filtered and concentrated, in vacuo, at 40°C to a syrup. The syrup was taken up in dry methanol (15ml.) and deacetylated by addition of a small piece of sodium metal (24 hours, in the refrigerator). The deacetylated sugars were deionised with IR 120(H^+) and IRA 400(OH^-) resins and were subjected to paper chromatography in solvent (b). The sugars were then separated in solvent (b) on a No.3 Whatman paper, eluted from the paper with distilled water, and subjected to electrophoresis in borate and (after reduction) molybdate electrolytes. Chromatograms and electrophoretograms were developed with spray reagent (a).

(e) Incubation of the oligosaccharides from AD-1299 S dextran with P. lilacinum dextranase

The experimental procedure is outlined in General Procedure (xiii).

(f) Partial acid hydrolysis of the oligosaccharides from AD-1299 S dextran

The oligosaccharide (approximately 50mg.) was heated for 2.5 hours, in a capped tube, at 70°C with N-sulphuric acid (4ml.).

The hydrolysate was neutralised with excess barium carbonate and

the insoluble barium salts centrifuged off. The supernatant solution was deionised with IR 120(H^+) and IRA 400(OH^-) resins, evaporated to a syrup and subjected to paper chromatography in solvent (b). The chromatograms were developed with spray reagent (a).

The degradation products from components 26 and 27 were, in addition, fractionated on a Whatman No.3 paper in solvent (b), and the pentasaccharide and tetrasaccharide fractions from component 27 and the tetrasaccharide fraction from component 26 were eluted from the papers.

These fractions were incubated, together with isomaltotetraose and isomaltopentaose standards, with dextranase - see General Procedure (xiii). The results are discussed in Section III.D(iii).

(g) Methylation of the oligosaccharides from AD-1299 S dextran[93,94]

The oligosaccharide (approximately 12mg.) was dissolved in N,N dimethyl formamide (redistilled from calcium oxide; 1.5ml.) and treated with dry silver oxide (1gm.) and redistilled methyl iodide (3ml.). The methylation mixture was shaken at room temperature (24 hours) in a darkened, sealed flask.

The insoluble silver salts were then filtered off, washed with chloroform and the filtrate and washings concentrated, by rotary evaporation, in vacuo at 40°C. The concentrated solution was retreated with dry silver oxide (1gram) and redistilled methyl iodide (3ml.) and shaken (24 hours) at room temperature in the dark. The insoluble salts were again removed by filtration and the filtrate and washings concentrated, in vacuo, at 40°C and then placed on a high vacuum line at 30°C to remove the last traces of dimethyl formamide.

The syrupy residue was dissolved in methyl iodide (5ml.), dry silver oxide added (0.5gm.) and the mixture refluxed on a water bath for 6 hours.

The insoluble material was filtered off and the filtrate and washings were concentrated to a syrup (in vacuo at 40°C) which was methanolysed (5 hours) with 4% methanolic hydrogen chloride (4ml.) - see expt. 13(c).

The solution was neutralised with silver carbonate, filtered, and the filtrate concentrated, in vacuo, at 30°C to a syrup.

The syrup was taken up in sodium dried ether (1ml.) and examined by vapour phase chromatography. Samples (0.6µl.) were injected onto the following columns:

I. Butan-1,4-diol succinate polyester at 175°C.[61]

II. Polyphenyl ether (m-bis[m-phenoxy phenoxy]) benzene at 200°C.[61]

The retention times (RT_{TMG}), and probable identities of the methyl glucosides found in the methanolysates of methylated components 25, 26 and 27 are shown in Tables V.27., V.28. and V.29. respectively.

The retention times of some standard methyl glucosides, chromatographed on columns I and II, are included in Table V.26.

Table V.26. Retention times of some standard o-methyl, methyl glucosides

o-methyl, methyl glucoside	Retention time (RT TMG)			
	Column I		Column II	
2,3,4,6 tetra	1.00	1.43	1.00	1.32
2,3,4 tri	2.50	3.52	1.35	1.83
3,4,6 tri	2.95	3.50	1.73	2.28
3,4 di	-	-	2.43	-

Table V.27. Retention times of the degradation products from the methanolysed methylated component 25

Retention time (RT TMG)				Identity*
Column I		Column II		
1.00 L	1.43 L	1.00 L	1.33 L	2,3,4,6 tetra
2.50 L	3.50 L	1.33 L	1.83 L	2,3,4 tri
2.93 M	3.50 L	1.70 Sh	2.30 M	3,4,6 tri
4.40 T	-	-	-	Under methylation?

* of the -o-methyl, methyl glucoside

KEY for Tables V.27., V.28. and V.29.

L large peak
M medium peak
S small peak
Sh shoulder
T trace

Table V.28. Retention times of the degradation products from the methanolysed methylated component 26

Retention time (RT TMG)				Identity*
Column I		Column II		
1.00 L	1.43 L	1.00 L	1.33 L	2,3,4,6 tetra
2.53 L	3.50 L	1.33 L	1.83 L	2,3,4 tri
2.93 S	3.50 L	1.70 Sh	2.30 S	3,4,6 tri
4.50 T	-	-	-	Under methylation?

* of the -o-methyl, methyl glucoside

Table V.29. Retention times of the degradation products from the methanolysed methylated component 27

Retention time (RT TMG)				Identity*
Column I		Column II		
1.00 L	1.43 L	1.00 L	1.33 L	2,3,4,6 tetra
2.53 L	3.53 L	1.33 L	1.83 L	2,3,4 tri
-	3.53 L	-	2.34 S	3,4,6 tri
-	-	2.34 S	-	3,4 di
4.50 T	-	-	-	Under methylation?

* of the -o-methyl, methyl glucoside

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