

INFORMATION TO USERS

This reproduction was made from a copy of a document sent to us for microfilming. While the most advanced technology has been used to photograph and reproduce this document, the quality of the reproduction is heavily dependent upon the quality of the material submitted.

The following explanation of techniques is provided to help clarify markings or notations which may appear on this reproduction.

1. The sign or "target" for pages apparently lacking from the document photographed is "Missing Page(s)". If it was possible to obtain the missing page(s) or section, they are spliced into the film along with adjacent pages. This may have necessitated cutting through an image and duplicating adjacent pages to assure complete continuity.
2. When an image on the film is obliterated with a round black mark, it is an indication of either blurred copy because of movement during exposure, duplicate copy, or copyrighted materials that should not have been filmed. For blurred pages, a good image of the page can be found in the adjacent frame. If copyrighted materials were deleted, a target note will appear listing the pages in the adjacent frame.
3. When a map, drawing or chart, etc., is part of the material being photographed, a definite method of "sectioning" the material has been followed. It is customary to begin filming at the upper left hand corner of a large sheet and to continue from left to right in equal sections with small overlaps. If necessary, sectioning is continued again—beginning below the first row and continuing on until complete.
4. For illustrations that cannot be satisfactorily reproduced by xerographic means, photographic prints can be purchased at additional cost and inserted into your xerographic copy. These prints are available upon request from the Dissertations Customer Services Department.
5. Some pages in any document may have indistinct print. In all cases the best available copy has been filmed.

**University
Microfilms
International**

300 N. Zeeb Road
Ann Arbor, MI 48106

8407062

Cote, Gregory L.

THE FORMATION OF ALPHA-(1 → 3) D-GLUCOSIDIC LINKAGES BY
EXOCELLULAR ALPHA-D-GLUCANSUCRASES FROM LEUCONOSTOC
MESENEROIDES AND STREPTOCOCCUS MUTANS

Iowa State University

Ph.D. 1983

University
Microfilms
International 300 N. Zeeb Road, Ann Arbor, MI 48106

PLEASE NOTE:

In all cases this material has been filmed in the best possible way from the available copy.
Problems encountered with this document have been identified here with a check mark ✓.

1. Glossy photographs or pages ✓
2. Colored illustrations, paper or print _____
3. Photographs with dark background _____
4. Illustrations are poor copy _____
5. Pages with black marks, not original copy _____
6. Print shows through as there is text on both sides of page _____
7. Indistinct, broken or small print on several pages _____
8. Print exceeds margin requirements _____
9. Tightly bound copy with print lost in spine _____
10. Computer printout pages with indistinct print _____
11. Page(s) _____ lacking when material received, and not available from school or author.
12. Page(s) _____ seem to be missing in numbering only as text follows.
13. Two pages numbered _____. Text follows.
14. Curling and wrinkled pages _____
15. Other _____

University
Microfilms
International

The formation of α -(1 \rightarrow 3) D-glucosidic linkages
by exocellular α -D-glucansucrases from *Leuconostoc mesenteroides*
and *Streptococcus mutans*

by

Gregory L. Côté

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of the
Requirements for the Degree of
DOCTOR OF PHILOSOPHY

Department: Biochemistry and Biophysics

Major: Biochemistry

Approved:

Signature was redacted for privacy.

In Charge of Major Work

Signature was redacted for privacy.

For the Major Department

Signature was redacted for privacy.

For the Graduate College

Iowa State University

Ames, Iowa

1983

TABLE OF CONTENTS

	<u>Page</u>
ABBREVIATIONS USED	v
NOTES ON THE NOMENCLATURE OF SUGARS	vii
HAWORTH PROJECTIONS OF SOME SUGAR STRUCTURES	viii
GENERAL INTRODUCTION	1
Explanation of Dissertation Format	2
LITERATURE REVIEW	4
SECTION I. ISOLATION AND PARTIAL CHARACTERIZATION OF AN EXTRACELLULAR GLUCANSUCRASE FROM <i>Leuconostoc</i> <i>mesenteroides</i> NRRL B-1355 THAT SYNTHESIZES AN ALTERNATING (1→6), (1→3)- α -D-GLUCAN	13
ABSTRACT	14
INTRODUCTION	15
MATERIALS AND METHODS	17
Growth Conditions	17
Preparation of Crude Enzyme Mixture	17
Enzyme Assays	17
Carbohydrate and Protein Determination	18
Poly(ethylene glycol) Precipitations	18
pH-Activity Assays	18
Dextranase Digests	18
Chromatography	19
Preparation of Polysaccharides	20
Periodate Oxidations	20
Preparation of 3-Deoxy-3-fluoro- α -D-glucopyranosyl Fluoride	20
RESULTS	22
DISCUSSION	42
ACKNOWLEDGMENT	48
REFERENCES	49

SECTION II. ACCEPTOR REACTIONS OF ALTERNANSUCRASE FROM <i>Leuconostoc mesenteroides</i> NRRL B-1355	52
ABSTRACT	53
INTRODUCTION	54
MATERIALS AND METHODS	55
Enzymes and Substrates	55
Chromatography	55
Acceptor-reaction Conditions	56
Determination of Relative Strengths of Acceptors	56
Structural Analysis of Oligosaccharide Acceptor-products	57
RESULTS	58
DISCUSSION	74
ACKNOWLEDGMENT	78
REFERENCES	79
SECTION III. THE FORMATION OF α -(1 \rightarrow 3) BRANCH LINKAGES BY AN EXOCELLULAR GLUCANSUCRASE FROM <i>Leuconostoc</i> <i>mesenteroides</i> NRRL B-742	82
ABSTRACT	83
INTRODUCTION	84
MATERIALS AND METHODS	86
Organism	86
Crude Enzyme Mixture	86
Chromatography	86
Hydrolysis by Dextranase	87
Synthesis of Samples of D-Glucan	87
¹³ C-Nuclear Magnetic Resonance Spectrometry	88
RESULTS	89
DISCUSSION	104
ACKNOWLEDGMENTS	108
REFERENCES	109

SECTION IV. DISPROPORTIONATION REACTIONS CATALYZED BY <i>Leuconostoc</i> AND <i>Streptococcus</i> GLUCANSUCRASES	112
ABSTRACT	113
INTRODUCTION	114
MATERIALS AND METHODS	115
Carbohydrates	115
Enzymes	115
Chromatography	116
Reaction Conditions	116
RESULTS	117
DISCUSSION	136
REFERENCES	139
 SECTION V. THE FORMATION OF α -(1 \rightarrow 3) BRANCH LINKAGES BY A SOLUBLE-GLUCAN-PRODUCING GLUCANSUCRASE FROM <i>Streptococcus mutans</i> 6715	 143
ABSTRACT	144
INTRODUCTION	145
MATERIALS AND METHODS	146
Enzymes	146
Carbohydrates	147
Analytical Methods	147
Production of Enzymatically Modified D-Glucans	148
RESULTS	149
DISCUSSION	160
ACKNOWLEDGMENTS	164
REFERENCES	165
 GENERAL DISCUSSION	 170
SUMMARY	176
ADDITIONAL REFERENCES	178
ACKNOWLEDGMENTS	188

ABBREVIATIONS USED

ara	arabinose
ATCC	American Type Culture Collection
c.p.m.	counts per minute
CSDC	culture supernatant, dialyzed and concentrated
DEAE	diethylaminoethyl
d.p.	degree of polymerization
F.t.	Fourier transform
fru	fructose
gal	galactose
glc	glucose
GTF-I	glucosyltransferase which synthesizes water-insoluble glucan
GTF-S	glucosyltransferase which synthesizes water-soluble glucan
h	hour(s)
im	isomaltose
im _n	isomaltooligosaccharides of n glucose units
IU	International Units of enzyme activity
L	liter(s)
leu	leucrose
M	molar (moles per liter)
mal, malt	maltose
man	mannose
mg	milligram(s)
MHz	megahertz
min	minute(s)
mL	milliliter(s)
mM	millimolar
mol	mole(s)
N	number of replicate samples

nig	nigerose
nm	nanometers
n.m.r.	nuclear magnetic resonance
NRRC	Northern Regional Research Center
NRRL	Northern Regional Research Laboratory
PA	phenoxyacetyl
pan	panose
PEG	poly(ethylene glycol)
R _{glc}	distance migrated by compound ÷ distance migrated by glucose
raff	raffinose
r.p.m.	revolutions per minute
S.D. _x	standard deviation
S.E. _{\bar{x}}	standard error of the mean
suc	sucrose
THAM	tris(hydroxymethyl)aminomethane
t.l.c.	thin layer chromatography
V _o	void volume
v/v	volume/volume
w/v	weight/volume
xyl	xylose
μg	microgram(s)
μL	microliter(s)

NOTES ON THE NOMENCLATURE OF SUGARS

For disaccharides, the following system is used:

$a-O-b-D$ -substituent- D -glycose

- a: The numeral indicates the position of the oxygen through which the substituent attaches to the glycose unit.
- b: α or β indicates the type of linkage.

For example: $4-O-\alpha-D$ -glucopyranosyl- D -glucose is the nomenclature for maltose.

For oligosaccharides larger than a disaccharide and containing more than one type of sugar, the following system is used:

$a^b-O-c-D$ -substituent-remainder of the oligosaccharide

- a: The numeral indicates the position of the oxygen through which the substituent is attached to the remainder of the oligosaccharide.
- b: the superscript is an abbreviation of the sugar to which the substituent is attached.
- c: α or β indicates the type of linkage by which the substituent is attached to the remainder of the molecule.

For example: $6^G-O-\alpha-D$ -galactopyranosylsucrose is the nomenclature for raffinose.

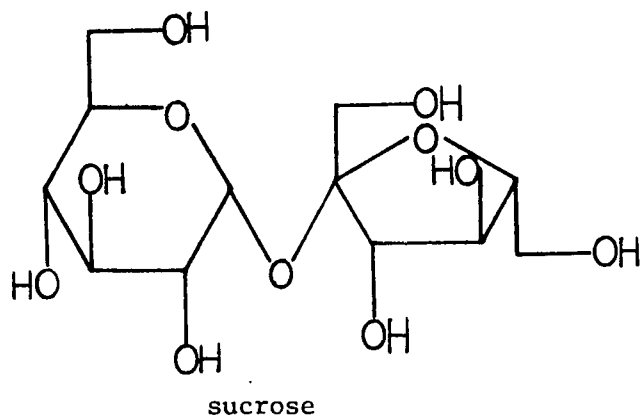
For isomaltooligosaccharides possessing other types of linkages besides α -(1 \rightarrow 6) linkages, the following system is used:

$a^b-O-c-D$ -(substituent) isomaltooligosaccharide

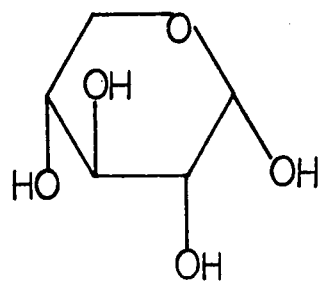
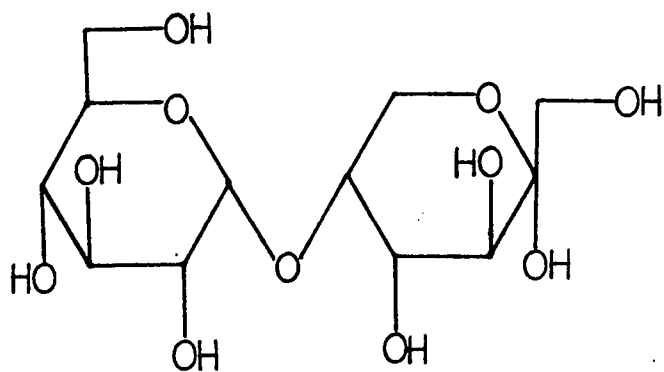
- a: The numeral indicates the position of the oxygen through which the substituent attaches to the isomaltooligosaccharide.
- b: The superscript is a numeral which identifies the glucose unit to which the substituent is attached. The numbering starts with 1 at the reducing end.
- c: α or β indicates the type of linkage between the substituent and the remainder of the molecule.

For example: $3^3-O-\alpha-D$ -glucopyranosyl isomaltotriose is the nomenclature for a tetrasaccharide consisting of a glucose unit linked to the C_3 -OH of the glucose unit at the nonreducing end of isomaltotriose.

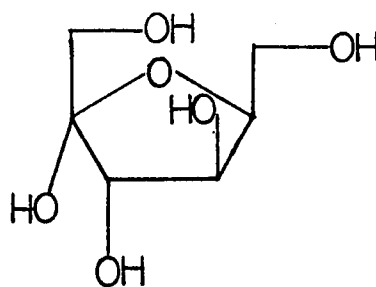
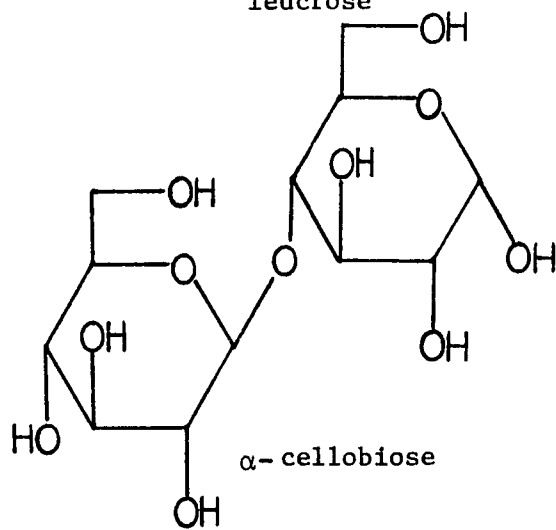
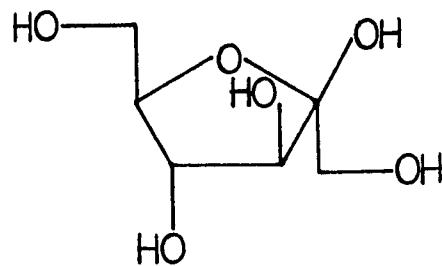
HAWORTH PROJECTIONS OF SOME SUGAR STRUCTURES

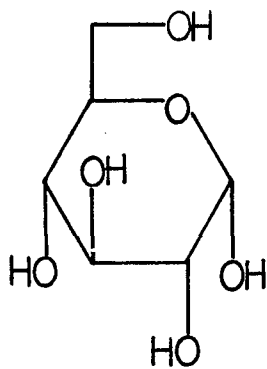
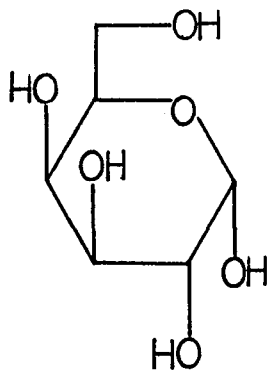
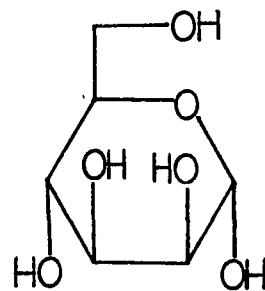
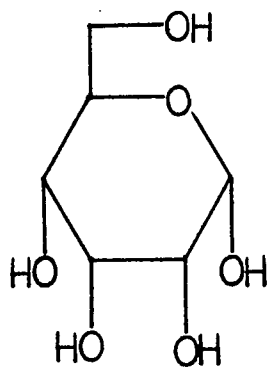
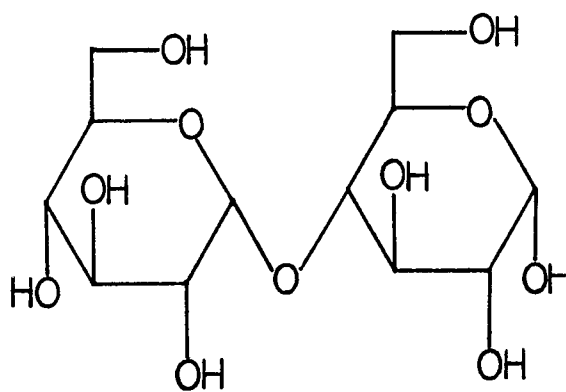
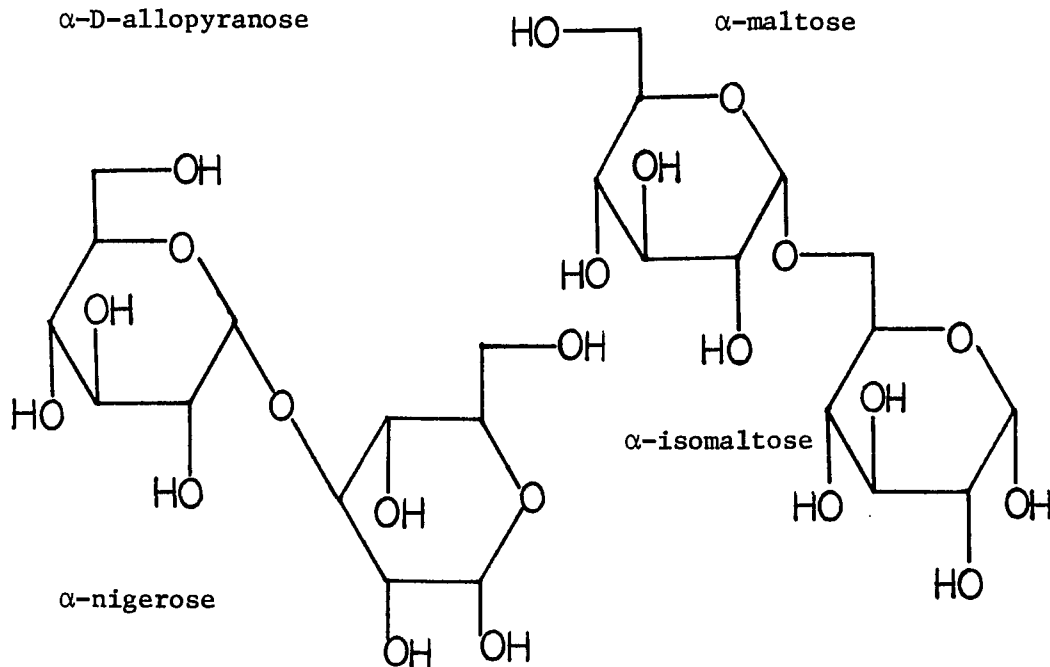
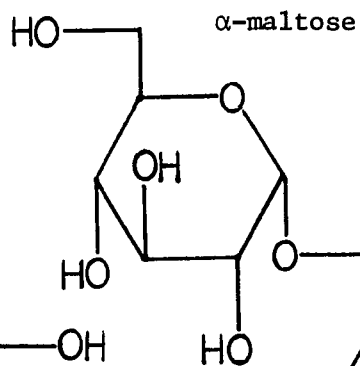


sucrose

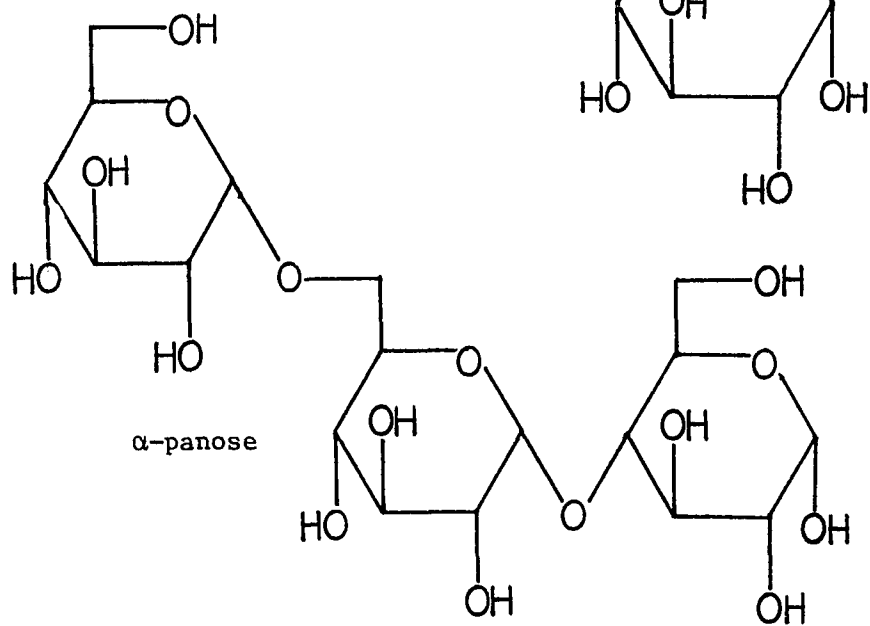
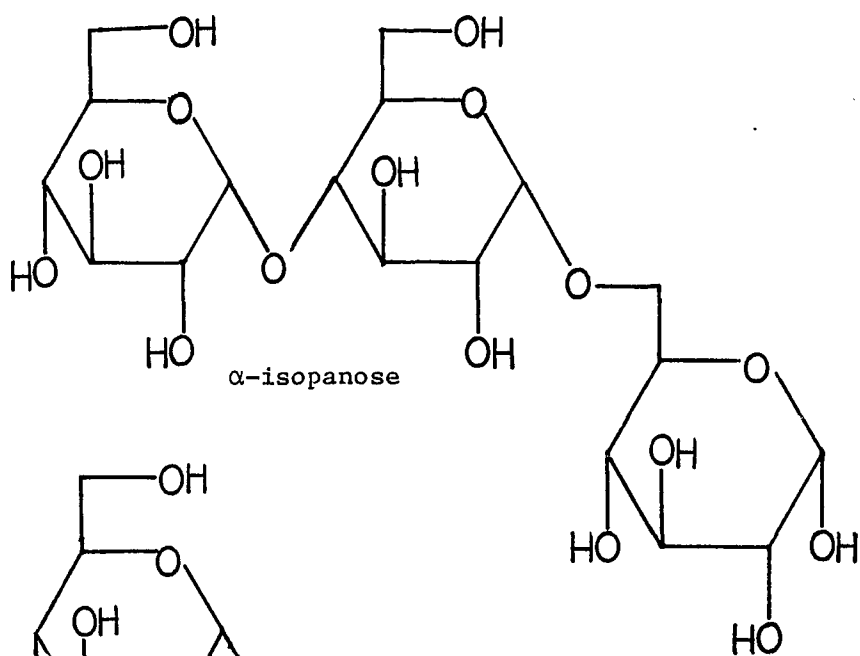
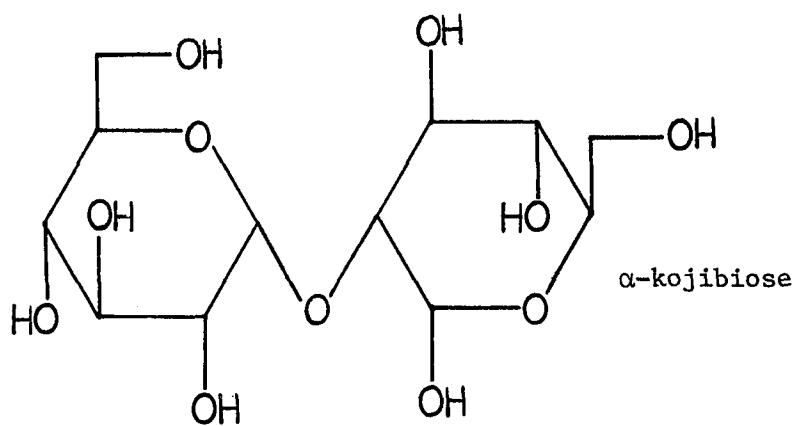
 α -D-xylopyranose

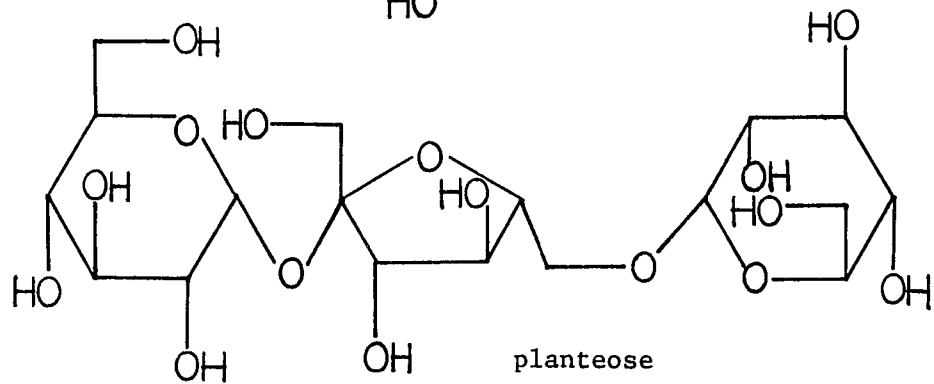
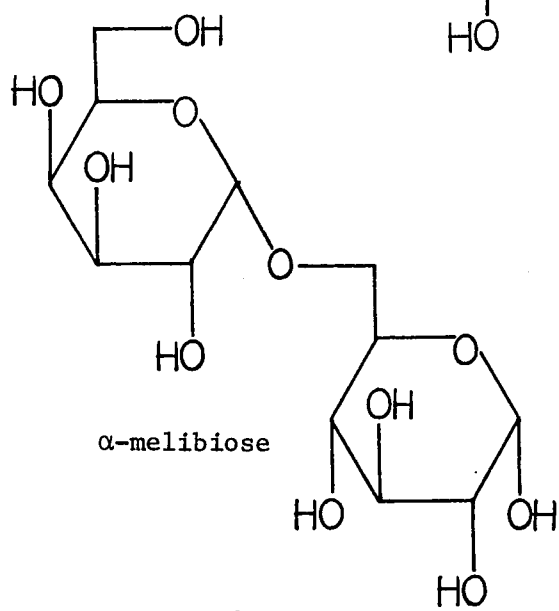
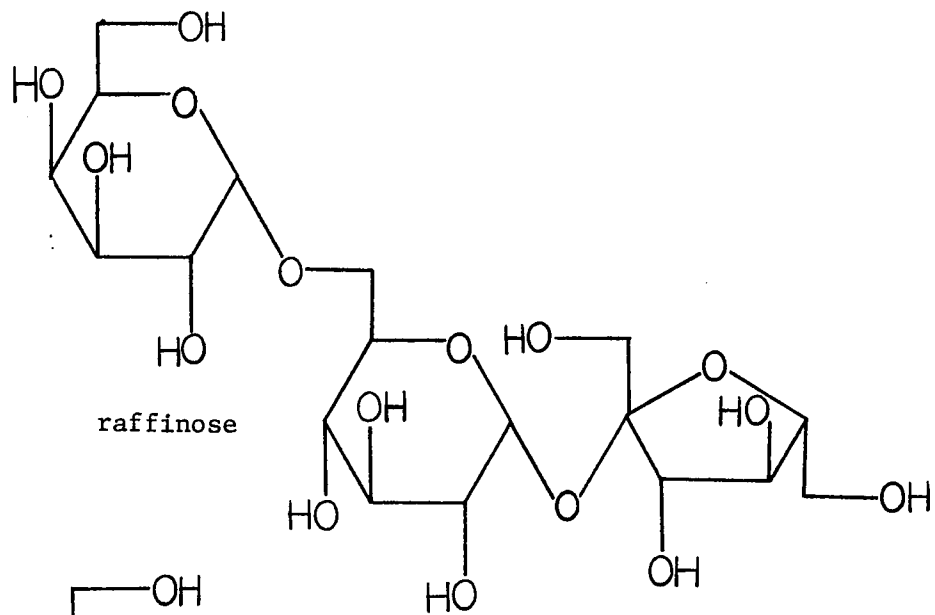
leucrose

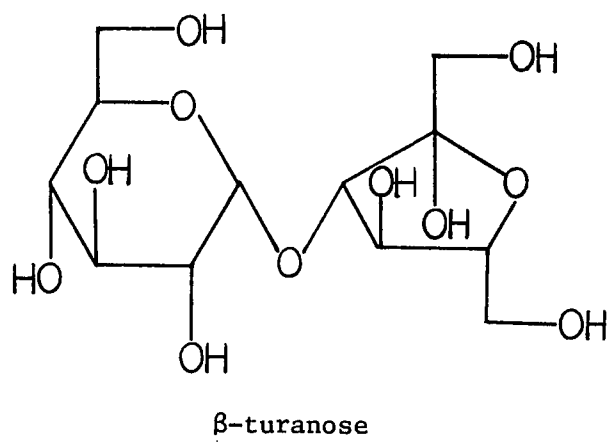
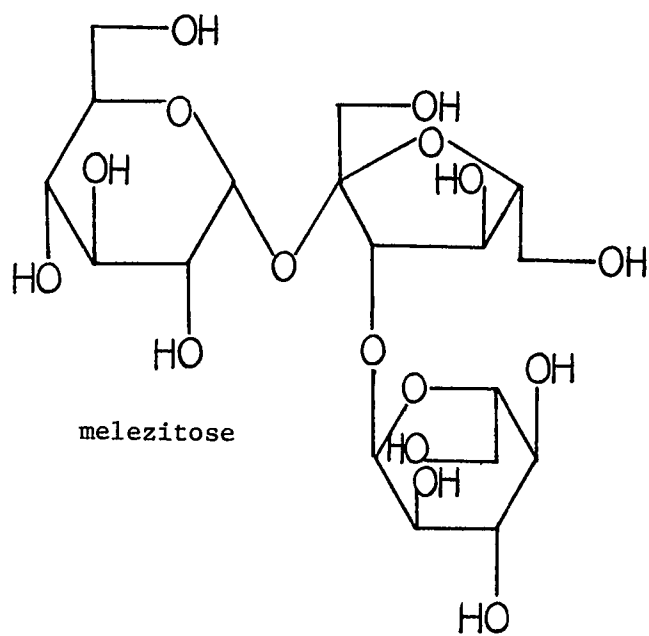
 α -L-sorbofuranose α -cellobiose β -D-fructofuranose

 α -D-glucopyranose α -D-galactopyranose α -D-mannopyranose α -D-allopyranose α -maltose α -nigerose α -isomaltose

x







GENERAL INTRODUCTION

Dextran is the name given to a class of α -D-glucans produced by exo-cellular enzymes from a number of bacteria in the family Streptococcaceae. More specifically, a dextran has been traditionally defined as a polysaccharide consisting of a linear chain of α -(1 \rightarrow 6)-linked D-glucopyranosyl units, with varying amounts of branches linked α -(1 \rightarrow 2), α -(1 \rightarrow 3), or α -(1 \rightarrow 4) to the main chains (1-3). The enzymes which synthesize them from sucrose are known by the generic term "dextransucrase".

Dextrans produced by *Streptococcus mutans* contain only α -(1 \rightarrow 6) and α -(1 \rightarrow 3) linkages (4), while many produced by *Leuconostoc mesenteroides* contain α -(1 \rightarrow 2) or α -(1 \rightarrow 4) branch linkages as well (3). While it is the presence of α -(1 \rightarrow 6) linkages that defines a dextran, it is the type, distribution, and relative amount of these secondary α -(1 \rightarrow 2), α -(1 \rightarrow 3), and α -(1 \rightarrow 4) branch linkages that differentiate one dextran from another. The work described in this dissertation involves some studies aimed at trying to better understand how some of these secondary linkages are formed.

Dextrans play a number of important roles in science, medicine, and industry (5). The production of dextran and related glucans by certain streptococci has been shown to be one of the key processes involved in the formation of dental plaque and the decay of teeth (6,7).

Dextran production by *Leuconostoc mesenteroides* has been a major problem for sugar manufacturers (5). Infection of sugar cane and beets during harvesting and processing may lead to the formation of sticky, gummy masses of dextran, which can foul processing equipment and prevent crystallization of the sugar.

In addition, dextran produced by *Leuconostoc mesenteroides* NRRL strain B-512F has been used as a blood plasma extender, and dextran sulfate is often used as an artificial anticoagulant, acting much like heparin (5).

Dextrans have also been used in a number of research applications, perhaps the best known of which is as Sephadex, a cross-linked dextran from *L. mesenteroides* NRRL B-512F, which is used in molecular-exclusion chromatography. DEAE-Dextran and other dextran derivatives are also used for a variety of chromatographic and immobilization procedures in the

laboratory (5). Various other types of dextrans are sometimes utilized in immunological research, where they are helpful in characterizing the specificity of certain carbohydrate-binding proteins (8).

The present study is a continuation of ongoing research in our laboratory which has been aimed at understanding the mechanism by which dextrans and related polysaccharides are enzymatically synthesized. Previous workers have shown that the glucansucrases produced by *L. mesenteroides* NRRL B-512F and *S. mutans* 6715 synthesize their respective glucans by an insertion mechanism (9,10), in which D-glucopyranosyl units are added to the reducing ends of growing dextran chains (11). The branch linkages of *L. mesenteroides* NRRL B-512F dextran were shown to be formed by what are known as acceptor reactions (12). Acceptor reactions are enzymic reactions in which glucosyl units or glucanosyl chains are transferred to a hydroxyl group of an acceptor sugar (13,14). In the case of *L. mesenteroides* NRRL B-512F dextransucrase, when dextran acts as an acceptor, the glucosyl units or glucanosyl chains are transferred to 3-hydroxyl groups on the glucose residues of the acceptor dextran, to form an α -(1 \rightarrow 3) branch linkage (12). The insertion mechanism, thus, can explain the formation of the α -(1 \rightarrow 6) main chain and the few (5% of the total linkages) α -(1 \rightarrow 3) branch linkages in B-512F dextran (9,12), but there are a large number of other dextrans and related α -D-glucans which contain far more non- α -(1 \rightarrow 6) linkages (15).

The goal of the research described herein has been to better understand the formation of the secondary linkages in three α -D-glucans which, in addition to α -(1 \rightarrow 6) linkages, contain relatively large proportions of α -(1 \rightarrow 3) linkages. These glucans are produced by exocellular glucansucrases from *L. mesenteroides* NRRL strains B-742 and B-1355, and *S. mutans* strain 6715.

Explanation of Dissertation Format

The experimental work described in this dissertation is presented in five sections, each of which is an individual paper which has been published in a scientific journal or has been submitted for publication. In

Sections I, II, III, and V, the work was performed entirely by myself, under the guidance of my major professor, Dr. John F. Robyt. In Section IV, the experiments on the streptococcal glucansucrase were performed by me, while the experiments on the *L. mesenteroides* NRRL B-512F dextransucrase were performed by Thomas Binder, a fellow graduate student also working in Dr. Robyt's research group. All five manuscripts were prepared by myself, with assistance from Dr. Robyt and other colleagues in editing and making appropriate revisions when necessary.

LITERATURE REVIEW

Dextran has been known to man for many years, and it was recognized as a polymer of glucose as early as 1876 (16). Aside from some early reports by microbiologists, however, there was only limited interest in dextrans until 1945, when it was found that solutions of dextran could be used as artificial blood plasma substitutes (17). In the early 1950s, scientists at the Northern Regional Research Laboratory (NRRL) of the U. S. Department of Agriculture began an intensive investigation into the production and properties of dextran (18). These workers characterized the dextrans produced by nearly a hundred different strains of bacteria (15), most of which were *Leuconostoc mesenteroides*, and studied their structures and factors which influenced their production, as well as some of their physical properties (15,18-22). This work showed, among other things, that there existed great structural diversity in the dextrans produced by various strains (15) and that some strains produced more than one type of dextran (20). After the dextran project at the NRRL was terminated in 1958, other workers continued to make progress in determining the nature of the secondary linkages in dextrans by physical (23), chemical (24-27), and enzymic (28-32) means.

Since 1976, a great deal of structural work has been done on dextrans from *L. mesenteroides* and related strains by Seymour and his coworkers (33-46), using chemical and spectroscopic methods. Besides firmly establishing ^{13}C -n.m.r. as a useful tool for determining the structure of dextrans and related glucans, they also provided the first comprehensive survey of various dextran structures since 1954 (15,39). According to Seymour and Knapp (45), *L. mesenteroides* dextrans can be grouped into three classes, based on structural features: Class 1 dextrans, which contain a main chain of α -(1 \rightarrow 6)-linked glucosyl units, with branching through either position 2, 3, or 4; Class 2 dextrans, which contain 3-mono-O-substituted glucosyl residues in nonconsecutive positions, as well as 6-mono- and 3,6-di-O-substituted residues; and Class 3 dextrans, which contain consecutive 3-mono-O-substituted glucosyl residues in addition to 6-mono- and 3,6-di-O-substituted residues. Class 1 contains the majority of

polysaccharides which are usually thought of as dextrans, while there are only three known examples of Class 2 dextrans. These are the fraction S glucans from *L. mesenteroides* strains B-1355, B-1498, and B-1501, which consist of alternating sequences of α -(1 \rightarrow 3)- and α -(1 \rightarrow 6)-linked glucosyl units (40). The author agrees with the statement by Seymour *et al.* (42) that these three glucans "are apparently not true dextrans...", since they consist of alternating α -(1 \rightarrow 3), α -(1 \rightarrow 6) sequences, rather than an α -(1 \rightarrow 6) chain of glucosyl residues. For this reason, the term "alternan" has been used to refer to these glucans of alternating structure (47). Misaki *et al.* (48) have published the results of structural studies done on alternan from *L. mesenteroides* NRRL B-1355, confirming its unique structure. They found that branching may occur at both positions 3 and 6 on main-chain glucosyl residues, and that both types of linkages are fairly evenly distributed throughout the molecule.

L. mesenteroides NRRL B-742 fraction S dextran, a Class 1 dextran, also contains a high proportion of α -(1 \rightarrow 3) linkages, but unlike alternan, they all occur as branch-point linkages (35,36). The structure of B-742 fraction S dextran has been postulated to be a "comb-like" polymer of α -(1 \rightarrow 6)-linked glucosyl residues, each of which bears a single α -D-glucosyl branch residue on position 3 (36). As will be shown in Section III, the degree of branching may be variable, depending on the conditions of synthesis (49).

There is one report of two *L. mesenteroides* glucans which contain consecutive α -(1 \rightarrow 3) linkages (45) and are thus Class 3 dextrans. These are produced by NRRL strains B-523 and B-1149, and tend to be relatively insoluble in water. Little is known about these unusual polysaccharides.

The extracellular glucans produced by *S. mutans* consist of only α -(1 \rightarrow 6) and α -(1 \rightarrow 3) linkages, and are generally of two distinct types: water-soluble glucans, which belong to Class 1; and water-insoluble glucans, which belong to Class 3 (50-62). It has been noted that there is a close relationship between the structures of the soluble α -D-glucans produced by *S. mutans* and the dextrans produced by *L. mesenteroides* (3, 45), but there are a few differences which should be kept in mind. For example, the glucansucrases produced by *S. mutans* are constitutive (6,7),

whereas *L. mesenteroides* glucansucrases are produced only when sucrose is present in the growth medium (3,63). It has also been noted that *L. mesenteroides* dextrans tend to be higher in apparent molecular weight than *S. mutans* soluble glucans (3,6,21,52,57), but there can be much variation in the apparent size, depending on the conditions of synthesis and the particular strain. Another difference is that all known *S. mutans* dextrans contain only α -(1 \rightarrow 3) and α -(1 \rightarrow 6) linkages, while *L. mesenteroides* dextrans may also contain either α -(1 \rightarrow 2) or α -(1 \rightarrow 4) branch linkages (45). All things considered, however, these differences only serve to highlight the many similarities between *L. mesenteroides* and *S. mutans* dextrans. On the other hand, *S. mutans* is distinct in that it also produces mutan, an insoluble α -(1 \rightarrow 3) D-glucan. It is this glucan which many believe to be the chief reason for the ability of *S. mutans* to form insoluble plaque on teeth (7), although the actual reasons are probably more complex than that (6).

Before the mechanism of an enzyme can be studied, it is usually necessary to first isolate and purify that enzyme, and dextransucrase is no exception. Most early studies on dextransucrase were done using crude cell-free culture filtrates (1-3), usually from *L. mesenteroides* NRRL B-512. One disadvantage of this was that such preparations invariably contained large amounts of dextran, since the organism requires sucrose for dextransucrase production (63). In the past ten years, a number of authors have described purification schemes for dextransucrases from *L. mesenteroides* strains NRRL B-1299 (64-67), IAM 1046 (68,69), and NRRL B-512F (70,71). Kobayashi and Matsuda (64-67) discovered several isozymic forms of B-1299 dextransucrase, and found that there existed complex interactions among the different forms and their aggregates. Bewildered by these complex interactions, other workers have attempted to first understand simpler, single-enzyme systems. Dextransucrase from *L. mesenteroides* IAM 1046 has been purified by ion-exchange and gel-filtration chromatography (68) and it was found that the purified enzyme required calcium ions for full branching activity (69). Others have described methods for the purification of *L. mesenteroides* B-512F dextransucrase

(70,71), and Robyt and Walseth (70) showed that this enzyme requires calcium ions for full catalytic activity.

The purification of streptococcal glucansucrases is simplified by the fact that, since these enzymes are constitutive (6,7), no sucrose is required in the growth medium, and thus no glucans are present in the culture filtrates. Numerous methods have been used to separate and purify the exocellular glucansucrases from *S. mutans*. These have included various combinations of hydroxyapatite chromatography (62,72-80), ion-exchange chromatography (81-84), affinity chromatography (85-90), gel-filtration chromatography (91-93), chromatofocusing (94), gel-electrophoresis (95), fractional ethanol precipitation (96-98), and precipitation with poly(ethylene glycol) (99). Since different workers have used different strains grown under different conditions, it is difficult to compare the results obtained by one group with those obtained by another. Montville *et al.*, (6) have reviewed many of these methods. The purification procedures are also complicated by the presence of many different forms of the enzyme in a single preparation, which often tend to interact with one another (96,97).

The purest preparation of the soluble-glucan-producing glucansucrase (GTF-S) from *S. mutans* 6715 reported to date seems to be that described by Shimamura *et al.* (84). Their procedure involved the following steps, in order: ethanol precipitation, Bio-Gel A-15m chromatography, ion-exchange chromatography on aminoethyl Bio-Gel P-60, Bio-Gel A-5m chromatography, and affinity chromatography on immobilized concanavalin-A. Their total purification factor was 10,000-fold, to give a 3.8% yield of enzyme with a specific activity of 36 IU/mL. The enzyme was shown to be electrophoretically homogeneous, with a molecular weight of $149,000 \pm 5000$. *S. mutans* 6715 also produces a glucansucrase which synthesizes a water-insoluble glucan (mutan) consisting almost exclusively of α -(1 \rightarrow 3) linkages. This enzyme (GTF-I) has been purified to electrophoretic homogeneity, to give a single protein with a molecular weight of 180,000 (79). The purification procedure consisted of molecular-exclusion chromatography, followed by chromatography on hydroxyapatite.

Early work on the mechanism of action of dextransucrase was carried out using the enzyme produced by *L. mesenteroides* NRRL B-512F. In 1963, Eisenberg and Hestrin (100) demonstrated that dextran synthesis occurred by transfer of glucosyl units, rather than by glucosido-transfer. Later, Genghof and Hehre (101) found that this enzyme, as well as a streptococcal glucansucrase, could synthesize glucosidic linkages *de novo* from the substrate analogue α -D-glucopyranosyl fluoride.

It was long assumed that dextran synthesis occurred by the transfer of glucosyl units from sucrose to the C-6 hydroxyl group at the nonreducing ends of growing dextran chains (102). This view was challenged by Ebert and Schenk (103,104), who proposed an alternate mechanism in which α -D-glucosyl units were inserted between the enzyme and the reducing end of the growing dextran chain. Robyt *et al.* (9,10) have provided experimental evidence for this type of mechanism using pulse-chase procedures. In the mechanistic model proposed by Robyt *et al.*, there exist two nucleophilic groups at the active site of the enzyme. These nucleophiles attack C-1 of the glucosyl moieties of sucrose molecules, forming a covalent β -D-glucopyranosyl-enzyme complex (11). The C-6 oxygen of one glucosyl unit then acts as a nucleophile, displacing the enzyme off of the other glucosyl unit, forming a β -isomaltosyl-enzyme intermediate. The second nucleophile of the enzyme is then free to form another β -D-glucosyl intermediate from sucrose, and the C-6 oxygen of this glucosyl unit is then able to attack C-1 of the β -linked glucosyl moiety of the isomaltosyl unit, displacing it from the nucleophile on the enzyme, forming a new α -(1 \rightarrow 6) linkage and giving a β -isomaltotriosyl-enzyme complex. The process can then continue, to yield a covalent dextranosyl-enzyme complex. This elongation process is terminated when the dextranosyl chain is transferred to an acceptor, such as fructose, dextran, or water. Figure 1 shows this two-site insertion mechanism in schematic form.

When glucosyl units or dextranosyl chains are transferred to the hydroxyl group of an acceptor molecule, this is known as an acceptor reaction (14). If the transfer is made to a 3-hydroxyl group on a dextran chain, this leads to the formation of an α -(1 \rightarrow 3) branch linkage (12,105)

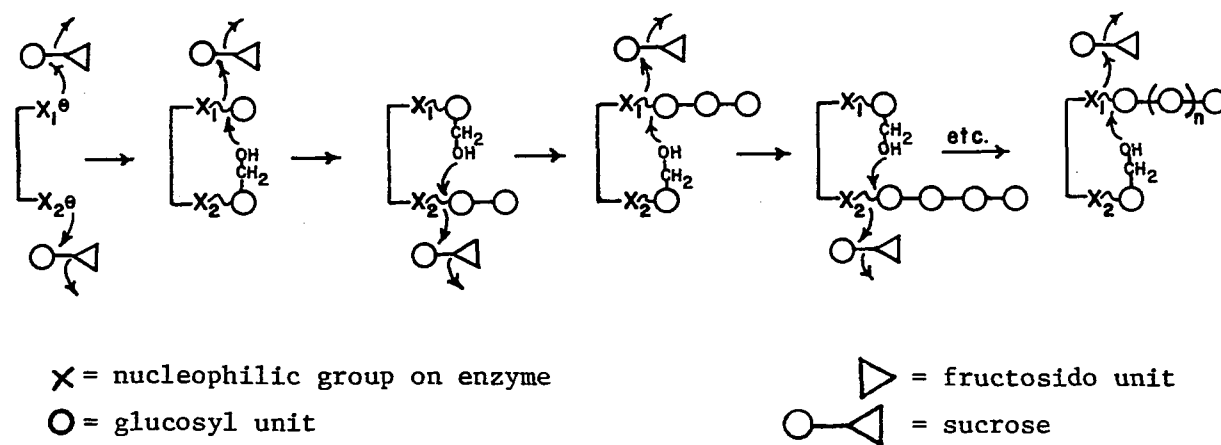


Figure 1. Two-site reducing-end insertion mechanism for dextran synthesis by dextransucrase (9)

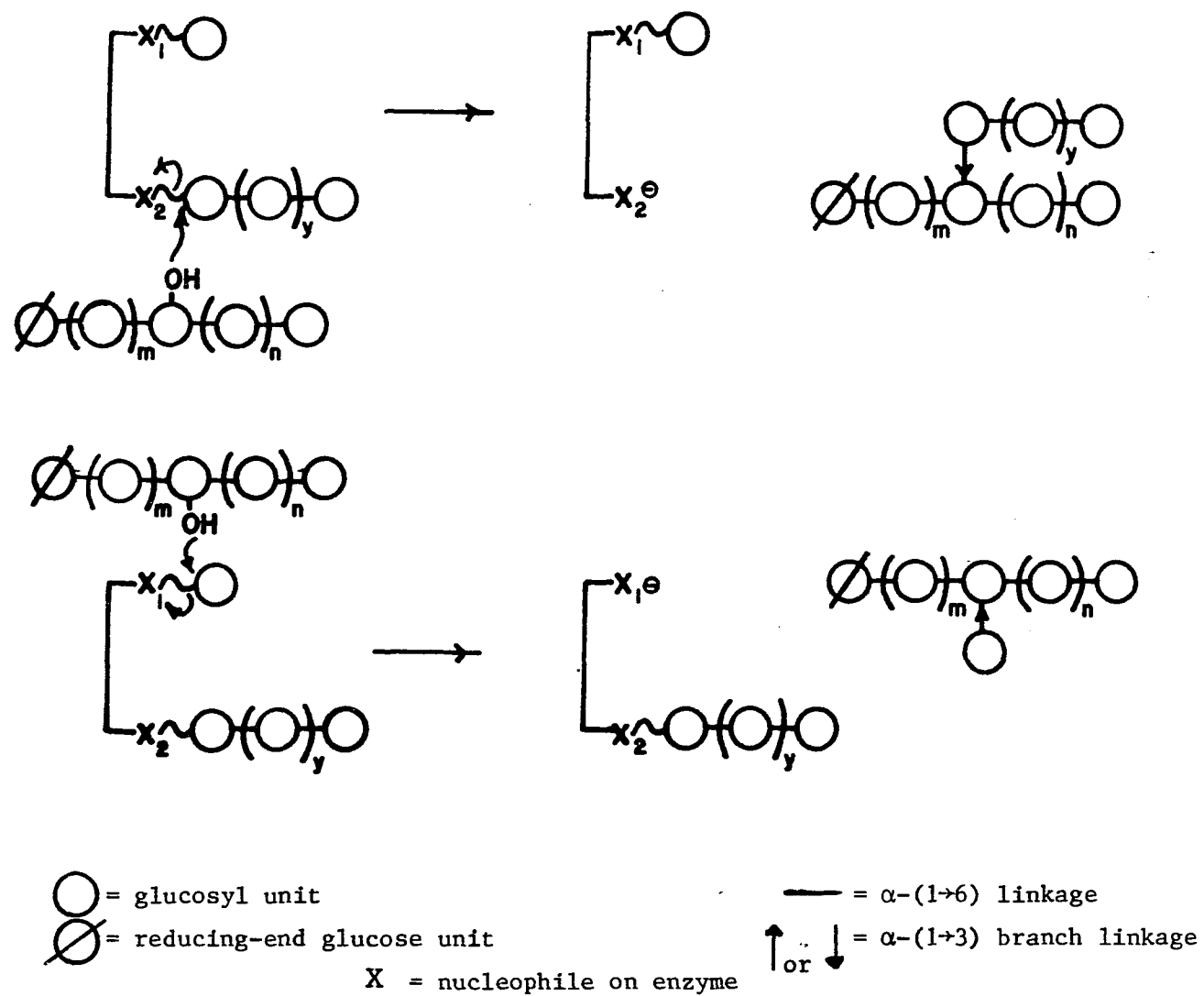


Figure 2. Mechanism of formation of branch linkages by *Leuconostoc mesenteroides* NRRL B-512F dextran-sucrase via acceptor reactions with dextran (12)

(Fig. 2). Robyt and Taniguchi (12) showed that this is how branches are formed in *L. mesenteroides* B-512F dextran, but until recently (49,106), it was not known if the same mechanism for branch formation occurred for the more highly branched glucans. The insoluble-glucan-producing glucan-sucrase from *S. mutans* can also transfer glucosyl units or glucanosyl chains to acceptor dextran, to give a graft copolymer of α -(1 \rightarrow 3) glucan linked to C-3 of the acceptor dextran (10,59,79,83,107-109). Many investigators have observed this transfer of glucosyl units to acceptor dextran (59,73,79,83,84,98,102,107-109), and it is often attributed to the dextran's acting as a "primer". Robyt and Corrigan (107) showed, however, that the dextran was not acting as a necessary primer, but simply as an acceptor. (For a discussion regarding the difference between primers and acceptors, see Section V.) This was done by chemically modifying the non-reducing ends of the dextran so as to make them unavailable for "priming" reactions. They found that the glucansucrase from *S. mutans* OMZ 176 was still activated by this modified dextran, indicating that the activation could not be due to glucosyl transfer to non-reducing ends. It was concluded that the increased activity was due to increased transfer of glucosyl units or glucanosyl chains to secondary hydroxyl groups along the dextran chain, to form branch points or graft copolymers (107).

Dextran is not the only carbohydrate which can act as an acceptor for glucansucrases. Many low-molecular-weight sugars can also act as acceptors, and the structures of the products have been investigated in great detail (3,11,13,14,110-125). Robyt and Eklund (11) have reviewed the acceptor products formed by *L. mesenteroides* B-512F dextransucrase, and proposed a possible mechanism for their formation, which is consistent with the two-site insertion mechanism (11,14). Other than branching, acceptor reactions may also serve to terminate glucan synthesis. It has been shown that dextran chains formed from sucrose are often terminated at the reducing end by a fructose residue (14,126). The fact that this fructose is reducing in nature indicates that it is not in a sucrose-type linkage. This suggests that fructose, released from sucrose during dextran synthesis, can act as an acceptor to terminate chain elongation,

resulting in a dextran chain with a leucrose (112) moiety at the reducing end. Although it is known that different glucansucrases have different specificities with regard to acceptor reactions with low-molecular-weight acceptors (3,11,13,14,122,124,125,127), the relationship of this specificity to the formation of specific dextran structures is not yet well-understood.

SECTION I

ISOLATION AND PARTIAL CHARACTERIZATION OF AN EXTRACELLULAR
GLUCANSUCRASE FROM *Leuconostoc mesenteroides* NRRL B-1355
THAT SYNTHESIZES AN ALTERNATING (1→6), (1→3)- α -D-GLUCAN

Gregory L. Côté and John F. Robyt
Department of Biochemistry and Biophysics
Iowa State University, Ames, Iowa 50011

ABSTRACT

Leuconostoc mesenteroides NRRL B-1355 grows on sucrose to produce two extracellular α -D-glucans. Although both are termed dextrans, they are chemically and physically distinct, and can be separated by fractional ethanol precipitation into fractions designated L and S. Fraction L is similar to B-512F dextran, having 95% α -(1 \rightarrow 6) linkages and 5% α -(1 \rightarrow 3) branch linkages, but Fraction S has an alternating sequence of α -(1 \rightarrow 6) and α -(1 \rightarrow 3) linkages. Because of its structural differences from dextran, its different physical characteristics, and its resistance to hydrolysis by endodextranase, we have named glucan S, alternan, and the enzyme that synthesizes it from sucrose, alternansucrase. Alternansucrase has been isolated by two different methods. The first involves removal of the fraction L glucan from the culture fluid via hydrolysis by an endodextranase, followed by chromatography on Bio-Gel A-5m. The void-volume fraction synthesizes only alternan, whereas the slower-migrating, second fraction synthesizes mainly dextran, together with some alternan. The second method utilized hydrophobic chromatography on *O*-(phenoxyacetyl)cellulose; a portion of the alternansucrase did not bind, whereas the bound portion, removed by eluting with detergent, contained both alternansucrase and dextransucrase. The glucans were identified by physical appearance, the concentration of ethanol required for precipitation, periodate-oxidation behavior, and susceptibility to hydrolysis by endodextranase. Also studied was the inhibition of the enzymes by 3-deoxy-3-fluoro- α -D-glucopyranosyl fluoride, tris(hydroxymethyl)aminomethane, 2-aminoethanol, and octyl β -D-glucopyranoside.

INTRODUCTION

In 1954, Jeanes *et al.* (1) reported the production, separation, and characterization of a number of extracellular α -D-glucans synthesized by various bacteria grown in the presence of sucrose. These included two glucans produced from sucrose by *Leuconostoc mesenteroides* NRRL B-1355. They could be separated by fractional ethanol precipitation into an L-fraction, which was precipitated at 37-38% ethanol, and an S-fraction, which precipitated at 39-40% ethanol (2). These two glucans, commonly referred to as dextrans, show markedly different chemical and physical characteristics (1,2).

Much work has been done on the structures of these two glucans (1-14), especially fraction S (14). Periodate oxidation (1,2), acetolysis (3,4), and Smith degradation (3) have all shown that the S fraction contains a high proportion of α -(1 \rightarrow 3)-linked residues. Partial hydrolyzates of the S glucan have yielded two trisaccharides, 6²- α -D-glucosylnigerose (3), and 3²- α -D-glucosylisomaltose (5). This work and the recent studies of Seymour *et al.*, using methylation (6,7) and ¹³C-n.m.r. (8-13), all suggest a structure of alternating sequences of α -(1 \rightarrow 6) and α -(1 \rightarrow 3)-linked D-glucose residues. Thus, B-1355 fraction S glucan seems not to be a true dextran, as the α -(1 \rightarrow 3) linkages are part of the linear chains and there are not any consecutive α -(1 \rightarrow 6) linkages as are present in dextran. Further, the α -(1 \rightarrow 3) linkages in B-512F dextran are exclusively found as branch points. Others have also commented on the unique nature of the B-1355 S glucan (11,13,14). Because of its unique structure and properties, we propose the name "alternan" for this glucan.

The B-1355 fraction L glucan, on the other hand, seems to be a typical dextran, having ~95% of α -(1 \rightarrow 6)-linked D-glucose residues, with the remainder being α -(1 \rightarrow 3)-linked branch points (8).

Until now, it was not known whether a single enzyme synthesizes the two extracellular B-1355 glucans, or if two distinct enzymes are responsible. One difficulty in studying the enzyme(s) is that the culture medium must contain sucrose in order to induce production of the enzyme(s). This results in large amounts of polysaccharide being produced in conjunction

with the enzyme(s) responsible for their syntheses. These apparently high-molecular-weight complexes of enzyme and polysaccharide make it difficult to separate the enzyme(s) by conventional means.

The nature of these complexes may, however, be used to separate the enzyme(s) if one of the polysaccharides can be specifically degraded while leaving the other intact. We have separated the two enzymes by hydrolyzing the L-fraction dextran with an endodextranase, leaving the alternan-alternansucrase intact as a high-molecular-weight complex. The enzymes were then separated, by the difference in molecular size, on a Bio-Gel A-5m column.

Another, entirely different method of separating alternansucrase from dextransucrase involves hydrophobic chromatography with *O*-(phenoxyacetyl)-cellulose. The mixture of enzymes is adsorbed onto a column, and a portion that contains both glucans and only alternansucrase is not adsorbed. When the column is eluted with detergent, dextransucrase and alternansucrase are eluted relatively free of glucans.

We have studied the activity of the enzymes in the presence of various compounds. Of these, the most interesting is 3-deoxy-3-fluoro- α -D-glucopyranosyl fluoride, which gives 100% inhibition of alternansucrase at concentrations as low as 5 mM. Also acting as inhibitors, although to a much lesser degree, are tris(hydroxymethyl)aminomethane and 2-aminoethanol. The nonionic detergents, Triton X-100 and Tween-80, had no noticeable effect on these enzymes, but octyl β -D-glucopyranoside, also a nonionic detergent, was inhibitory to a significant degree.

MATERIALS AND METHODS

Growth Conditions

Leuconostoc mesenteroides B-1355 was grown in the medium described by Hehre (15). Cultures were incubated on a rotary shaker for 12 h at 25°. Successive transfers of 10% were made into larger volumes until 1 L was used to inoculate 10 L of medium in a fermentor at 25° with 150 r.p.m. agitation and aeration of 0.5 L per min per 10 L of culture. Cells were removed by continuous centrifugation in a Sharples centrifuge.

Preparation of Crude Enzyme Mixture

All preparation and purification steps were conducted at 4° unless otherwise stated. After centrifugation, the clear amber supernatant fluid was dialyzed and concentrated ten fold, as previously described (16) for *Leuconostoc mesenteroides* B-512F. This preparation, referred to as CSDC (culture supernatant, dialyzed, and concentrated), was stable when frozen, but slowly lost activity when stored for several weeks at 4°. A significant amount of material precipitated on prolonged standing at 4°, possible because of aggregation of the enzymes and polysaccharide.

Enzyme Assays

Glucansucrase activity was measured by a radiochemical method (16, 17), in which a sample of the enzyme solution was incubated at room temperature at pH 5.1-5.4 with 150 mM [U-¹⁴C]sucrose (New England Nuclear). Timed aliquots were absorbed onto 1.5-cm squares of Whatman 3MM filter paper. The paper squares were immediately dropped into methanol, washed with methanol five times, dried, and counted by liquid scintillation to determine the amount of ¹⁴C incorporated into polysaccharide. An enzyme unit is defined as the amount of enzyme that incorporates 1 μmol of monosaccharide into polysaccharide in one min. Separate assays using [¹⁴C]-fructose-labeled sucrose showed no fructansucrase present in the CSDC.

Carbohydrate and Protein Determination

Total carbohydrate was measured either manually, by the phenol-sulfuric acid method (18), or by the orcinol-sulfuric acid method with a Technicon AutoAnalyzer (19,20). Protein determinations were attempted by the Coomassie Blue method of Bradford (21), but the results are not reliable, because of the relatively large proportions of polysaccharide present in most of the preparations. As the high concentrations of polysaccharide produce significant interference in any spectrophotometric determination of protein, no values for protein concentration are given here.

Poly(ethylene glycol) Precipitations

Poly(ethylene glycol) (PEG), of various molecular weights was used to precipitate proteins and protein-carbohydrate complexes (22-24) by adding a volume of aqueous PEG of twice the desired final concentration to an equal volume of protein-containing solution. After mixing thoroughly, the mixture was kept for 15-20 min at 4-8°. The mixture was then centrifuged, the clear supernatant fluid poured off, and the precipitate redissolved in the original volume of cold buffer.

pH-Activity Assays

The activity of alternansucrase was measured at various pH values by precipitating all of the enzyme out of the A-5mV₀ concentrate (Fig. 2b) by using a 6% concentration of PEG-6000, and then redissolving the precipitate in the original volume of the appropriate buffer. The following buffers were used, with the final concentrations in the assay all at 20 mM: phosphate (pH 2.3-3.0 and pH 5.6-8.9), and pyridine acetate (pH 3.9-5.3 and pH 6.1).

Dextranase Digests

CSDC was treated with *endo*-dextranase as previously described (16), with one of the following preparations: (1) Purified Dextranase S (Sigma Chem. Co., St. Louis, MO); (2) Crude Dextranase S (Swiss Ferment, Basle,

Switzerland); (3) Purified Dextranase S (Worthington Biochemical Corp., Freehold, NJ); and (4) An unidentified *endo*-dextranase, purchased from ICN Nutritional Biochemicals, Cleveland, OH, which gave markedly different products as compared with Dextranase S (see discussion).

Chromatography

Thin-layer chromatography was performed on 0.25-mm silica-gel plates (Merck). The oligosaccharides of the isomaltose series were separated by two ascents in 3:1:1 (v/v) ethanol - nitroethane - water. Compounds were detected by spraying the plates with sulfuric acid and charring for 10 min at 120°. Standard isomalto-oligosaccharides were prepared by partial acid hydrolysis of B-512F dextran with 0.07 M trifluoroacetic acid for 30 min at 130° (16).

Molecular-exclusion gel chromatography employed a 2.5 cm x 75 cm column of Bio-Gel A-5m (Bio-Rad) at 4°. The eluant was 20 mM pyridine-acetate buffer at pH 5.1-5.4, containing 0.01% sodium azide and 1 mM calcium chloride. Fractions of 3 mL were collected, and the void volume was ~60 mL.

O-(Phenoxyacetyl)cellulose (PA-cellulose) was prepared by the method described by Butler (25), using phenoxyacetyl chloride to derivatize either fibrous or microcrystalline cellulose. In order to obtain maximum flow-rate and surface area, columns contained 1:1 (w/w) microcrystalline and fibrous PA-cellulose. Columns were eluted with 20 mM pH 5.1-5.4 pyridine-acetate buffer until nonbinding polysaccharide and protein was eluted, at which point the eluent was changed to detergent in buffer in order to remove bound protein. Tween 80 (0.5%) and Triton X-100 were used initially, but were found difficult to remove by dialysis. Zwittergent 3-10, (*N*-decyl-*N,N*-dimethyl-3-ammonio-1-propanesulfonate) (1%, Calbiochem) was subsequently used, as it is more readily removed by dialysis.

Column fractions were assayed by a modification of the usual technique (16), in which only a single time-point was checked for synthesis of [¹⁴C]glucan. Pooled fractions were concentrated as described previously (16), with an Amicon ultrafiltration cell and a PM 10 membrane.

Preparation of Polysaccharides

Relatively pure polysaccharides were prepared by incubating a buffered (pH 5.1-5.4) solution of 0.3 M sucrose with a solution of one or both of the enzymes studied. After 1-5 days at room temperature, when the mixture had become viscous and opalescent, it was diluted to give a polysaccharide concentration of ~2%. Absolute ethanol was then added at room temperature, slowly and with continuous stirring, until the desired concentration of ethanol had been reached (1,2). Typically, B-1355 fraction L glucan precipitated at 37-38% ethanol, and fraction S glucan at 39-40% ethanol.

To purify the glucans further, they were redissolved in water, dialyzed overnight against distilled water, and precipitated as before. The precipitates were washed with ethanol, dried *in vacuo* for 24 h at 40° and stored *in vacuo* over phosphorus pentaoxide at room temperature.

Periodate Oxidations

Polysaccharide structures were verified by periodate oxidation and periodate consumption was monitored spectrophotometrically (26). Mixtures contained the following final concentrations: polysaccharide, 1 mM as glucose residues, 2.5 mM sodium metaperiodate, and 60 mM sodium acetate buffer, pH 5.1.

Periodate absorbance was measured at 290 nm against a blank of water. A reagent blank containing only sodium metaperiodate and buffer showed no decrease in absorbance at 290 nm after being kept in the dark for 2 weeks at 20°. A value of $0.22 \text{ mM}^{-1} \text{ cm}^{-1}$ was used for the absorptivity coefficient of periodate (26).

Preparation of 3-Deoxy-3-fluoro- α -D-glucopyranosyl Fluoride

Oxidation of 1,2:5,6-di-O-isopropylidene- α -D-glucofuranose (27) with chromium trioxide - pyridine (28) gave 1,2:5,6-di-O-isopropylidene- α -D-ribohexofuranos-3-ulose, which was reduced with sodium borohydride to 1,2:5,6-di-O-isopropylidene- α -D-allofuranose (29). The latter was mesylated with methanesulfonyl chloride in pyridine to give 1,2:5,6-di-O-isopropylidene-3-O-(methanesulfonyl)- α -D-allofuranose. Heating this compound to 200° in

acetamide with potassium fluoride gave as a major product 3-deoxy-3-fluoro-1,2:5,6-di-*O*-isopropylidene- α -D-glucofuranose (30), isolated by column chromatography on silica gel with 9:1 (v/v) benzene-diethyl ether (30). The *O*-isopropylidene groups were removed by using Amberlite IR-120 (H^+) ion-exchange resin (31,32). The free deoxyfluoro sugar was benzoylated (33,34), fluorinated by using liquid HF (34,35), and deesterified with sodium methoxide in dry methanol, to give 3-deoxy-3-fluoro- α -D-glucopyranosyl fluoride, with the free deoxyfluoro sugar as a major contaminant (31-35). The identities of all intermediates and products were verified by chromatographic mobility, melting points, proton n.m.r. and ^{19}F -n.m.r. spectroscopy.

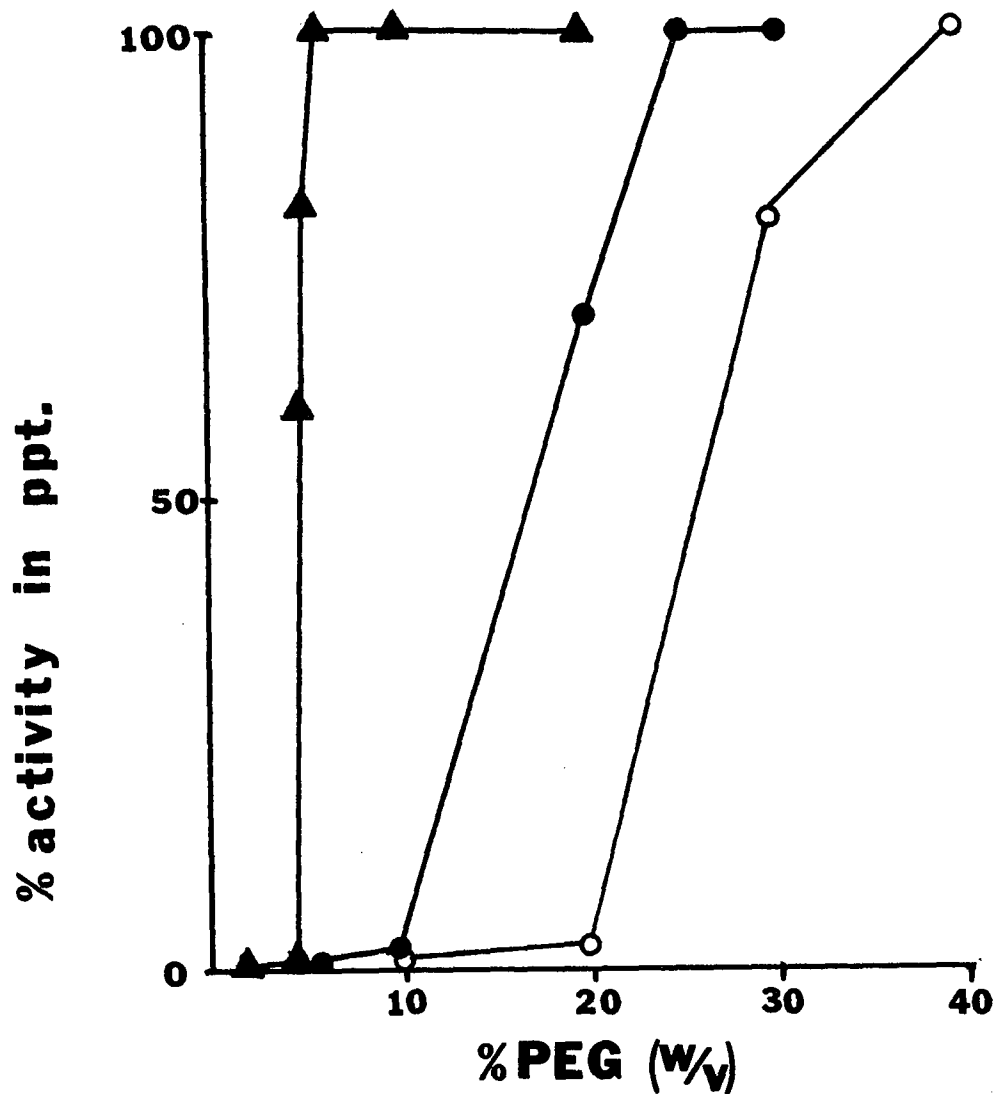
RESULTS

The culture supernatant fluid showed an activity of 0.2 unit of enzyme per mL. After dialysis and concentration, the CSDC contained 0.84 unit/mL. There was a high amount of polysaccharide associated with these preparations, and the CSDC was found to contain 20 mg/mL of total carbohydrate, as determined by the phenol-sulfuric acid method.

Initial attempts at separating the enzymes present in the CSDC consisted of fractional precipitation of the glucan-enzyme complexes with ethanol. This method, however, gave very low yields of activity (< 15%) and did not separate the two activities from each other.

Further attempts consisted of fractional precipitation of the proteins in the CSDC by using poly(ethylene glycol) of various molecular weights (22,23). This technique has been used in the past to isolate two different glucansucrases from other proteins in *Streptococcus mutans* (24), although it did not separate them from each other. In the *L. mesenteroides* B-1355 system, no separation of the two activities present could be accomplished by this procedure, although it was possible to precipitate out and subsequently recover all of the activity of the CSDC by precipitation with PEG. Fig. 1 shows the amount of enzyme activity recovered from the precipitate at various concentrations of PEG, using PEG of molecular weights 400, 600, and 6000. Although the two activities could not be separated from each other in this manner, it was observed that some extraneous carbohydrate, and small amounts of protein as well, could be removed by this procedure.

Alternansucrase was isolated from the mixture by a procedure which makes use of the fact that both enzymes occur as large, high-molecular-weight protein-polysaccharide complexes. It was found that dextranase S and ICN dextranase both were capable of hydrolyzing B-1355 fraction L dextran into small oligosaccharides and D-glucose, but they gave no significant amount of similar products from alternan under the same conditions, although it has been reported (14) that dextranase gave a trace of isomaltose from alternan. This difference apparently arises from the fact that alternan has alternating sequences of α -(1 \rightarrow 6) and α -(1 \rightarrow 3)-linked



▲ = PEG-6000

● = PEG-600

○ = PEG-400

Figure 1. Precipitation of glucansucrase activity from the dialyzed and concentrated culture supernatant by three poly(ethylene glycol)s. The vertical axis is the percent of activity precipitated

glucosyl residues and that the α -(1 \rightarrow 3) linkages of alternan are part of the linear chains, whereas dextrans, such as fraction L and B-512F glucans, have consecutive α -(1 \rightarrow 6)-linked glucose residues as part of their main chains and their α -(1 \rightarrow 3) linkages are exclusively branch linkages. It was also found that an α -(1 \rightarrow 3) glucanase (mutanase), obtained from Novo Industries, hydrolyzed neither dextran nor alternan.

With this selectivity of dextranase action in mind, we incubated a sample of the CSDC with ICN dextranase until no fraction L dextran remained. Products of low molecular weight were removed by conducting the digestion in a dialysis bag (16), dialyzing the mixture against 20 mM pH 5.1-5.4 pyridine acetate buffer. The digest was then applied to a column of Bio-Gel A-5m and eluted with pH 5.1-5.4 buffer. The elution profiles for CSDC before and after action of dextranase (Figs. 2A and 2B) show two activity peaks. The void volume in both cases contained tangible amounts of polysaccharide. In order to determine the nature of the two activities represented by the two peaks in Fig. 2B, the appropriate fractions were pooled and concentrated by ultrafiltration. These two pooled samples, referred to as A-5mV₀C and A-5mIIC, were each incubated with 0.3 M sucrose in pH 5.1-5.4 pyridine acetate buffer. When these mixtures had become opalescent and somewhat viscous, the polysaccharides were precipitated with ethanol. Most of the glucan produced from A-5mIIC precipitated at an ethanol concentration of 38%, although a small amount did not precipitate out until the concentration reached 40-41%. The polysaccharide produced by the A-5mV₀C precipitated at an ethanol concentration of 40%. These observations, together with the physical appearances of the two polysaccharides (see Fig. 3), suggested that the void volume was producing only alternan, whereas fraction II was synthesizing mainly fraction L dextran, as well as a small amount of alternan. To verify this, both of the glucans, together with samples of alternan and dextran, were incubated with dextranase S and ICN dextranase. After 48 h at 20°, t.l.c. (see Fig. 4) showed that the A-5mV₀ glucan was not significantly hydrolyzed by dextranase, nor was the sample of alternan. The fraction L dextran was completely hydrolyzed by the dextranases, leaving only a very small trace of material at the origin. However, the A-5mII glucan, while giving a large

Figure 2. Bio-Gel A-5m chromatography of B-1355 CSDC before and after dextranase treatment.

A: Elution of dialyzed and concentrated culture supernatant on Bio-Gel A-5m. The vertical axis is the c.p.m. of [^{14}C]glucose incorporated into methanol-insoluble glucan, which is directly proportional to the glucansucrase activity. Each fraction was 3.8 mL.

B: Elution of dextranase-treated, dialyzed and concentrated, culture supernatant on Bio-Gel A-5m. Same conditions as in A

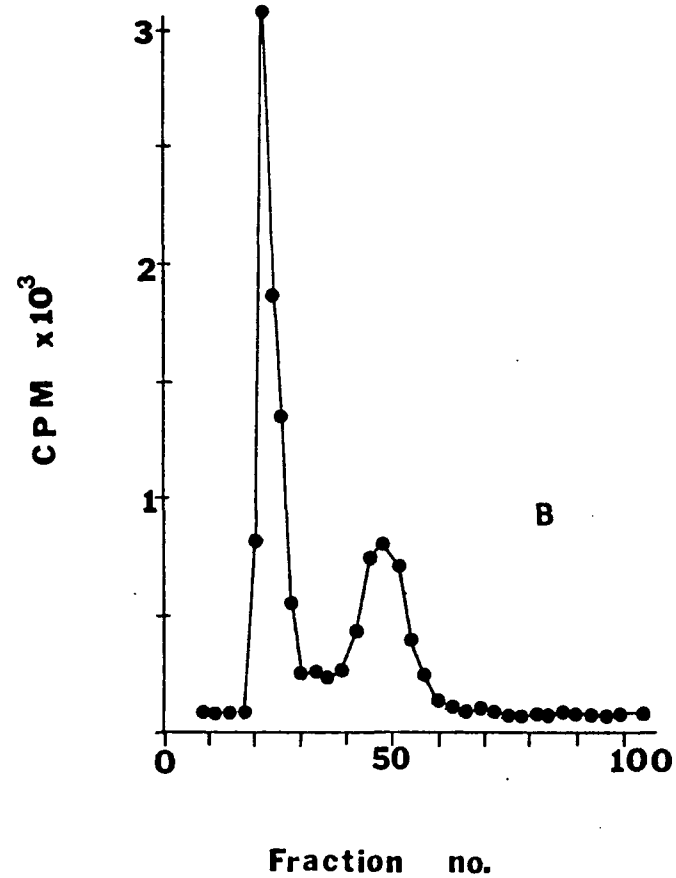
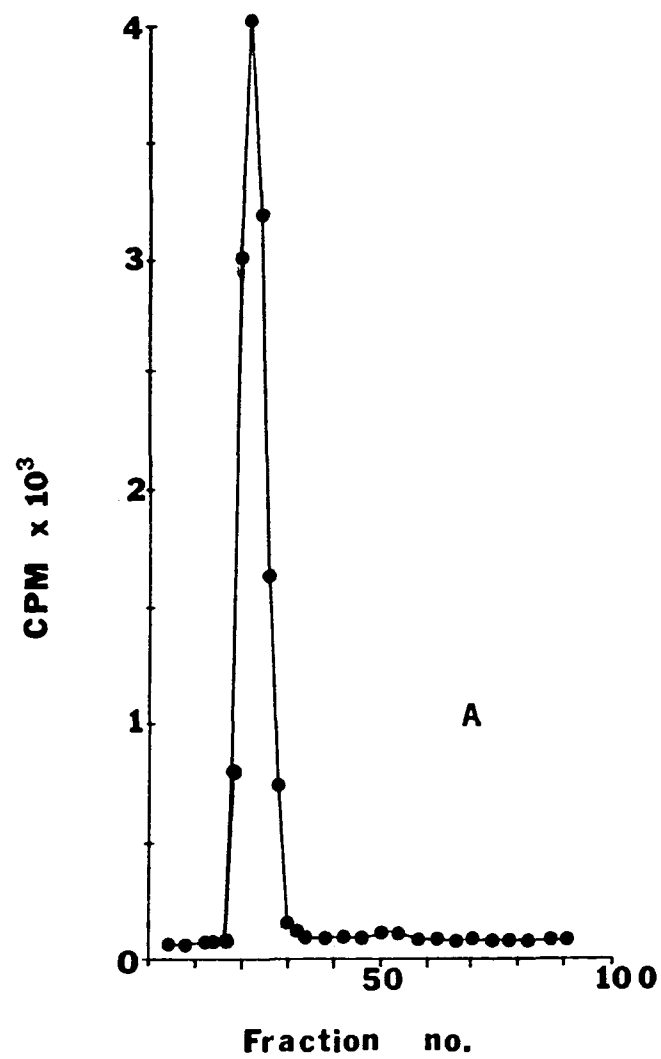
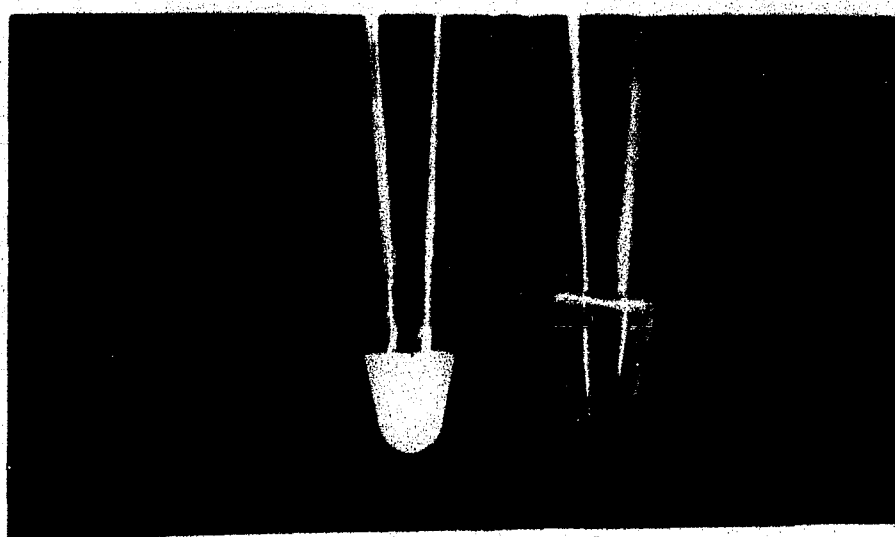


Figure 3. Differences in physical appearance of alternan (fraction S glucan) and dextran (fraction L glucan) after precipitation with 40 and 38% ethanol, respectively

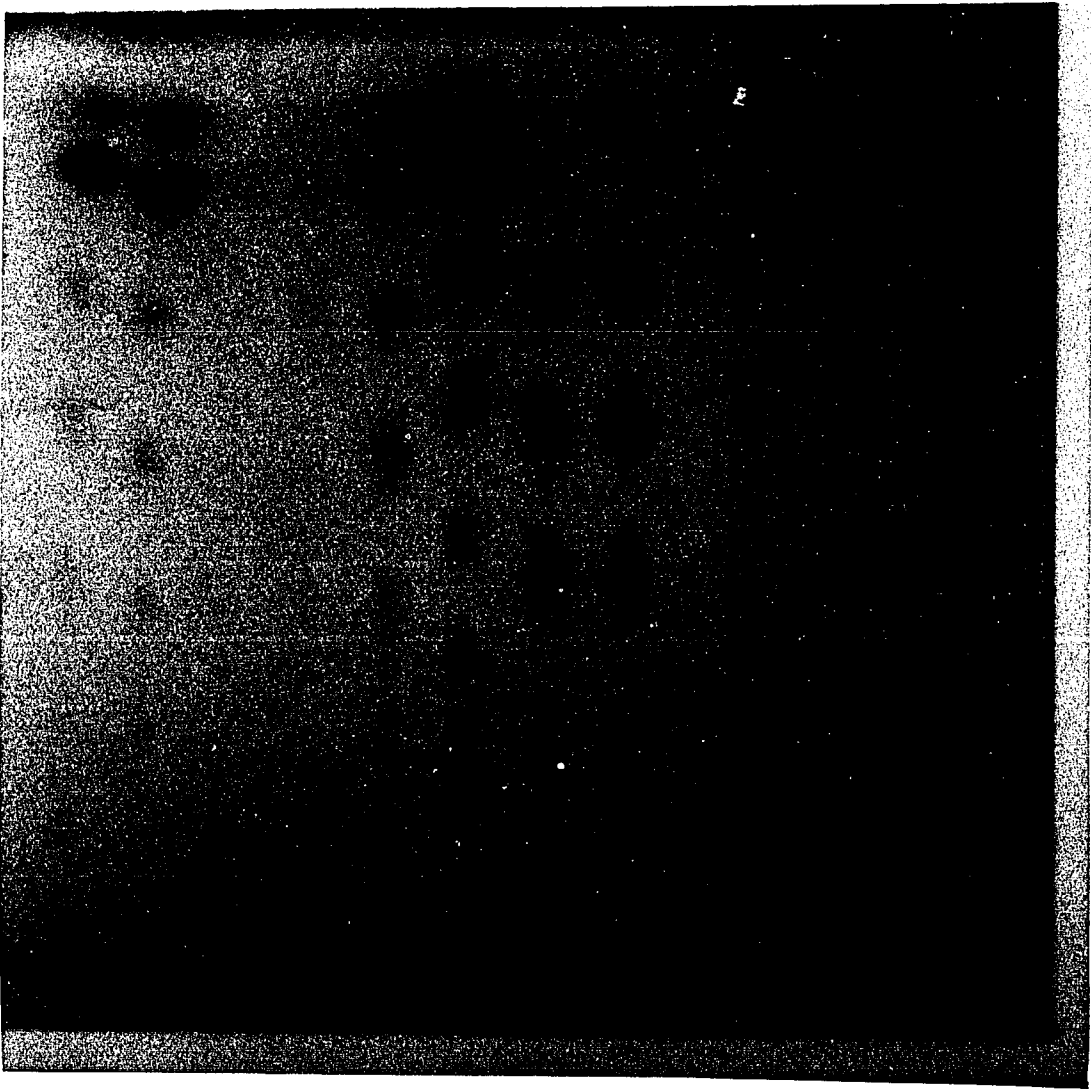


S

L

B-1355 Glucans

Figure 4. Thin-layer chromatography of endo-dextranase products from various glucans. A, B, C, D, and E denote action of Sigma dextranase on B-512F dextran, B-1355 L dextran, B-1355 S glucan, A-5mV_O glucan, and A-5m fr. II glucan; F, isomalto-oligosaccharide standards; G, H, I, J, and K denote action of ICN dextranase on B-512F dextran, B-1355 L dextran, B-1355 S glucan, A-5mV_O glucan, and A-5m fr. II glucan



amount of hydrolysis products identical to those from B-1355 L-dextran, also showed a significant amount of material remaining at the origin, indicating that some alternan is produced by A-5mIIC, in addition to the dextran that is produced. The B-1355 fraction L dextran appears to give products on dextranase hydrolysis identical to those produced from B-512F dextran, indicating the close structural similarity between the two.

Periodate oxidations were also performed on the glucans produced by the A-5m fractions. The results, shown in Table I, indicate that alternan and the glucan produced by the A-5mV₀ both consumed the same amount of periodate per glucose residue. Furthermore, this figure is exactly as expected for a glucan of the alternating structure described by others (3-14). Likewise, the periodate consumption by fraction L dextran and A-5mII glucan are also both, within experimental error, the same as expected from the usual dextrans (10). This result indicates that, although a small amount of alternan is produced by fraction II, most of the activity in A-5mII is dextransucrase.

A totally different method of separating alternansucrase from dextranase involves hydrophobic chromatography on *O*-(phenoxyacetyl)cellulose (PA-cellulose) as described in the Methods section. When a sample of CSDC was applied to a column of PA-cellulose and washed through with buffer, the first several fractions contained considerable polysaccharide and enzyme activity. If the column was then washed with buffered detergent, such as Tween 80 or Triton X-100, a second peak of activity was eluted (see Fig. 5). The first peak produced a glucan that precipitated at 40% ethanol. This glucan was not hydrolyzed by dextranase, as shown by two different methods. The first method was t.l.c. of the dextranase digest, which showed no oligosaccharide products. The second, slightly more sensitive technique, consisted of using [¹⁴C]sucrose to obtain a ¹⁴C-labeled sample of this first glucan. Following treatment of this sample with dextranase, timed aliquots of the digest were spotted on paper, washed with methanol five times, dried, and counted by liquid scintillation as described in the assay method for glucansucrase. As Fig. 6 shows, there was no decrease in the amount of ¹⁴C-labeled, methanol-insoluble glucan on treatment by dextranase of both the A-5mV₀ glucan and the PA-cellulose un-

Table I. Periodate-oxidation data

Sample	Moles IO_4^- consumed per mole glucose residues (theoretical from Seymour <i>et al.</i> (8))	Moles IO_4^- consumed per mole glucose residues (experimental) \pm S.E. \bar{x} ^a
Alternan (glucan S) ^b	1.1	1.1 \pm 0.1
A-5mV ₀ glucan ^c		1.0 \pm 0.1
Dextran (glucan L) ^b	1.9	1.7 \pm 0.2
A-5m fraction II glucan ^c		2.1 \pm 0.2

^aS.E. \bar{x} = S.D. $_x$ / \sqrt{N} , where S.D. $_x$ \equiv standard deviation for x for N runs.

^bGlucans S and L from B-1355, isolated and purified by the methods of Wilham *et al.* (2).

^cGlucans synthesized by the void volume (V₀) fraction and a slower-migrating fraction (Fr. II) from a column separation of dextranase-treated CSDC on Bio-Gel A-5m.

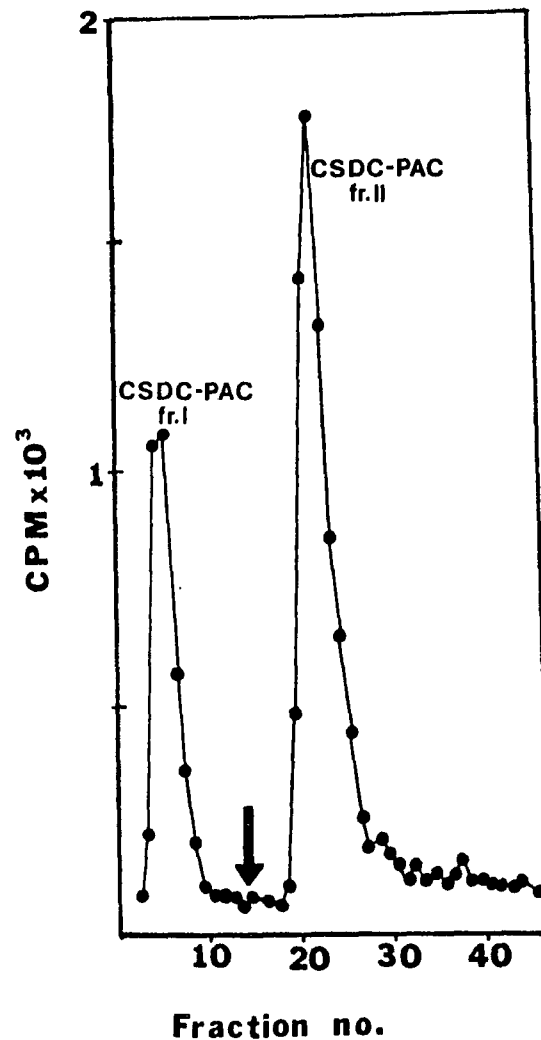
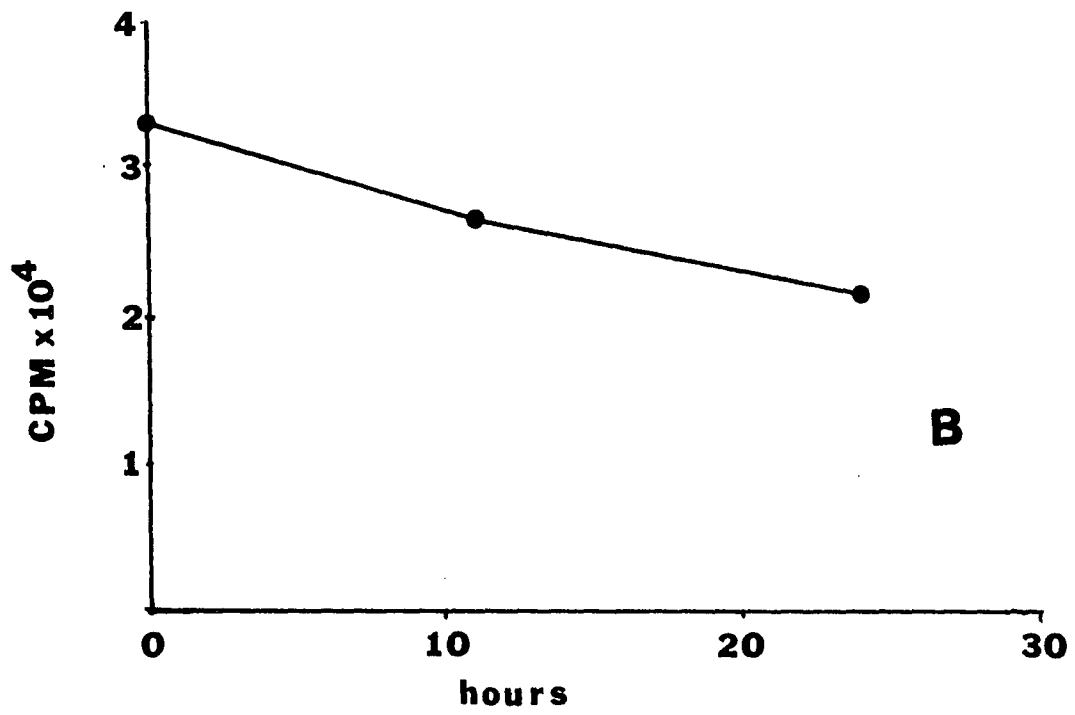
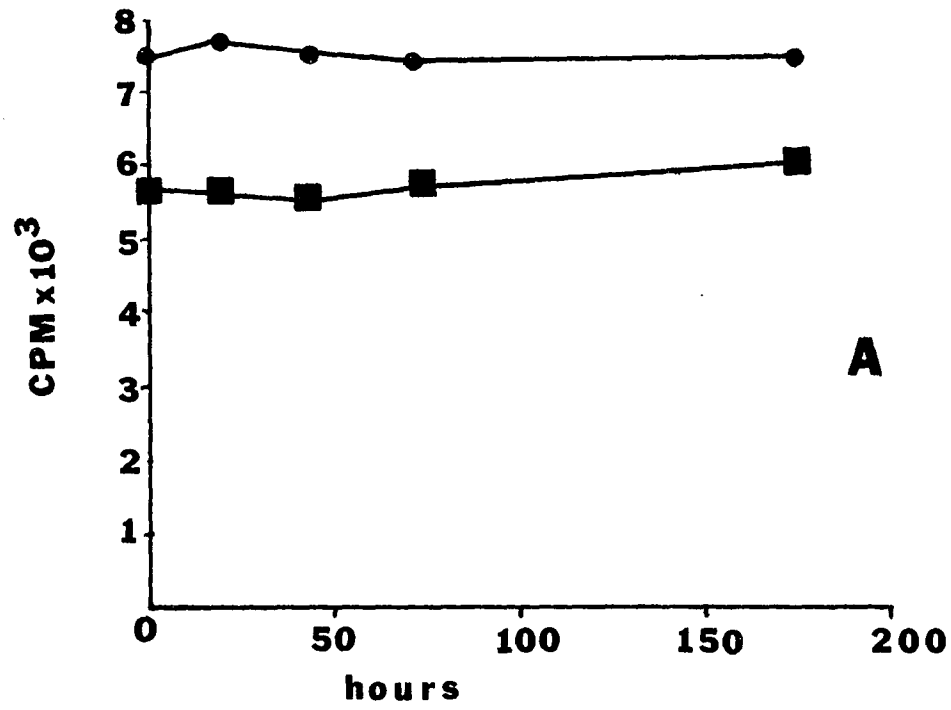


Figure 5. Elution of dialyzed and concentrated culture supernatant from *O*-(phenoxyacetyl)cellulose. Vertical axis refers to c.p.m. of D-[¹⁴C]glucose from [¹⁴C]sucrose incorporated into methanol-insoluble glucan and is directly proportional to the glucan-sucrase activity. Eluent was 20 mM pH 5.3 pyridine-acetate buffer until fraction 15 (arrow), when the eluent was changed to 0.5% Triton X-100 in buffer. Column size was 1 x 6 cm, sample size was 1.5 mL, and each fraction was 1 mL

Figure 6. Action of ICN dextranase on [^{14}C]glucans, with methanol-insoluble [^{14}C]glucan remaining as a function of time.

A: Action of dextranase on the glucan produced by CSDC-PAC I (—●—); action on the glucan produced by A-5mV₀C (—■—).

B: Hydrolysis of B-512F dextran by dextranase



bound-enzyme glucan. This result indicates that the activity that is not bound to the PA-cellulose is alternansucrase.

The enzyme activity that did bind to the PA-cellulose, on the other hand, and which was removed by detergent, produced two polysaccharides from sucrose. One of these precipitated at 38% ethanol, whereas the other precipitated at 40% ethanol. There were labeled as fractions II-B and II-A, respectively. Hydrolysis by dextranase, followed by t.l.c. of the products, showed that fraction II-A was not hydrolyzed by ICN dextranase. Fraction II-B gave products identical to those produced by action of ICN dextranase on a known sample of B-1355 fraction L dextran (see Fig. 7). This indicates that both activities are bound to the PA-cellulose and are removable by detergents. It should be noted that the unbound fraction contained high amounts of polysaccharide, whereas there was only a trace of carbohydrate in the bound fractions. An ionic detergent (Zwittergent 3-10) also removed the enzymes from PA-cellulose.

The pH optimum of alternansucrase was determined by assays as described in the Methods section. Fig. 8 shows the pH optimum for alternansucrase to be 5.5 ± 0.5 . Preliminary work has shown that the CSDC has a pH-activity curve identical to that of alternansucrase alone (results not shown). All preparative steps and enzyme assays were thus performed in the pH range 5.1-5.5, using 20 mM pyridine acetate buffer.

The activity of the enzymes in the presence of certain compounds (Table II) was also studied. Tris(hydroxymethyl)aminomethane (THAM) and 2-aminoethanol are both inhibitors of *L. mesenteroides* B-512F dextranase (unpublished results). These two compounds were also found to be inhibitors of the B-1355 enzymes (Table II). For this reason, they were not used as buffers in the regions of higher pH when determining the pH optimum for the enzymes. It was also shown that L-serine and L-threonine do not inhibit these enzymes, even though these two amino acids both con-

tain the $\begin{array}{c} | \quad | \\ \text{HO}-\text{CH}-\text{C}-\text{NH}_3^+ \\ | \end{array}$ moiety common to both THAM and 2-aminoethanol.

When selecting detergents to use with these enzymes, octyl β -D-glucopyranoside was considered, but proved unsuitable, as it was found to be an

Figure 7. Action of ICN dextranase on three glucans. Thin-layer chromatogram of products; 2 ascents on silica-gel plates in 3:1:1 (v/v/v) ethanol - nitroethane - water. L, action of dextranase on B-1355 fraction L glucan. IIA, action of dextranase on glucan that precipitates at 40% ethanol and was produced by fraction II (Fig. 4) of chromatography of CSDC on *O*-(phenoxyacetyl)cellulose. IIB, same as IIA, but on glucan that precipitates at 38% ethanol



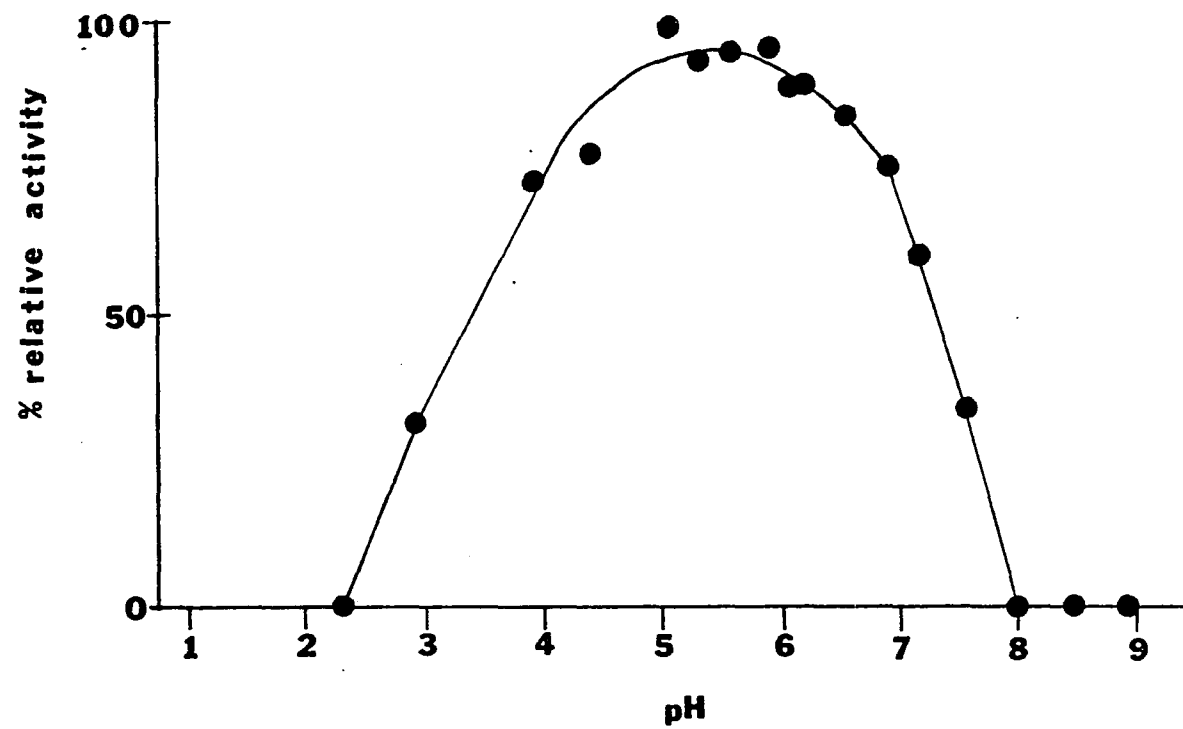


Figure 8. pH-Activity curve for alternansucrase, A-5mV₀C

Table II. Activity of glucansucrases in the presence
of various compounds^a

Compound	Concentration (mM)	Activity remaining (%)	
		in CSDC	in A-5mV ₀ C
3-Deoxy-3-fluoro- α -D-glucopyran- osyl fluoride	5	ND ^b	0
	25	25	0
	50	0	0
3-Deoxy-3-fluoro-D-glucose	70	100	100
3-O-Methyl-D-glucose	33	100	ND ^b
Potassium fluoride	100	100	ND ^b
EDTA, disodium salt	15	100	ND ^b
THAM	50	40	70
2-Aminoethanol	50	90	80
L-Serine	50	100	100
L-Threonine	50	100	100
Octyl β -D-glucopyranoside	1 ^c	70	ND ^b
Zwittergent 3-10	1 ^c	100	100
Triton X-100	1 ^c	100	100
Tween 80	1 ^c	100	100
Sodium dodecyl sulfate	0.5	30	0

^aSucrose concentration in each case was 0.1 M.

^bND = not determined.

^cPercent (w/v).

inhibitor of the enzyme mixture, as was sodium dodecyl sulfate. On the other hand, Triton X-100, Tween 80, Zwittergent 3-10, and sodium taurocholate all had no effect on the activity of the enzymes.

Perhaps the most interesting compound studied was the substrate analogue, 3-deoxy-3-fluoro- α -D-glucopyranosyl fluoride. In previous work, Genghof and Hehre (35) and Figures and Edwards (36) established that dextranases from other organisms were able to synthesize dextran from α -D-glucopyranosyl fluoride. For this reason, we considered that 3-deoxy-3-fluoro- α -D-glucopyranosyl fluoride might act as a substrate analogue and inhibit the production of alternan. To test this idea, assays were conducted in which the concentration of sucrose was 0.1 M and the inhibitor concentration ranged from 5 to 50 mM. The results (Table II) show that this compound is a fairly potent inhibitor of alternansucrase, but is less inhibitory to the dextranase as it occurs in the CSDC. This inhibition was found to be at least partially reversible. A sample of CSDC that had been inhibited by 3-deoxy-3-fluoro- α -D-glucopyranosyl fluoride was dialyzed overnight against buffer, and was found to have regained half of its original activity.

It should be noted that the sample of 3-deoxy-3-fluoro- α -D-glucopyranosyl fluoride used in these assays contained significant amounts of fluoride ion, as well as the free sugar 3-deoxy-3-fluoro-D-glucose. Neither of these materials, however, has any effect on the activity of the enzymes (Table II).

DISCUSSION

While relatively pure B-1355 fractions L and S glucans can be obtained by fractional precipitation with ethanol (2), the enzymes that synthesize them cannot be separated in this way. By ethanol fractionation, no more than 12% of the activity could be recovered. This is most likely due to denaturation of the enzymes at ethanol concentrations of 35 to 40%.

In contrast, poly(ethylene glycol) (PEG), of various molecular weights, could precipitate out all of the enzyme activity, as shown in Fig. 1, but did not separate the two enzymes. The concentrations of PEG required for precipitation were inversely proportional to the molecular weight of the PEG. It was also found that PEG precipitates dextran; the amount needed for this depends on the amount of dextran present and the molecular weight of the PEG (data not shown).

In studying the effects of various glucanases on these two polysaccharides, it was found that dextranase S was capable of completely hydrolyzing B-1355 fraction L glucan. The major products, shown in Fig. 4, were glucose and isomaltose. Dextranase S produced no detectable low-molecular-weight products from fraction S glucan (alternan).

Another α -(1 \rightarrow 6)-endodextranase, obtained from ICN Pharmaceuticals, was also able to hydrolyze fraction L dextran, but gave different products. In this instance, very small amounts of glucose and isomaltose were produced, and the major products were isomaltotriose and tetra-, penta-, and hexa-saccharides containing α -(1 \rightarrow 3) linkages that arise from hydrolysis around α -(1 \rightarrow 3) branch points. This dextranase also was unable to hydrolyze alternan (see Fig. 4). Mutanase (an α -(1 \rightarrow 3) endoglucanase from Novo Industries) showed no hydrolytic action on either of the glucan fractions.

The inability of any of these endoglucanases to hydrolyze alternan is apparently due to its unique alternating α -(1 \rightarrow 6), α -(1 \rightarrow 3)-D-glucose structure. This result shows that these dextranases require consecutively linked α -(1 \rightarrow 6)-D-glucose residues as a substrate. Likewise, mutanase seems to require consecutive α -(1 \rightarrow 3) linkages.

Isolation of alternansucrase from CSDC was accomplished by using ICN dextranase to hydrolyze the B-1355 fraction L dextran, thus removing this

polysaccharide from the enzyme mixture. This left the alternan-alternan-sucrase complex intact as a complex of very high molecular weight. This mixture, when passed through a column of Bio-Gel A-5m, gave two activity peaks, one at the void volume and another in a slower-moving fraction. These are referred to as the A-5m void volume fraction (A-5mV₀) and A-5m fraction II (A-5mII). The methods used to characterize the activity in each fraction are described in the Results section. The results in Figs. 4 and 6A show that the glucan produced from sucrose by the A-5mV₀ is not hydrolyzed by any of the dextranases, indicating it to be alternan. This conclusion is verified by the physical characteristics, namely precipitation occurs at 40% ethanol to give a dense white precipitate (see Fig. 3) that dissolves very readily in water. Periodate-oxidation data (Table I) further demonstrate that this glucan is alternan. Calculating the theoretical amount of periodate that would be consumed by alternan, using the methylation results of Seymour *et al.* (7), there should be 1.1 mol of periodate consumed per mol of glucose residues. The results shown in Table I, thus, stand as proof of the identity of the glucan produced by the A-5mV₀ enzyme. Likewise, the same types of evidence indicate that the glucan produced by A-5mII enzyme is a mixture of both glucans. As this mixture gives approximately the same results upon periodate oxidation as does fraction L dextran, it is assumed that the A-5mII produces alternan in much lower amounts than dextran. The precipitation from fraction II of only a small amount of glucan at 40-41% ethanol also indicates that the predominant activity in this fraction is dextranase. This second A-5m fraction lacks any significant amount of polysaccharide, and is very unstable, losing virtually all of its activity on being kept for more than a few h, even at 4°. Apparently, the removal of polysaccharide destabilizes these enzymes and causes loss of activity. Dextran has been shown to stabilize dextranase from *L. mesenteroides* B-512F (16). It should be noted that if the CSDC is not treated with dextranase, only a single peak emerges from the Bio-Gel A-5m column (Fig. 2A). This implies that the second peak in Fig. 2B represents material whose molecular size has been decreased by removal of dextran. From this, it may be concluded that both enzymes exist

in the CSDC as extremely large complexes. The CSDC enzymes have also been shown to be excluded from Bio-Gel A-15m. Previous workers (9), using light scattering, have estimated the average alternan molecule to have a molecular weight of $\sim 4 \times 10^7$.

It is interesting to consider the presence of alternansucrase in the A-5m fraction II after treatment with dextranase. Apparently, enough alternan is removed by dextranase action to allow some small amount of the alternansucrase complex to be decreased in size to $< 5 \times 10^6$ daltons. Misaki *et al.* (14) report up to 7.3% hydrolysis of B-1355 S glucan by *Penicillium sp.* dextranase. If this hydrolysis did result in a decrease in size of some alternan molecules to a value less than 5×10^6 daltons, this may account for the presence of a small amount of alternansucrase in fraction II. This decrease in molecular size would not be noted by our t.l.c. method, as any oligosaccharides larger than d.p. ~ 20 would remain at the origin.

Another method of isolating alternansucrase utilized hydrophobic chromatography on *O*-(phenoxyacetyl)cellulose (25). This material has been used to immobilize a number of proteins by non-covalently binding them. The proteins may be removed by such non-ionic detergents as Tween 80 and Triton X-100.

When a sample of CSDC is chromatographed on PA-cellulose, a peak of activity that does not bind is eluted in the first few fractions (Fig. 5), and contains a large amount of polysaccharide. If the column is then eluted with detergent, a second peak emerges that contains appreciable amounts of enzyme activity, but only a trace of carbohydrate.

The portion of the CSDC which does not bind to PA-cellulose is referred to as CSDC-PAC-fraction I, whereas the portion that binds and is removed by detergent is referred to as CSDC-PAC-fraction II. CSDC-PAC-fraction I was incubated with [^{14}C]sucrose, and the resultant [^{14}C]glucan was precipitated with 40% ethanol and redissolved in water. A digest of this glucan with ICN dextranase showed no decrease in the amount of methanol-insoluble [^{14}C]glucan after 172 h (Fig. 6A). A sample of [^{14}C]A-5mV₀ glucan also showed no decrease in methanol-insoluble glucan after dextranase digestion for 172 h. A dextranase digest of [^{14}C]B-512F dextran, on the

other hand, showed a significant decrease in the amount of methanol-insoluble glucan after only 24 h under the same conditions. These results indicate that it is only alternansucrase which does not bind to PA-cellulose under these conditions.

CSDC-PAC-fraction II, however, produced both types of glucans. This was demonstrated by allowing the fraction II enzymes to synthesize glucan(s) from sucrose, and fractionating the products with ethanol. A sample that precipitated at 40% ethanol was isolated and is referred to as CSDC-PAC fraction II-A glucan. The second glucan precipitated at 38% ethanol and is referred to as CSDC-PAC fraction II-B glucan. Glucan II-A was readily soluble in water, whereas II-B dissolved only with some difficulty, further suggesting that II-A is alternan and II-B is dextran. This was confirmed when it was shown that II-B, on hydrolysis by ICN dextranase, gave the same products as B-1355 fraction L dextran, while CSDC-PAC fraction II-A glucan was not hydrolyzed by ICN dextranase (Fig. 7). The overall recovery of activity from the PAC is usually between 30 and 40%.

The pH-activity curve (Fig. 8) is discussed in the Results section. The pH optimum for alternansucrase occurs between pH 5 and 6, which is similar to that of B-512F dextransucrase (16).

Of special interest is the inhibition of one or both of the B-1355 enzymes by some of the compounds shown in Table II. Figures and Edwards have previously described the effects of a number of sugars and sugar derivatives on *Streptococcus mutans* FA1 dextransucrase (36). Included were some α -D-glucopyranosyl fluorides. As α -D-glucopyranosyl fluoride has been shown to be a substrate for B-512F dextransucrase (35) and FA1 dextransucrase (36), it was our feeling that analogues of this compound could act as inhibitors of the B-1355 enzymes. It has also been demonstrated that α -D-xylopyranosyl fluoride inhibits dextransucrase from *L. mesenteroides* B-512F (37). As Table II shows, 3-deoxy-3-fluoro- α -D-glucopyranosyl fluoride proved to be a potent inhibitor of alternansucrase, but was much less effective as an inhibitor of the CSDC, a mixture of both enzymes. This result is not entirely unexpected, as O-3 in alternan is directly involved in every other glucosidic linkage in the main chain,

whereas in B-1355 L-dextran, 0-3 of D-glucose is only involved in branching. Interestingly, 3-deoxy-3-fluoro- α -D-glucopyranosyl fluoride does not inhibit B-512F dextranase (unpublished results), possibly for this same reason. Fluoride ions and 3-deoxy-3-fluoro-D-glucose were also present as major contaminants, but were found to have no effect on either of the two B-1355 enzymes. 3-O-Methyl-D-glucose also had no effect on the activity of the CSDC.

Experiments showed that when a sample of CSDC, which had been 100% inhibited by 3-deoxy-3-fluoro- α -D-glucopyranosyl fluoride, was dialyzed overnight, approximately half of the lost activity was regained. This suggests that at least part of the inhibition is reversible.

Sodium EDTA [(ethylenedinitrilo) tetraacetate] was also checked as a possible inhibitor to determine whether divalent metal cations were required for activity, but the lack of inhibition shown in Table II should not be taken to mean that these metal ions are not required. At the pH studied, EDTA has a much lower affinity for divalent metal ions than it does at higher pH values. It may be that, if divalent metal ions are required, the enzyme may simply bind the metal more strongly than does EDTA. Work by Robyt and Walseth (16) has shown that B-512F dextranase does have a binding site for divalent metal cations.

Previous workers (38-40) have shown that certain glucanohydrolases can be inhibited by 2-aminoethanol and tris(hydroxymethyl)aminomethane (THAM). As these compounds, especially THAM, are often used as buffers in many biochemical systems, we checked them as possible inhibitors of the B-1355 enzymes. As Table II shows, THAM and 2-aminoethanol both inhibit these glucanases, and though they are not particularly potent inhibitors, they should be avoided when working with these enzymes, and possibly with other glucosyltransferases. The amino acids L-serine and L-

threonine both contain the same structural moiety $(\text{HO}-\text{CH}-\overset{\overset{|}{|}}{\underset{|}{\text{C}}}-\overset{+}{\text{NH}}_3)$ as is

found in THAM and 2-aminoethanol. However, they do not inhibit the two enzymes studied here. One possible explanation for this may be that the negatively charged carboxyl group of the amino acids is repelled from a

negatively charged nucleophile at the active site of the enzyme.

When choosing detergents for use in removing activity from PA-cellulose, it was discovered that octyl β -D-glucopyranoside is an inhibitor of the CSDC enzyme-mixture. As no other detergents studied were inhibitors (Table II), it seems that this inhibition is specific and may arise from the structural similarity of octyl β -D-glucopyranoside to sucrose and α -D-glucopyranosyl fluoride.

In summary, we have described methods suitable for the separation and at least partial purification of the extracellular glucansucrase responsible for the synthesis of the fraction S, α -(1 \rightarrow 6), α -(1 \rightarrow 3) alternately linked D-glucan, produced by *Leuconostoc mesenteroides* B-1355. We have also described some characteristics of the enzyme, including the pH-activity relationship of alternansucrase, as well as inhibition by a number of compounds.

Because B-1355 fraction S glucan does not have the characteristics of a true dextran, that is, it consists of an alternating sequence of α -(1 \rightarrow 6) and α -(1 \rightarrow 3) D-glucose residues, is not significantly hydrolyzed by endodextranases, and has physical properties distinct from dextran, we propose the name "alternan" for this type of polysaccharide and the name "alternansucrase" for the enzyme that synthesizes it from sucrose. Similar alternating α -(1 \rightarrow 6) and α -(1 \rightarrow 3)-glucans are also produced by enzymes from *Leuconostoc mesenteroides* strains B-1498 and B-1501 (11).

ACKNOWLEDGMENT

We thank Dr. John N. Zikopoulos for his generous advice and assistance in the preparation of the 3-deoxy-3-fluoro- α -D-glucopyranosyl fluoride.

REFERENCES

1. A. Jeanes, W. C. Haynes, C. A. Wilham, J. C. Rankin, E. H. Melvin, M. J. Austin, J. E. Cluskey, B. E. Fisher, H. M. Tsuchiya, and C. E. Rist. Characterization and classification of dextrans from ninety-six strains of bacteria. *J. Amer. Chem. Soc.* 76, 5041-5052 (1954).
2. C. A. Wilham, B. H. Alexander, and A. Jeanes. Heterogeneity in dextran preparations. *Arch. Biochem. Biophys.* 59, 61-75 (1955).
3. I. J. Goldstein and W. J. Whelan. Structural studies of dextrans. Part I. A dextran containing α -1,3-glucosidic linkages. *J. Chem. Soc.* 170-175 (1962).
4. M. Torii and K. Sakakibara. Column chromatographic separation and quantitation of α -linked glucose oligosaccharides. *J. Chromatogr.* 96, 255-257 (1974).
5. T. Sawai, T. Tohyama, and T. Natsume. Hydrolysis of fourteen native dextrans by *Arthrobacter* isomaltodextranase, and correlation with dextran structure. *Carbohydr. Res.* 66, 195-205 (1978).
6. F. R. Seymour, R. D. Knapp, and S. H. Bishop. Determination of the structure of dextran by ^{13}C -nuclear magnetic resonance spectroscopy. *Carbohydr. Res.* 51, 179-194 (1976).
7. F. R. Seymour, M. E. Slodki, R. D. Plattner, and A. Jeanes. Six unusual dextrans: methylation structural analysis by combined g.l.c.-m.s. of per-O-acetyl aldononitriles. *Carbohydr. Res.* 53, 153-166 (1977).
8. F. R. Seymour, E. C. M. Chen, and S. H. Bishop. Methylation structural analysis of unusual dextrans by combined gas-liquid chromatography - mass spectrometry. *Carbohydr. Res.* 68, 113-121 (1979).
9. F. R. Seymour, R. D. Knapp, S. H. Bishop, and A. Jeanes. High-temperature enhancement of ^{13}C -n.m.r. chemical shifts of unusual dextrans and correlation with methylation structural analysis. *Carbohydr. Res.* 68, 123-140 (1979).
10. A. Jeanes and F. R. Seymour. The α -D-glucopyranosidic linkages of dextrans: comparison of percentages from structural analysis by periodate oxidation and by methylation. *Carbohydr. Res.* 74, 31-40 (1979).
11. F. R. Seymour, R. D. Knapp, E. C. M. Chen, S. H. Bishop, and A. Jeanes. Structural analysis of *Leuconostoc* dextrans containing 3-O- α -D-glucosylated α -D-glucosyl residues in both linear-chain and branch-point positions, or only in branch-point positions, by methylation and by ^{13}C -n.m.r. spectroscopy. *Carbohydr. Res.* 74, 41-62 (1979).
12. F. R. Seymour and R. L. Julian. Fourier-transform infrared difference spectrometry for structural analysis of dextrans. *Carbohydr. Res.* 74, 63-75 (1979).

13. F. R. Seymour, R. D. Knapp, and S. H. Bishop. Correlation of the structure of dextrans to their ^1H -n.m.r. spectra. *Carbohydr. Res.* 74, 77-92 (1979).
14. A. Misaki, M. Torii, T. Sawai, and I. J. Goldstein. Structure of the dextran of *Leuconostoc mesenteroides* B-1355. *Carbohydr. Res.* 84, 273-285 (1980).
15. E. J. Hehre. Polysaccharide synthesis from disaccharides. *Methods Enzymol.* 1, 178-180 (1955).
16. J. F. Robyt and T. F. Walseth. Production, purification, and properties of dextransucrase from *Leuconostoc mesenteroides* NRRL B-512F. *Carbohydr. Res.* 68, 95-111 (1979).
17. G. R. Germaine, C. F. Schachtele, and A. M. Chludzinski. Rapid filter paper assay for the dextransucrase activity from *Streptococcus mutans*. *J. Dent. Res.* 53, 1355-1360 (1974).
18. M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith. Colorimetric method for determination of sugars and related substances. *Anal. Chem.* 28, 350-352 (1956).
19. R. B. Kesler. Rapid quantitative anion-exchange chromatography of carbohydrates. *Anal. Chem.* 39, 1416-1421 (1967).
20. M. John, G. Trenel, and H. Dellweg. Quantitative chromatography of homologous glucose oligomers and other saccharides using polyacrylamide gel. *J. Chromatogr.* 42, 476-479 (1969).
21. M. M. Bradford. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 248-250 (1976).
22. A. Polson, G. M. Potgieter, J. F. Largier, G. E. F. Mears, and F. J. Joubert. The fractionation of protein mixtures by linear polymers of high molecular weight. *Biochim. Biophys. Acta* 82, 463-475 (1964).
23. S. I. Miekka and K. C. Ingham. Influence of self-association of proteins on their precipitation by poly(ethylene glycol). *Arch. Biochem. Biophys.* 191, 525-536 (1978).
24. R. R. B. Russell. Purification of *Streptococcus mutans* glucosyltransferase by polyethylene glycol precipitation. *FEMS Microbiol. Lett.* 6, 197-199 (1979).
25. L. G. Butler. Enzyme immobilization by adsorption on hydrophobic derivatives of cellulose and other hydrophilic materials. *Arch. Biochem. Biophys.* 171, 645-650 (1975).
26. T. Ikenaka. Studies on the mechanism of the enzymatic action of Taka-amylase. A. Isolation and identification of O- α -glucopyranosyl (1 \rightarrow 4) phenyl-6-O-acetyl- α -D-glucopyranoside. *J. Biochem. (Tokyo)* 54, 328-333 (1963).

27. O. T. Schmidt. Isopropylidene derivatives. *Methods Carbohydr. Chem.* 2, 318-325 (1963).
28. P. Garegg and B. Samuelsson. Oxidation of primary and secondary alcohols in partially protected sugars with the chromium trioxide-pyridine complex in the presence of acetic anhydride. *Carbohydr. Res.* 67, 267-270 (1978).
29. J. D. Stevens. β -D-Allose. From D-glucose by oxidation of 1,2:5,6-di-O-isopropylidene- α -D-glucofuranose and reduction of 1,2:5,6-di-O-isopropylidene- α -D-*ribo*-hexofuranos-3-ulose. *Methods Carbohydr. Chem.* 6, 123-128 (1972).
30. U. Reichman, K. A. Watanabe, and J. J. Fox. A practical synthesis of 2-deoxy-2-fluoro-D-arabinofuranose derivatives. *Carbohydr. Res.* 42, 233-240 (1975).
31. A. B. Foster and R. Hems. Deoxyfluoro sugars via displacement of sulfonyloxy groups with tetrabutylammonium fluoride. *Methods Carbohydr. Chem.* 6, 197-200 (1972).
32. A. B. Foster; R. Hems, and J. M. Webber. Fluorinated carbohydrates. Part I. 3-Deoxy-3-fluoro-D-glucose. *Carbohydr. Res.* 5, 292-301 (1967).
33. A. B. Foster, R. Hems, and L. D. Hall. Fluorinated carbohydrates. Part V. Nuclear magnetic resonance data on pyranose and furanose derivatives of 3-deoxy-3-fluoro-D-glucose. *Can. J. Chem.* 48, 3937-3945 (1970).
34. L. D. Hall, R. N. Johnson, A. B. Foster, and J. H. Westwood. Fluorinated carbohydrates. Part VIII. Studies of some 3-deoxy-3-fluoro-D-pyranosyl fluorides. *Can. J. Chem.* 49, 236-240 (1971).
35. D. S. Genghof and E. J. Hehre. De novo glycosidic linkage synthesis by glycosylases: α -D-glucosyl fluoride polymerization by dextransucrase. *Proc. Soc. Exp. Biol. Med.* 140, 1298-1301 (1972).
36. W. R. Figures and J. R. Edwards. α -D-Glucopyranosyl fluoride as a D-glucopyranosyl donor for a glycosyltransferase complex from *Streptococcus mutans* FA1. *Carbohydr. Res.* 48, 245-253 (1976).
37. J. N. Zikopoulos. Chemical synthesis of sucrose analogues and the study of their inhibition of dextransucrase. Ph. D. dissertation, Iowa State Univ., 1980. *Diss. Abstr.* 41, 943-B (1980).
38. M. U. Keleman and W. J. Whelan. Inhibition of glucosidases and galactosidases by polyols. *Arch. Biochem. Biophys.* 117, 423-428 (1966).
39. A. Dahlquist. Determination of maltase and isomaltase activities with a glucose oxidase reagent. *Biochem. J.* 80, 547-549 (1961).
40. J. Larner and R. E. Gillespie. Gastrointestinal digestion of starch. II. Properties of the intestinal carbohydrates. *J. Biol. Chem.* 223, 709-713 (1956).

SECTION II

ACCEPTOR REACTIONS OF ALTERNANSUCRASE
FROM *Leuconostoc mesenteroides* NRRL B-1355

Gregory L. Côté and John F. Robyt
Department of Biochemistry and Biophysics
Iowa State University, Ames, Iowa 50011

ABSTRACT

Extracellular glucansucrases from various bacterial sources, including *Leuconostoc mesenteroides*, have been shown to catalyze the transfer of glucosyl groups from sucrose to low-molecular-weight acceptor sugars, forming a series of oligosaccharides. An extracellular glucansucrase recently isolated from *Leuconostoc mesenteroides* NRRL B-1355 synthesizes a polysaccharide consisting of alternating α -(1 \rightarrow 6)- and α -(1 \rightarrow 3)-linked D-glucose residues. We have found that this enzyme preparation is capable of forming both α -(1 \rightarrow 6)- and α -(1 \rightarrow 3)-linked acceptor-products in the presence of a number of low-molecular-weight acceptor sugars. D-Glucose gave isomaltose only, no nigerose being formed. Similarly, methyl α -D-glucoside, methyl β -D-glucoside, maltose, and nigerose gave methyl α -isomaltoside, methyl β -isomaltoside, panose, and 6²-O- α -D-glucosylnigerose, respectively. However, isomaltose gave both isomaltotriose and 3²-O- α -D-glucosylisomaltose. These initial acceptor-products may also act as acceptors, and the structures of the products of higher d.p. show that a (1 \rightarrow 3)- α -D-glucosidic bond is formed only when the nonreducing glucose acceptor group is linked through an α -(1 \rightarrow 6) bond to another glucose residue. Experiments using [¹⁴C]sucrose and unlabeled acceptors showed that acceptor reactions occur by transfer of a glucosyl group from sucrose to O-3 or O-6 of the glucosyl group at the nonreducing end of the acceptor saccharide. If a "good" acceptor is defined as one that gives rise to a greater amount of oligosaccharide and less polysaccharide, then the acceptors tested may be listed as follows: maltose \geq nigerose > methyl α -D-glucoside > isomaltose > D-glucose > methyl β -D-glucoside.

INTRODUCTION

Koepsell *et al.* (1) showed that dextransucrase from *Leuconostoc mesenteroides* NRRL B-512, in the presence of low-molecular-weight sugars, catalyzed the transfer of D-glucosyl groups from sucrose to these "acceptor" sugars, forming a series of oligosaccharide acceptor-products. The acceptor reaction competes with the normal formation of high-molecular-weight glucan, and different sugars compete to different degrees. Maltose and isomaltose served as the best acceptors, giving large proportions of oligosaccharide products, and only a small fraction of the dextran was formed as compared with the proportion formed when acceptors were absent. Yamauchi and Ohwada (2) studied the formation of acceptor products from all eleven glucobioses, and showed that, for D-glucose-containing mono- and di-saccharides in general, B-512F dextransucrase effected acceptor reactions by transferring a glucosyl group from sucrose to O-6 of the nonreducing end of the acceptor sugar. A notable exception to this general observation was the formation of 2¹-O- α -D-glucosylcellobiose from cellobiose.

Recently, we have described (3) the separation of two different, extracellular glucansucrases produced by *Leuconostoc mesenteroides* NRRL B-1355. One of these produces a glucan consisting of alternating α -(1 \rightarrow 6)- and α -(1 \rightarrow 3)-linked D-glucose residues. This glucan has commonly been referred to as B-1355 fraction S dextran, but from its unusual structure and properties, we have proposed the name alternan for this glucan and alternansucrase for the enzyme that synthesizes it from sucrose (3). We have studied the structures of a number of oligosaccharide acceptor-products formed by alternansucrase in the presence of sucrose and acceptors, and show that this enzyme forms, via acceptor reactions, both α -(1 \rightarrow 6) and α -(1 \rightarrow 3) linkages to a variety of acceptor sugars. We have also determined the relative abilities of some of these acceptors to divert glucosyl groups away from formation of alternan and into the formation of oligosaccharide acceptor-products.

MATERIALS AND METHODS

Enzymes and Substrates

Dextranucrase from *Leuconostoc mesenteroides* B-512F was prepared as described by Robyt and Walseth (4), and was used to prepare reference oligosaccharides. Alternansucrase was prepared from the culture fluid of *Leuconostoc mesenteroides* B-1355 and was separated from dextranucrase as previously described (3). Exo-isomaltodextranase was prepared from *Arthrobacter globiformis* T6 by the method of Sawai *et al.* (5).

^{14}C -Uniformly labeled sucrose was obtained from New England Nuclear, Boston, MA. Reagent-grade maltose was purchased from EM Laboratories, Elmsford, NY. Methyl α -D-glucoside was purchased from Eastman Kodak Co., Rochester, NY, and was recrystallized from ethanol before use. Methyl β -D-glucoside was purchased from Sigma Chemical Co., St. Louis, MO. Panose was kindly provided by Dr. Dexter French. Isomaltose was isolated, from a partial acid-hydrolyzate of commercial B-512F dextran, by column chromatography on silica gel with 80% acetonitrile in water. Nigerose and kojibiose were prepared by acetolysis of alternan and B-1299 dextran, respectively, followed by deacetylation and subsequent purification by charcoal-column chromatography (6). D-Allose was prepared by oxidation of 1,2:5,6-di-O-isopropylidene- α -D-glucofuranose with chromium trioxide (7), followed by sodium borohydride reduction and acid-catalyzed removal of the acetal protective groups (8). 3-Deoxy-3-fluoro-D-glucose was prepared as previously described (3). All other materials were commercially available preparations.

Chromatography

Descending paper-chromatography was performed on Whatman 3MM paper with either 10:4:3 (v/v/v) ethyl acetate - pyridine - water or 7:3 (v/v) 1-propanol - water. Irrigation was carried out at 37° for 20-40 h, depending on the compounds being separated. For preparative paper-chromatography, the sample was streaked along the origin of a 23 x 56-cm sheet of paper. After irrigation, guide strips (1 cm) were cut from each edge

and developed by the alkaline silver nitrate dip-method (9); corresponding bands were cut out and the compound was eluted with water. For radioactive compounds, autoradiographic detection was used. T.l.c. was performed on either Whatman K5 0.25 mm silica gel plates, or on E. Merck plates (0.25 mm) of 1:1 silica gel 60 - kieselguhr. Multiple ascents were made in one of the following solvent-mixtures: *A*, 4:1 (v/v) acetonitrile - water; *B*, 9:1 (v/v) acetonitrile - water; *C*, 4:1:3:2 (v/v/v/v) acetonitrile - nitroethane - ethanol - water; and *D*, 5:2:4:4 (v/v/v/v) 1-propanol - nitromethane - acetonitrile - water. Detection was by 20% sulfuric acid in methanol, 5% orthonilic acid in pyridine (10), or 0.2% 1-(1-naphthyl)ethylenediamine dihydrochloride (Marshall's reagent) in 3% sulfuric acid in methanol (11), with heating for 10-15 min at 100-110°.

Acceptor-reaction Conditions

Acceptor reactions were performed at room temperature, with final concentrations of 42 mM sucrose, 100 mM acceptor, and 0.13 IU/ml of alternansucrase. The reactions were allowed to proceed until sucrose had disappeared (~48 h), as judged by t.l.c. with two ascents of solvent *A*. When acceptor products were to be isolated, an equal volume of ethanol was added to precipitate the polysaccharide. Enzymic reactions were conducted in 20 mM pyridinium acetate buffer (pH 5.3) containing 0.01% sodium azide and 2 mM calcium chloride. Control mixtures containing glucansucrase and acceptor sugars, but no sucrose, showed no reaction, indicating that acceptors do not serve as glycosyl donors.

Determination of Relative Strengths of Acceptors

The relative strengths of the acceptors were determined by treating alternansucrase with 42 mM [U-¹⁴C]sucrose (460 c.p.m./μg) and 100 mM acceptor as already described until all sucrose had been consumed. Aliquots (20 μL) of the reaction were spotted at the origin of a 23 x 56 cm paper chromatogram, which was irrigated with 7:3 (v/v) 1-propanol - water, for 24 h at 37°. Channels 3 cm wide were cut into 1-cm segments from the origin to the end of the paper, and each segment was counted for ¹⁴C radio-

activity by liquid scintillation in toluene cocktails. This procedure is similar to that described by Mayer *et al.* (12), but the use of ^{14}C -labeled sucrose permits application to any acceptor, not just radioactive ones.

Structural Analysis of Oligosaccharide Acceptor-products

The structures of acceptor products isolated by paper chromatography were determined by chemical and physical means. For some products, comparison by t.l.c. with known compounds was sufficient. When the degree of polymerization (d.p.) of a sample was unknown, it was determined in one of two ways: the reducing value of unlabeled compounds was determined by the Somogyi-Nelson method (13) and the amount of total carbohydrate was determined by the phenol-sulfuric acid method (14); alternatively, the specific activity of ^{14}C -labeled compounds was determined and compared with the specific activity of the [^{14}C]sucrose used in the acceptor reaction.

The products from hydrolysis (15) of trisaccharides and larger oligosaccharides by isomaltodextranase (5) were examined by t.l.c. with three ascents of solvent A. As the specificity of this exolytic enzyme (5,15-17) has been well studied, we were able to use it in the determination of the structures of a number of oligosaccharides.

When partial acid-hydrolysis was used to study the composition of an oligosaccharide, 0.2 mL of a solution of the sugar (0.5-1.0 mg/mL) was added to 0.8 mL of 0.4 M trifluoroacetic acid in a glass ampule. The ampule was then flushed with nitrogen, sealed, and heated for 40 min at 90°. The cooled samples were evaporated to solids under diminished pressure at 45°, and the residues redissolved in 0.2 mL of water. A 5-10 μL sample of this hydrolyzate was chromatographed by t.l.c. with three ascents in solvent A.

^{13}C -N.m.r. spectrometry was used to aid in the determination of the structure of a tetrasaccharide acceptor-product from maltose (see Results). The spectrum was recorded with 2 mL of a 0.15 M solution of the tetrasaccharide in D_2O in a 10-mm tube, using a JEOL FX-90Q ^{13}C -n.m.r. spectrometer in the Fourier-transform, proton-decoupled mode at 22.5 MHz, with 1,4-dioxane as the internal reference standard.

RESULTS

When dextransucrase from *L. mesenteroides* B-512F reacts with sucrose, dextran is formed and D-fructose is released. Once its concentration becomes high enough, fructopyranose acts as an acceptor, and the disaccharide leucrose [*O*- α -D-glucopyranosyl-(1 \rightarrow 5)-D-fructopyranose] is formed (18). We have also found leucrose to be formed when alternansucrase reacts with sucrose. Leucrose was identified by its R_{Glc} value in two different t.l.c. systems (2 ascents with solvent *A* or 4 ascents with solvent *B*) and by its color reaction when orthanilic acid (10) was used for t.l.c. detection. D-Glucose, D-fructose, and sucrose all gave orange-brown spots with this reagent, whereas leucrose gave a brownish to olive-brown spot.

When D-glucose was added as the acceptor with alternansucrase, the only disaccharide product detected by t.l.c. (other than leucrose) was isomaltose. No spots corresponding to nigerose, maltose, or kojibiose were observed. A trace of leucrose was seen, as in all reactions where sucrose was a substrate. Products of higher d.p. were also observed with D-glucose, and they corresponded to the same products as when isomaltose was the added acceptor. These arise from the isomaltose, formed from D-glucose, also acting as an acceptor.

The first alternansucrase acceptor-product from methyl α -D-glucoside had the same chromatographic mobility as the first product formed by B-512F dextransucrase, namely methyl α -isomaltoside (19). In paper chromatography with 10:4:3 (v/v/v) ethyl acetate - pyridine - water, this product had R_{Glc} 0.62; in t.l.c., with four ascents in solvent *B*, its R_{Glc} value was 0.88. Orthanilic acid did not react with this compound, indicating that it was nonreducing. Prolonged treatment with isomaltodextranase gave a small but significant amount of isomaltose. These results show that the alternansucrase acceptor-product from methyl α -D-glucoside is methyl α -isomaltoside. Products of higher d.p. were also formed, but their structures were not studied. It was found that methyl β -D-glucoside could also serve as an acceptor for both B-1355 alternansucrase and B-512F dextransucrase. As with methyl α -D-glucoside, the disaccharide products from both enzymes

had the same R_{Glc} (0.9) in t.l.c., and are presumed to be methyl β -isomaltoside. Again products of higher d.p. were formed but were not studied; presumably they have the same linkages as the products from D-glucose and isomaltose acceptors.

One of the best acceptors was maltose. Only one trisaccharide was isolated from the acceptor reaction of alternansucrase with sucrose and maltose (see Fig. 1). This trisaccharide had the same R_{Glc} in t.l.c. as panose (6²-O- α -D-glucosylmaltose), which was produced by the acceptor reaction of B-512F dextranase with sucrose and maltose (1,2). Isomaltodextranase acted on this compound to give D-glucose and isomaltose (Fig. 2), confirming that this acceptor product was panose. No other trisaccharides were found in the acceptor reaction with maltose. Two tetrasaccharides were produced from maltose. The first of these (Fig. 1) migrated between panose and 6²-O- α -isomaltosylmaltose (20) in t.l.c., and was found to be a reducing tetrasaccharide. As it has been shown that acceptor products of higher d.p. arise from the lower-d.p. precursors (12,20), this tetrasaccharide must contain panose as part of its structure. Isomaltodextranase converted this material into D-glucose and a trisaccharide having an R_{Glc} value similar to, but not identical to, that of panose (Fig. 2). Partial acid-hydrolysis yielded D-glucose, plus a spot corresponding to either nigerose or maltose, isomaltose, a trisaccharide, and starting material. No kojibiose was found. Of particular importance in the ¹³C-n.m.r. spectrum of this tetrasaccharide (Table I) is the peak at 80.8 p.p.m. Only four types of glucobiose linkages give a peak in this region (21), namely: C-2 in a β -(1 \rightarrow 2) linkage, C-3 in a β -(1 \rightarrow 3) linkage, C-4 in a β -(1 \rightarrow 4) linkage, and C-3 in an α -(1 \rightarrow 3) linkage (21). The possibility of β -linkages in this tetrasaccharide can be ruled out, as isomaltodextranase would not act on such a compound, even if alternansucrase were capable of forming β -linked products. This means that the first tetrasaccharide acceptor-product from maltose contains a D-glucosyl group linked α -(1 \rightarrow 3) to panose, and must therefore have the structure O- α -D-glucopyranosyl-(1 \rightarrow 3)-O- α -D-glucopyranosyl-(1 \rightarrow 6)-O- α -D-glucopyranosyl-(1 \rightarrow 4)-D-glucopyranose.

Figure 1. Thin-layer chromatogram of various acceptor-reaction mixtures, 50% ethanol-soluble fractions. Whatman K5 0.25-mm silica gel, 2 ascents at 37° with solvent *D*, detection by sulfuric acid.

(a) Isomaltooligosaccharide standards.

(b-d) B-512F dextran sucrose acceptor-reactions in the presence of sucrose:

(b) maltose acceptor-reaction: 1 = 6²-O-α-isomaltosylmaltose; 2 = 6²-O-α-isomaltotriosylmaltose.

(c) nigerose acceptor-reaction: 1 = 6²-O-α-D-glucosylnigerose; 2 = 6²-O-α-isomaltosylnigerose; 3 = 6²-O-α-isomaltotriosylnigerose.

(d) isomaltose acceptor-reaction.

(e-g) B-1355 alternansucrase acceptor-reactions in the presence of sucrose:

(e) maltose acceptor-reaction: 1 = panose; 2 = 6²-O-α-nigerosylmaltose; 3 = 6²-O-α-isomaltosylmaltose; 4 = structure not determined.

(f) nigerose acceptor-products: 1 = 6²-O-α-D-glucosylnigerose; 2 = 6²-O-α-nigerosylnigerose; 3 = 6²-O-α-isomaltosylnigerose; 4 = structure not determined.

(g) isomaltose acceptor-products: 1 = 3²-O-α-D-glucosylisomaltose; 2 = structure not known; 3 = isomaltotriose; 4 = structure not known.

(Abbreviations used: Glc = D-glucose, Fru = D-fructose; Im = isomaltose, Im_n = isomaltose oligosaccharide of d.p. = n, Mal = maltose, Nig = nigerose, and Pan = panose.)

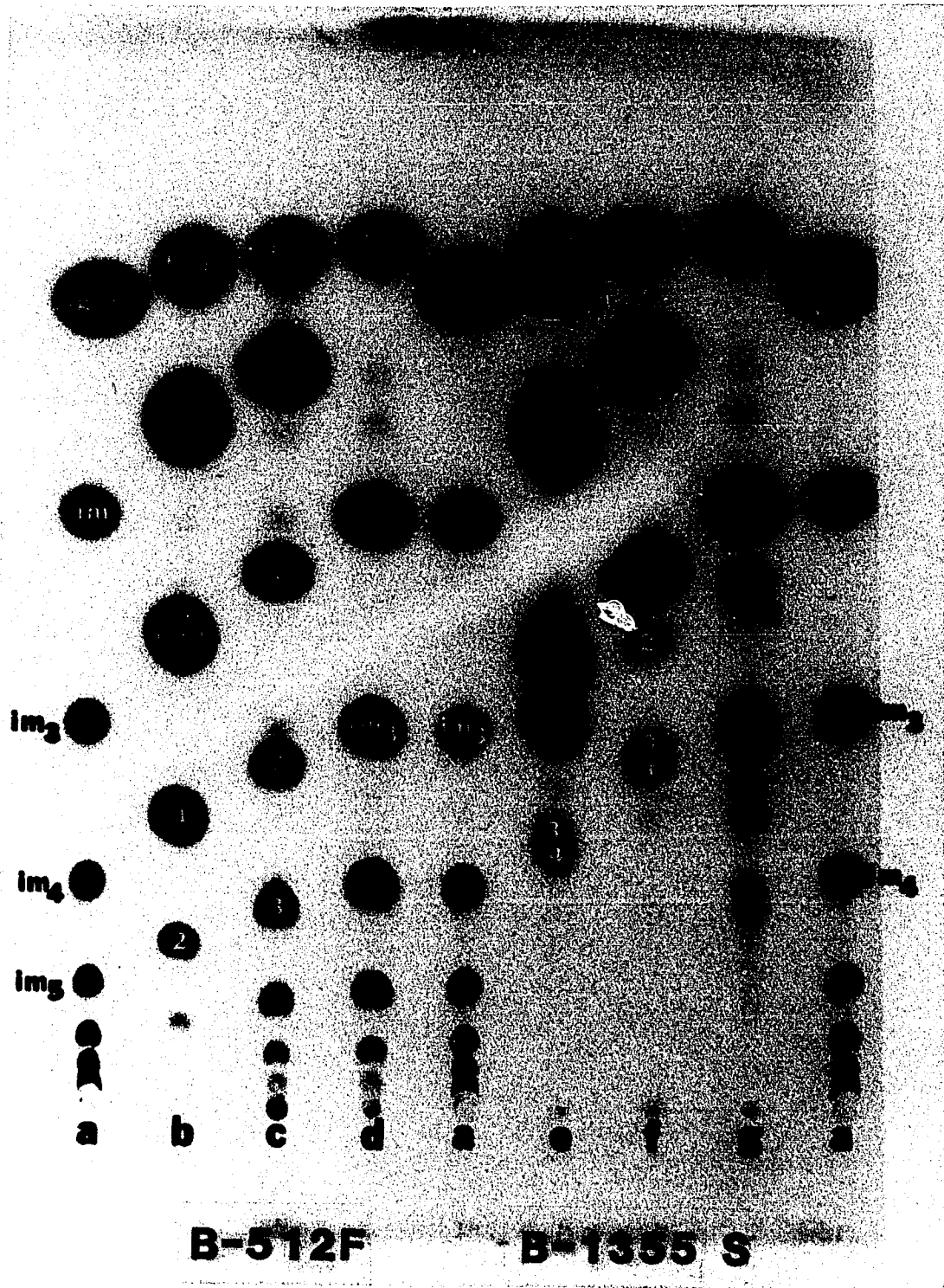


Figure 2. Isomaltodextranase degradation-products of alternansucrase-maltose acceptor-products. Thin-layer chromatogram on Whatman K5 0.25-mm silica gel, 3 ascents in solvent A at 25°; detection by sulfuric acid.

(a) Standards (top to bottom): fructose, glucose, sucrose, nigerose, isomaltose, and panose.

(b) first maltose acceptor-product, d.p. 3.

(c) isomaltodextranase hydrolyzate of (b).

(d) second maltose acceptor-product, d.p. 4.

(e) isomaltodextranase hydrolyzate of (d).

(f) standards (top to bottom): fructose, glucose, sucrose, nigerose, and isomaltose.

(g) standards: maltose (faster) and kojibiose (slower).

(h) third maltose acceptor product, d.p. 4.

(i) isomaltodextranase hydrolyzate of (h).

(j) fourth maltose acceptor-product(s).

(k) isomaltodextranase hydrolyzate of (j).

(Abbreviations used: Suc = sucrose, Koj = kojibiose; see also Fig. 1.)

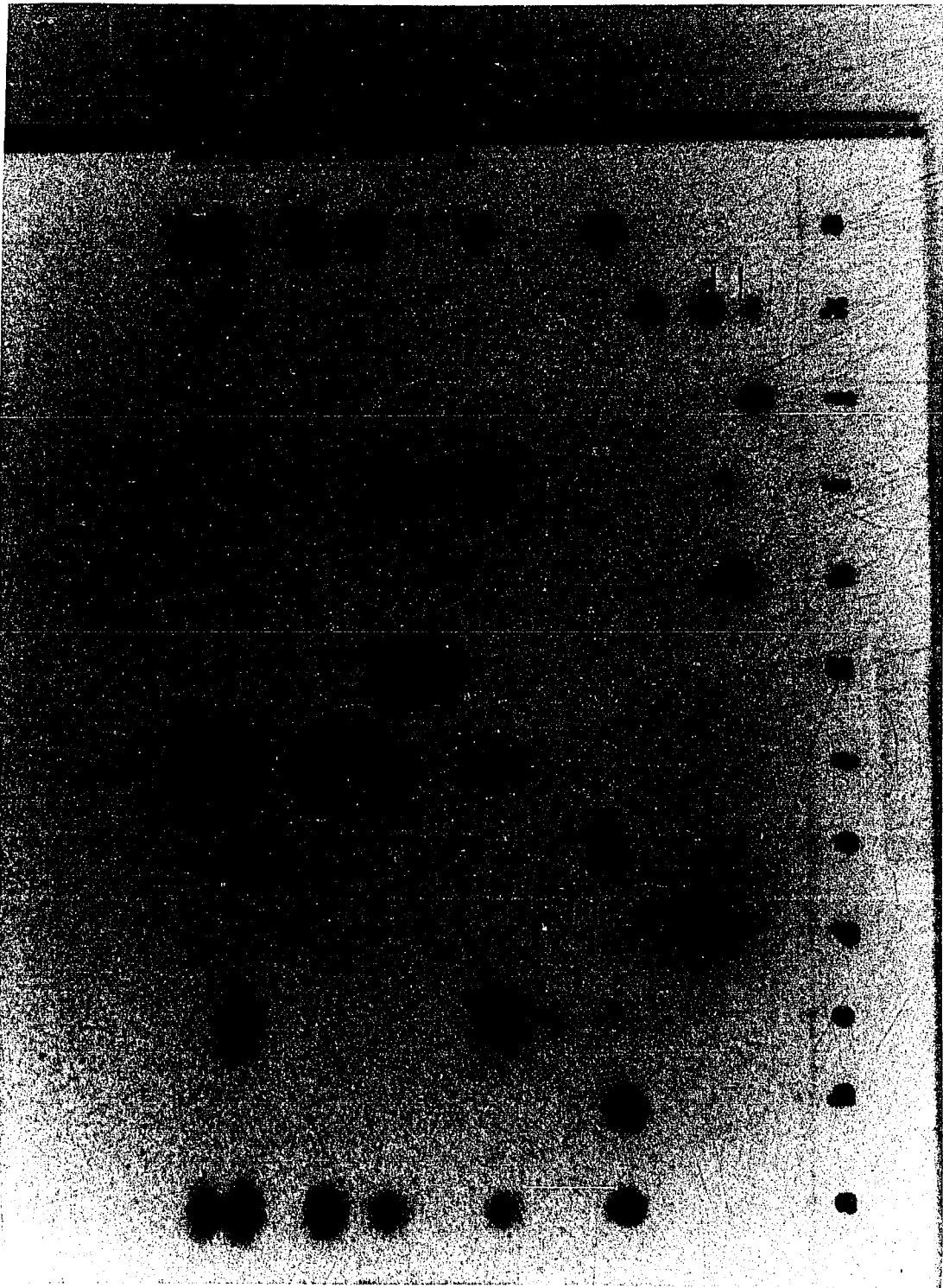


Table I. ^1H -Decoupled ^{13}C -n.m.r. spectrum of the faster-migrating tetrasaccharide alternansucrase-maltose acceptor-product

Chemical shift (p.p.m. relative to Me_4Si)	Intensity	Carbon atom ^a
100.6	1029	1 ²
100.1	1498	1 ⁴
98.9	1244	1 ³
96.6	838	1 β
92.7	384	1 α
80.8	1077	3 ³
78.2	607 ^b	
78.0	888 ^b	
77.0	863 ^b	
75.4	883 ^b	
74.8	1017 ^b	
74.0	1761 ^b	
73.7	1737 ^b	
72.5	3864 ^b	
72.1	1842 ^b	
70.8	2150 ^b	
70.2	2637 ^b	
69.9	327 ^b	
67.4 ^c	6969	
66.6	854	6 ²
61.5	906	}6 ¹ , 6 ³ , 6 ⁴
61.1	1955	

^aAssigned on the basis of resonances observed for panose and nigerose (22).

^bNot assigned.

^c1,4-Dioxane internal standard.

Table II. Alternansucrase acceptors and acceptor products

Acceptor	Acceptor products		
	d.p. 2	d.p. 3	d.p. 4
D-Fructose	leucrose	---	---
D-Glucose	isomaltose	(a) α -D-Glc-(1 \rightarrow 3)- α -D-Glc-(1 \rightarrow 6)-D-Glc (b) isomaltotriose (c) minor component ^a	a
Methyl α -D-glucoside	methyl α -isomaltoside	a	a
Methyl β -D-glucoside	methyl β -isomaltoside	a	a
Maltose	---	panose	(a) α -D-Glc-(1 \rightarrow 3)- α -D-Glc-(1 \rightarrow 6)- α -D-Glc-(1 \rightarrow 4)-D-Glc (b) α -D-Glc-(1 \rightarrow 6)- α -D-Glc-(1 \rightarrow 6)- α -D-Glc-(1 \rightarrow 4)-D-Glc
Nigerose	---	α -D-Glc-(1 \rightarrow 6)- α -D-Glc-(1 \rightarrow 3)-D-Glc	(a) α -D-Glc-(1 \rightarrow 3)- α -D-Glc-(1 \rightarrow 6)- α -D-Glc-(1 \rightarrow 3)-D-Glc (b) α -D-Glc-(1 \rightarrow 6)- α -D-Glc-(1 \rightarrow 6)- α -D-Glc-(1 \rightarrow 3)-D-Glc
Isomaltose	---	(a) α -D-Glc-(1 \rightarrow 3)- α -D-Glc-(1 \rightarrow 6)-D-Glc (b) isomaltotriose (c) minor component ^a	

^aStructure(s) not determined.

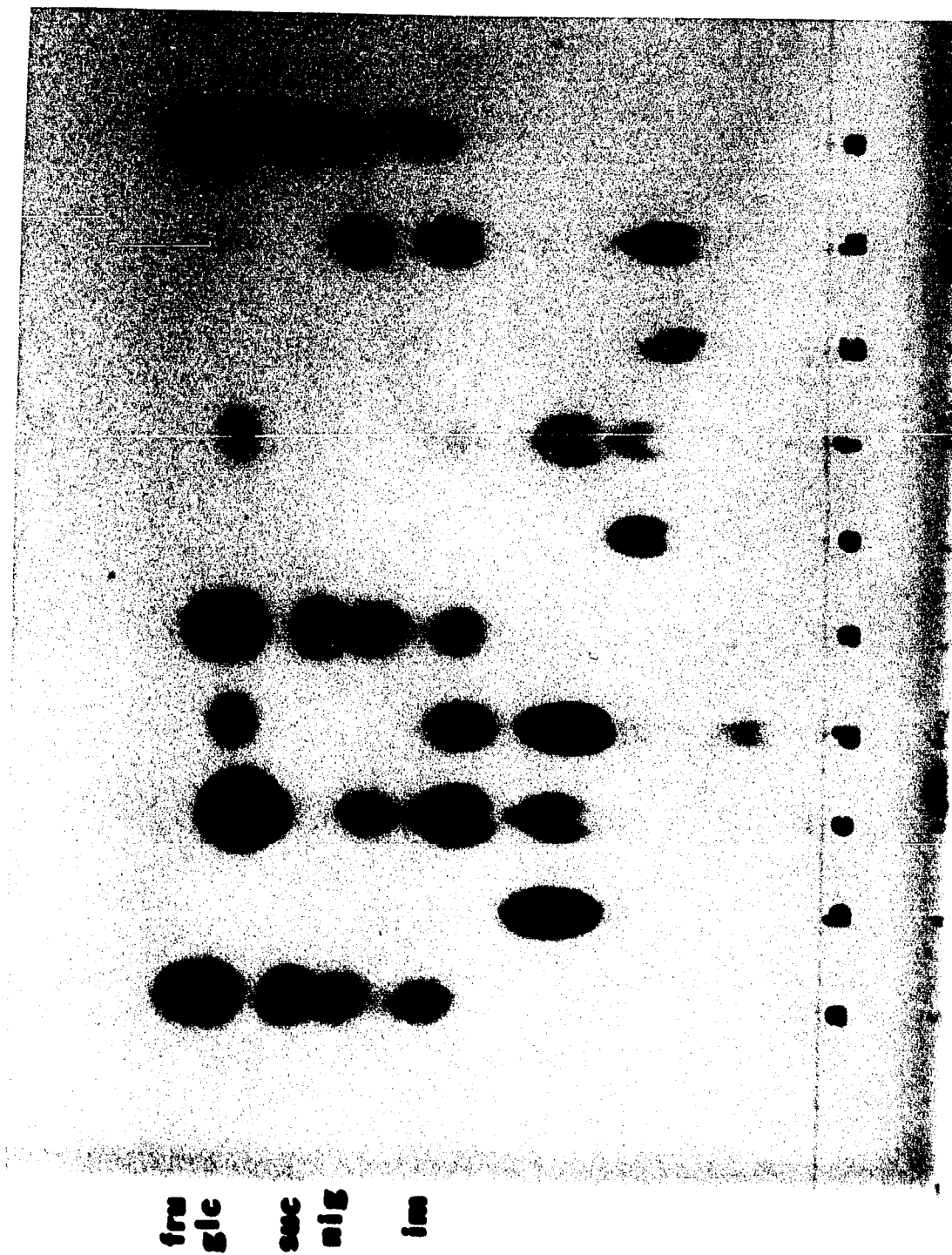
The second, slower-migrating tetrasaccharide, had the same R_{Glc} value in t.l.c. as the tetrasaccharide ($6^2-O-\alpha$ -isomaltosylmaltose (20)) produced by the B-512F acceptor-reaction with maltose (Fig. 1). Treatment with isomaltodextranase converted this alternansucrase product into maltose and isomaltose (Fig. 2), indicating that alternansucrase also produces $6^2-O-\alpha$ -isomaltosylmaltose from maltose in the presence of sucrose. The fourth acceptor-product from maltose has not been structurally identified, but isomaltodextranase hydrolyzed it to D-glucose and two oligosaccharides having d.p. 3 or 4 (see Fig. 2), whose identities have not been established. This pattern of products does not support any of the possible structures consisting of linear, consecutive, D-glucose residues, and so it may be a "branched" oligosaccharide acceptor-product.

Nigerose was approximately as good an acceptor as maltose, and the pattern of products from the two appears similar (Fig. 1). Hydrolysis of the first nigerose acceptor-product with isomaltodextranase gave D-glucose and isomaltose (Fig. 3). Partial acid-hydrolysis gave isomaltose, nigerose, and D-glucose, indicating that the nigerose acceptor-product is $6^2-O-\alpha$ -D-glucosylnigerose, which is the same trisaccharide as that formed from nigerose by B-512F dextranase (2).

The second alternansucrase product from nigerose is a tetrasaccharide having considerably greater mobility in t.l.c. (Fig. 1) than the tetrasaccharide produced by B-512F dextranase. This alternansucrase product was hydrolyzed rather slowly by isomaltodextranase to give D-glucose and a trisaccharide having the same R_{Glc} value as the first nigerose acceptor product. This result indicates that the acceptor product must be $6^2-O-\alpha$ -nigerosylnigerose. The third nigerose acceptor-product from alternansucrase had the same R_{Glc} value as the second nigerose acceptor-product from B-512F dextranase (Fig. 1), and, on treatment with isomaltodextranase, gave nigerose and isomaltose (Fig. 3). Thus, the third acceptor-product produced from nigerose by alternansucrase is the tetrasaccharide, $6^2-O-\alpha$ -isomaltosylnigerose. Also produced in the alternansucrase reaction with sucrose and nigerose was a saccharide of higher d.p. and unknown structure. No nigerotriose was formed.

Figure 3. Hydrolysis products of alternansucrase-nigerose acceptor-products. Thin-layer chromatogram on Merck 0.25-mm 50% silica gel 60 + 50% kieselguhr plate, 3 ascents in solvent A at 25°, detection by sulfuric acid.

- (a) Standards (top to bottom): fructose, glucose, sucrose, nigerose, and isomaltose.
 - (b) first nigerose acceptor-product, d.p. 3.
 - (c) partial acid hydrolyzate of (b).
 - (d) isomaltodextranase hydrolyzate of (b).
 - (e) second nigerose acceptor-product, d.p. 4.
 - (f) isomaltodextranase hydrolyzate of (e).
 - (g) third nigerose acceptor-product, d.p. 4.
 - (h) isomaltodextranase hydrolyzate of (g).
- (Abbreviations as in Figs. 1 and 2.)



The acceptor products arising from the reaction of alternansucrase with isomaltose and sucrose were more difficult to analyze, partly because of difficulties in separation. For example, the chromatogram in Fig. 1 shows two compounds migrating between isomaltose and isomaltotriose (g, spots 1 and 2), but these could not be resolved by paper chromatography, the method used to prepare the other pure acceptor-products. The only isomaltose acceptor-product thus far isolated pure has been a trisaccharide having the same R_{Glc} value as isomaltotriose (Fig. 1). A sample of this product, labeled with ^{14}C by using $[U-^{14}C]$ sucrose as the D-glucosyl donor, was subjected to hydrolysis by isomaltodextranase. Fig. 4 shows that both isomaltose and D-glucose were produced by this enzymic hydrolysis, but that only the isomaltose was ^{14}C -labeled. It was concluded that this acceptor product is isomaltotriose, which has arisen from transfer of a D-glucosyl group from sucrose to O-6 of the nonreducing end of isomaltose. Of the other major acceptor-products from isomaltose, the one having the highest mobility in t.l.c. has the same R_{Glc} value as an authentic sample of 3²-O- α -D-glucosylisomaltose, a trisaccharide also produced by the action of isomaltodextranase on alternan (16). T.l.c. with solvent *D* shows this trisaccharide to have a slightly different R_{Glc} value from 6²-O- α -D-glucosylnigerose, the first nigerose acceptor-product. Thus, it would be reasonable to assume that this product is 3²-O- α -D-glucosylisomaltose, and arises from the transfer of a D-glucosyl group from sucrose to O-3 of the nonreducing end of isomaltose. The identity of the minor compound migrating just behind this product (Fig. 1) is not known, nor are the identities of any of the compounds in Fig. 1 which arise from isomaltose and have R_{Glc} values less than that of isomaltotriose.

D-Allose, D-mannose, D-galactose, cellobiose, melibiose, raffinose, 3-deoxy-3-fluoro-D-glucose, D-xylose, and L-sorbose were also found to be acceptors, but the structures of the products were not determined. The last two were only weak acceptors. D-Arabinose was not an acceptor.

The relative activities of five acceptors were determined by adding acceptor to alternansucrase and $[^{14}C]$ sucrose, and measuring the amounts of $[^{14}C]$ alternan and $[^{14}C]$ oligosaccharide acceptor-products formed. Table III

Figure 4. Isomaltotriose produced by alternansucrase acceptor-reaction with isomaltose and [^{14}C]sucrose.

Left: thin-layer chromatogram, Whatman K5 0.25-mm silica gel, 3 ascents in solvent A at 25°, detection by sulfuric acid.

(a,a') isomaltodextranase hydrolyzate of [^{14}C]isomaltotriose.

(b,b') ^{14}C -labeled isomaltooligosaccharide standards.

(c) unlabeled standards (top to bottom): fructose, glucose, sucrose, nigerose, leucrose, isomaltose.

Right: autoradiogram of t.l.c. plate at left.

(Abbreviations used: Leu = leucrose, all others as in Figs. 1 and 2.)

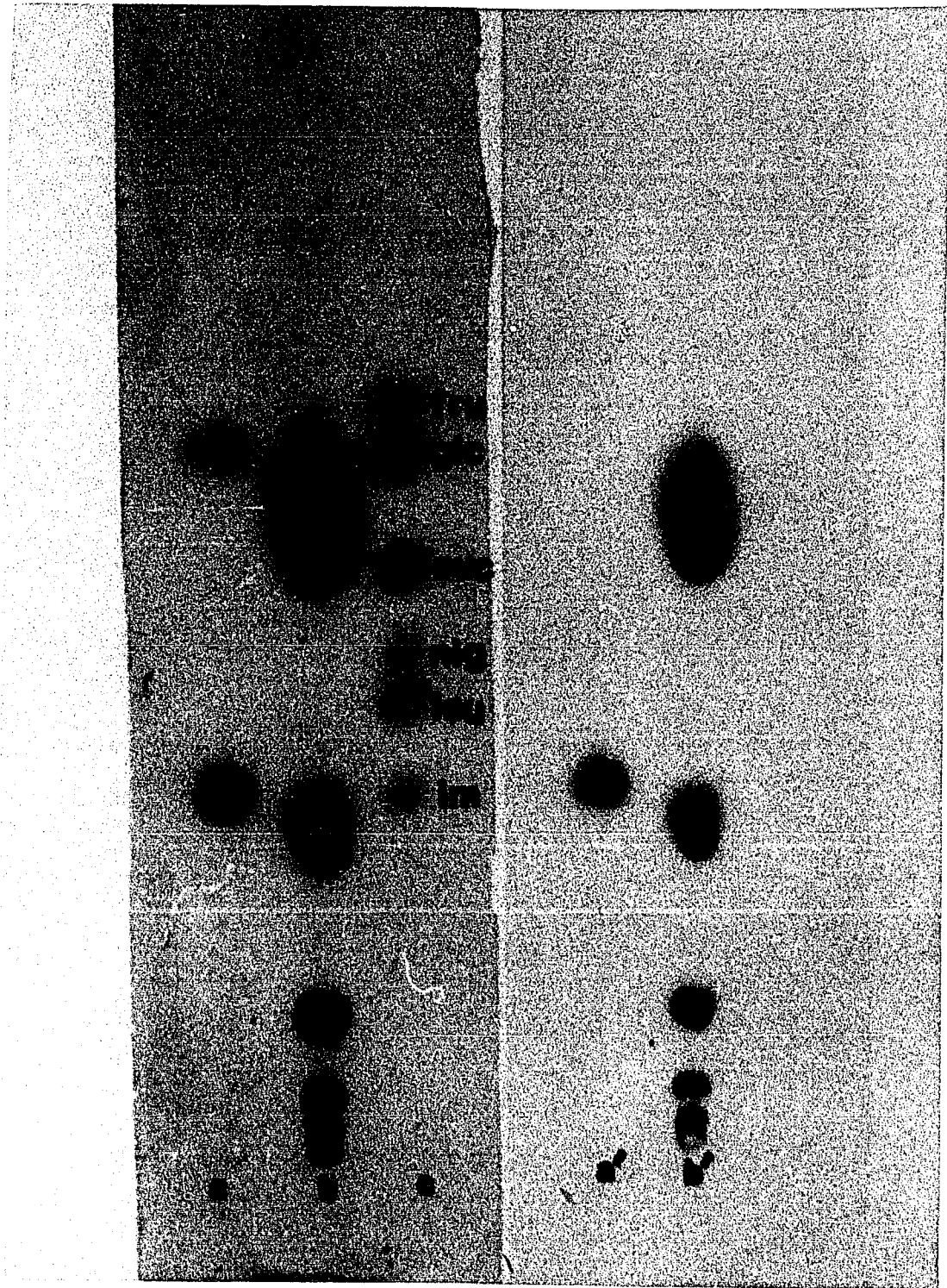


Table III. Distribution and amounts of acceptor products

Acceptor	D-[¹⁴ C]Glucose incorporated (%) into products from [U- ¹⁴ C]sucrose		
	Alternan ^a	Leucrose ^b	Oligosaccharide acceptor-products ^a
None	90	10	0
Methyl β-D-glucoside	75	6	19
Methyl α-D-glucoside	45	6	49
D-Glucose	66	5	29 { 22% isomaltose 7% d.p. ≥ 3 }
Isomaltose	52	7	41 { 13% 3 ² -O-α-D-glucosylisomaltose 12% isomaltotriose 14% d.p. 4 2% d.p. ≥ 4 }
Nigerose	15	5	80 { 69% d.p. 3 11% d.p. ≥ 4 }
Maltose	11	5	84 { 68% panose 16% d.p. ≥ 4 }

$$^a \text{For alternan and oligosaccharides, percent} = \frac{\text{CPM}_{\text{Product}} (\times 100)}{\text{CPM}_{\text{total}} - \text{CPM}_{\text{Fru}} - \frac{1}{2}\text{CPM}_{\text{Leu}}}$$

$$^b \text{For leucrose, percent} = \frac{\frac{1}{2}\text{CPM}_{\text{Leu}} (\times 100)}{\text{CPM}_{\text{total}} - \text{CPM}_{\text{Fru}} - \frac{1}{2}\text{CPM}_{\text{Leu}}}$$

gives the percentages of D-[^{14}C]glucose incorporated after [^{14}C]sucrose had all reacted. The percent of D-[^{14}C]glucose from [U- ^{14}C]sucrose incorporated into each product was calculated as follows:

For products containing glucose only,

percent of D-glucose incorporated =

$$\frac{\text{c.p.m. of product} \times 100}{\text{total c.p.m.} - \text{c.p.m. in fructose} - \frac{1}{2}(\text{c.p.m. in leucrose})}$$

For leucrose,

percent of D-glucose incorporated =

$$\frac{\frac{1}{2}(\text{c.p.m. in leucrose}) \times 100}{\text{total c.p.m.} - \text{c.p.m. in fructose} - \frac{1}{2}(\text{c.p.m. in leucrose})}$$

The relative activities of these acceptors, in increasing order of percent of the D-[^{14}C]glucose incorporated into oligosaccharide acceptor-products, are methyl β -D-glucoside (19%) < D-glucose (29%) < isomaltose (41%) < methyl α -D-glucoside (49%) < nigerose (80%) \approx maltose (84%).

DISCUSSION

Alternansucrase is elaborated, together with dextransucrase, by *Leuconostoc mesenteroides* NRRL B-1355. We have recently separated alternansucrase from dextransucrase, and showed that it synthesizes a glucan (alternan (3)) having alternating α -(1 \rightarrow 6) and α -(1 \rightarrow 3) linkages. Alternan differs significantly from other glucans that contain similarly high amounts of 1,3-disubstituted α -D-glucopyranose residues in that very few, if any, of these residues are adjacent or consecutively linked (22). Furthermore, the majority of (1 \rightarrow 3) linkages of alternan do not comprise branch points as they do in many other glucans synthesized by glucansucrases from sucrose.

Acceptor reactions occur with glucansucrases when monosaccharides, disaccharides, or oligosaccharides are added to the sucrose digests. The products are oligosaccharides containing one, two, three or more D-glucopyranosyl groups more than the acceptor. The structures of the products resulting from the reaction of acceptors with sucrose and B-512F dextransucrase have been previously determined (1,2,19). Robyt and Walseth (20) studied the mechanism of formation of the acceptor products and showed that a specific hydroxyl group on the acceptor acted as a nucleophile and attacked C-1 of an enzyme-glucosyl complex to give an α -D-glucosidic bond between glucose and the acceptor. Robyt and Taniguchi (23) showed that, when the acceptor is dextran, a 3-OH group acts as a nucleophile and an α -(1 \rightarrow 3) branch-linkage is formed.

Sidebotham (24) has stated that the specificity of acceptor reactions may account for the type and distribution of the linkages in the different glucans. This is true for the type and distribution of branch linkages, but not for the type and distribution of the linkages found in the main chains. Robyt *et al.* (25) have shown that the mechanism of elongation of the main chain of B-512F dextran is from the reducing end and that, even though the same enzyme is involved, the formation of linkages by the acceptor reaction (namely, branching) is not the same as elongation (20, 23).

In the present study, we found, as with other glucansucrases (18,26), that D-fructopyranose serves as an acceptor with alternansucrase and gives the disaccharide leucrose. D-Fructopyranose acceptor-products of higher d.p. were not detected, indicating that leucrose itself is a poor acceptor.

The fact that methyl β -D-glucoside acts as an acceptor for both alternansucrase and dextransucrase is interesting. This, along with the observation by Yamauchi and Ohwada (2) that β,β -trehalose acts as an acceptor for dextransucrase, refutes the statement by Sidebotham (24) that "dextransucrases do not transfer α -D-glucopyranosyl groups to β -D-glucopyranosyl residues". An α -D-glucopyranosyl-type configuration is not required for a sugar residue to act as an acceptor.

The present work shows that the acceptor reactions of alternansucrase thus far studied proceed by transfer of an α -D-glucopyranosyl group onto the nonreducing end-group of the di- and tri-saccharide acceptors. Whether or not the transfer to the nonreducing end occurs for higher oligosaccharide acceptors remains to be seen. Walker (27) reported that the glucansucrase of *Streptococcus mutans* transfers D-glucosyl groups onto O-3 of the D-glucose residue penultimate to the reducing end of isomaltooligosaccharide acceptors of d.p. 7 and higher.

A significant result of the present study is the observation that α -(1 \rightarrow 3) linkages are formed in oligosaccharide acceptor-products when the acceptor has the nonreducing D-glucose group linked by an α -(1 \rightarrow 6) linkage to another D-glucose residue. If the α -(1 \rightarrow 6) linkage is not present at the nonreducing end of the acceptor, the α -(1 \rightarrow 3) linkage is not formed. Thus, D-glucose gives isomaltose, and not nigerose, maltose gives only panose but not 3²-O- α -D-glucosylmaltose, and so forth; isomaltose, however, gives both isomaltotriose and 3²-O- α -D-glucosylisomaltose. The patterns may be clearly seen in Table II. In addition, once an α -(1 \rightarrow 6) linkage is present, two acceptor products are formed in approximately equal amounts, resulting from the transfer of D-glucose to either O-3 or O-6 of the nonreducing D-glucosyl group of the acceptor. This result suggests that acceptors having the nonreducing unit linked α -(1 \rightarrow 6) are

capable of binding in two equally favored ways.

It has been recognized that different acceptors give rise to different amounts of products (1,2,12). A "good" acceptor is one that will give rise to a greater amount of oligosaccharide acceptor-product(s) and less polysaccharide. For B-512F dextransucrase, Koepsell *et al.* (1) and Yamauchi and Ohwada (2) found isomaltose to be the best acceptor. Maltose was found to be the second best (1,2) followed by methyl α -D-glucoside. Leucrose was a very weak acceptor (1), and nigerose was moderate to weak (2). Mayer *et al.* (12) reported that, for a glucansucrase from *Streptococcus sanguis* ATCC 10558, maltose was a much better acceptor than isomaltose. Curiously, they also reported D-fructose to be a good acceptor, but identified the product as sucrose, rather than leucrose or isomaltulose. They did not identify the structures of most of their other acceptor-products except to show that the glucosyl group of sucrose was transferred onto the nonreducing end of the acceptor.

Our results with alternansucrase differ significantly from those reported for B-512F dextransucrase in two ways. One difference is in the structures of the products formed. The second is in the relative strengths of the acceptors with respect to their abilities to divert D-glucosyl groups from glucan synthesis into synthesis of oligosaccharides. Like the results of Mayer *et al.* (12) with *S. sanguis* glucansucrase, and in contrast to the results of Koepsell *et al.* (1) and Yamauchi and Ohwada (2) with *L. mesenteroides* B-512F dextransucrase, we have found that maltose serves as a better acceptor for alternansucrase than isomaltose. Nigerose is also a much better acceptor than isomaltose (Table III). These observations, together with the differences in the structures of the various acceptor-products between dextransucrase and alternansucrase, clearly indicate that acceptors do not react with all glucansucrases in the same manner.

D-Glucose is intermediate in acceptor strength between methyl α -D-glucoside and methyl β -D-glucoside. Considering that D-glucose in solution exists as ~37% in the α -pyranose form and 62% in the β -pyranose form, and taking the percentage of acceptor products obtained for methyl α -D-

glucoside (49%) and methyl β -D-glucoside (19%) from Table III, the weighted average, $(49\%)(0.37) + (19\%)(0.62) = 30\%$, is very close to the experimentally determined value of 29% for D-glucose. These results suggest that the acceptor reactivity of D-glucose may be a weighted average of the reactivities of the two anomeric forms present, and that the anomeric methyl groups of the D-glucosides have little effect on the reactivity of these acceptors.

Another phenomenon which has been observed by others (12,28), and which we have also observed for alternansucrase, is that at higher acceptor-to-sucrose ratios, most of the acceptor product results from addition of a single D-glucosyl group to the acceptor, whereas at lower ratios of acceptor to sucrose, relatively greater amounts of higher-d.p. acceptor-products are formed. This observation may be of use for determining the conditions to be used when acceptor reactions are used to prepare unusual saccharides. One unusual saccharide that may be prepared by these reactions is O - α -D-glucopyranosyl-(1 \rightarrow 3)- O - α -D-glucopyranosyl-(1 \rightarrow 6)- O - α -D-glucopyranosyl-(1 \rightarrow 4)-D-glucopyranose (6²- O - α -nigerosylmaltose), the faster-migrating alternansucrase acceptor-product arising from panose (Table II). This interesting tetrasaccharide, containing a sequence of three different α -linkages (namely, 1 \rightarrow 3, 1 \rightarrow 6, and 1 \rightarrow 4), has not previously been reported.

In summary, we have described some of the acceptor reactions of alternansucrase from *Leuconostoc mesenteroides* B-1355, and have shown that both α -(1 \rightarrow 6) and α -(1 \rightarrow 3) linkages are formed, although an α -(1 \rightarrow 6) bond must be present for an α -(1 \rightarrow 3) bond to be formed. We have also compared some of the acceptor reactions of alternansucrase with those of other bacterial glucansucrases.

ACKNOWLEDGMENT

We thank Mr. Steve Eklund for preparing the sample of nigerose and for his assistance in obtaining the ^{13}C -n.m.r. spectrum.

REFERENCES

1. H. J. Koepsell, H. M. Tsuchiya, N. N. Hellman, A. Kazenko, C. A. Hoffman, E. S. Sharpe, and R. W. Jackson. Enzymatic synthesis of dextran. Acceptor specificity and chain initiation. *J. Biol. Chem.* 200, 793-801 (1953).
2. F. Yamauchi and Y. Ohwada. Synthesis of oligosaccharides by growing culture of *Leuconostoc mesenteroides*. Part IV. Oligosaccharide formation in the presence of various types of glucobioses as acceptors. *Agric. Biol. Chem.* 33, 1295-1300 (1969).
3. G. L. Côté and J. F. Robyt. Isolation and partial characterization of an extracellular glucansucrase from *Leuconostoc mesenteroides* NRRL B-1355 that synthesizes an alternating (1→6), (1→3)- α -D-glucan. *Carbohydr. Res.* 101, 57-74 (1982).
4. J. F. Robyt and T. F. Walseth. Production, purification, and properties of dextransucrase from *Leuconostoc mesenteroides* NRRL B-512F. *Carbohydr. Res.* 68, 95-111 (1979).
5. T. Sawai, K. Toriyamo, and K. Yano. A bacterial dextranase releasing only isomaltose from dextrans. *J. Biochem. (Tokyo)* 75, 105-112 (1974).
6. K. Matsuda, H. Watanabe, K. Fujimoto, and K. Aso. Isolation of nigerose and kojibiose from dextrans. *Nature* 191, 278 (1961).
7. P. J. Garegg and B. Samuelsson. Oxidation of primary and secondary alcohols in partially protected sugars with the chromium trioxide-pyridine complex in the presence of acetic anhydride. *Carbohydr. Res.* 67, 267-270 (1978).
8. J. D. Stevens. β -D-Allose. From D-glucose by oxidation of 1,2:5,6-di-O-isopropylidene- α -D-glucofuranose and reduction of 1,2:5,6-di-O-isopropylidene- α -D-ribo-hexofuranos-3-ulose. *Methods Carbohydr. Chem.* 6, 123-128 (1972).
9. E. F. L. J. Anet and T. M. Reynolds. Isolation of mucic acid from fruits. *Nature* 174, 930-931 (1954).
10. F. Iinuma, Y. Hiraga, T. Kinoshita, and M. Watanabe. Simultaneous fluorometric and colorimetric detection of carbohydrates on silica gel plates using *o*-aminobenzenesulfonic acid. *Chem. Pharm. Bull.* 27, 1268-1271 (1979).
11. M. Bounias. *N*-(1-Naphthyl)ethylenediamine dihydrochloride as a new reagent for nanomole quantification of sugars on thin-layer plates by a mathematical calibration process. *Anal. Biochem.* 106, 291-295 (1980).
12. R. M. Mayer, M. M. Matthews, C. L. Futerman, V. P. Parnaik, and S. M. Jung. Dextransucrase: acceptor substrate reactions. *Arch. Biochem. Biophys.* 208, 278-287 (1981).

13. J. E. Hodge and B. T. Hofreiter. Determination of reducing sugars and carbohydrates. *Methods Carbohydr. Chem.* 1, 380-394 (1962).
14. M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith. Colorimetric method for determination of sugars and related substances. *Anal. Chem.* 28, 350-356 (1956).
15. M. Torii, K. Sakakibara, A. Misaki, and T. Sawai. Degradation of α -linked D-glucosaccharides and dextrans by an isomaltodextranase preparation from *Arthrobacter globiformis* T6. *Biochem. Biophys. Res. Commun.* 70, 459-464 (1976).
16. T. Sawai, T. Tohyama, and T. Natsume. Hydrolysis of fourteen native dextrans by *Arthrobacter* isomaltodextranase, and correlation with dextran structure. *Carbohydr. Res.* 66, 195-205 (1978).
17. T. Sawai, S. Ohara, Y. Ichimi, S. Okaji, K. Hisada, and N. Fukaya. Purification and some properties of the isomaltodextranase of *Actinomadura* strain R10 and comparison with that of *Arthrobacter globiformis* T6. *Carbohydr. Res.* 89, 289-299 (1981).
18. F. H. Stodola, E. S. Sharpe, and H. J. Koepsell. The preparation, properties, and structure of the disaccharide leucrose. *J. Amer. Chem. Soc.* 78, 2514-2518 (1956).
19. R. W. Jones, A. Jeanes, C. S. Stringer, and H. M. Tsuchiya. Crystalline methyl α -isomaltoside and its homologs obtained by synthetic action of dextransucrase. *J. Amer. Chem. Soc.* 78, 2499-2502 (1956).
20. J. F. Robyt and T. F. Walseth. The mechanism of acceptor reactions of *Leuconostoc mesenteroides* NRRL B-512F dextransucrase. *Carbohydr. Res.* 61, 433-445 (1978).
21. T. Usui, N. Yamaoka, K. Matsuda, K. Tuzimura, H. Sugiyama, and S. Seto. ^{13}C -N.m.r. spectra of glucobioses, glucotrioses, and glucans. *J. Chem. Soc. Perkin Trans. 1*, 2425-2432 (1973).
22. A. Misaki, M. Torii, T. Sawai, and I. J. Goldstein. Structure of the dextran of *Leuconostoc mesenteroides* B-1355. *Carbohydr. Res.* 84, 273-285 (1980).
23. J. F. Robyt and H. Taniguchi. The mechanism of dextransucrase action. Biosynthesis of branch linkages by acceptor reactions with dextran. *Arch. Biochem. Biophys.* 174, 129-135 (1976).
24. R. L. Sidebotham. Dextrans. *Adv. Carbohydr. Chem. Biochem.* 30, 371-444 (1974).
25. J. F. Robyt, B. K. Kimble, and T. F. Walseth. The mechanism of dextransucrase action. Direction of dextran biosynthesis. *Arch. Biochem. Biophys.* 165, 634-640 (1974).
26. E. S. Sharpe, F. H. Stodola, and H. J. Koepsell. Formation of isomaltulose in enzymatic dextran synthesis. *J. Org. Chem.* 25, 1062-1063 (1960).

27. G. J. Walker. Branching-enzyme activity of an α -D-glucosyltransferase of *Streptococcus mutans*. Carbohydr. Res. 82, 404-410 (1980).
28. G. J. Walker. Preparation of isomaltose oligosaccharides labelled with ^{14}C in the non-reducing terminal unit, and their use in studies of dextranase activity. Carbohydr. Res. 30, 1-10 (1973).

SECTION III.

THE FORMATION OF α -D-(1 \rightarrow 3) BRANCH LINKAGES
BY AN EXOCELLULAR GLUCANSUCRASE FROM
Leuconostoc mesenteroides NRRL B-742

Gregory L. Côté and John F. Robyt
Department of Biochemistry and Biophysics
Iowa State University, Ames, Iowa 50011

ABSTRACT

Leuconostoc mesenteroides NRRL B-742 produces two exocellular α -D-glucans, namely, fraction L, which consists of an α -D-(1 \rightarrow 6) backbone with α -D-(1 \rightarrow 4) branch-points, and fraction S, which consists of an α -D-(1 \rightarrow 6) backbone with α -D-(1 \rightarrow 3) branch-points. It was found that the percentage of α -D-(1 \rightarrow 3) branch-points in fraction S glucan is variable, depending on the conditions under which it is synthesized from sucrose by the exocellular glucansucrase, and that α -D-(1 \rightarrow 3) branch formation by this glucansucrase occurs by acceptor reactions in which α -D-glucosyl groups are transferred from sucrose to OH-3 groups on α -D-(1 \rightarrow 6)-linked D-glucan chains. Thus, any change in reaction conditions that affects the rate of acceptor reactions relative to chain elongation also affects the degree of branching in B-742 fraction S dextran. It was also found that this glucansucrase is capable of modifying other dextrans, such as B-512F and B-742 fraction L, by transferring D-glucosyl groups to OH-3 of D-glucosyl residues in these dextrans as well.

INTRODUCTION

In order to understand further the biosynthesis of secondary D-glucosidic linkages in dextrans, we have undertaken the study of such D-glucans that contain high relative proportions of α -D-(1 \rightarrow 3)-glucosidic linkages, both at the branch points and in linear positions of the polysaccharide chains. We have previously described enzymes that are capable of forming both types of α -D-(1 \rightarrow 3) linkage (1-3). In the present work, we discuss the formation of α -D-(1 \rightarrow 3) branch linkages by an exocellular glucansucrase from *Leuconostoc mesenteroides* NRRL B-742. As in the *L. mesenteroides* B-1355 system, this strain also produces two exocellular α -D-glucans from sucrose, namely, fraction L, which is precipitated at an ethanol concentration of 39%, and fraction S, precipitated at an ethanol concentration (4) of 45%. This particular strain was first isolated and described by Hucker and Pederson (5) in 1930. Subsequent workers described the growth of the bacteria and the production of exocellular D-glucan in detail (6-15), as well as some of the structural characteristics of the two dextrans.

Seymour *et al.* (16-24) described their structural analyses of a number of D-glucans, including those produced by *L. mesenteroides* B-742. According to their data (see Table I), B-742 dextran fraction S consists of a linear chain of α -D-(1 \rightarrow 6)-linked D-glucopyranosyl residues, each bearing a single α -D-glucopyranosyl group linked to O-3, to give a "comb-like" polymer. Nearly all of the D-glucosyl residues in the backbone chain would be substituted in this way, with approximately one in ten, or twenty, lacking a D-glucosyl group on O-3. It would be assumed that such a dextran would be completely resistant to hydrolysis by most endodextranases, such as that produced by *Penicillium funiculosum*. However, we have found that B-742 S dextran may not always be so highly branched. Our investigation has shown that branch formation and the degree of branching in this dextran can vary, depending on the conditions of synthesis. We have also found that branch formation by *L. mesenteroides* B-742 S dextransucrase can occur by acceptor reactions with less highly branched

dextran molecules, such as B-512F dextran (which has 5% of α -D-(1 \rightarrow 3) branches), to give a much more highly branched product. Branch formation by acceptor reactions can vary not only from one strain of dextran-producing bacteria to another, but can also vary depending on the reaction conditions.

Table I. Methylation data of Seymour and co-workers (16)

Dextran	Mole % of each methylated D-glucose ^a				
	2,3,4,6	2,3,4	2,4,6	2,3	2,4
B-742 S	45.4	4.4	---	---	50.2
B-742 L	14.4	72.5	0.7	12.4	---

^aNumbers refer to positions of *O*-methyl groups on D-glucose.

MATERIALS AND METHODS

Organism

A lyophilized sample of *Leuconostoc mesenteroides* strain B-742 was obtained from the Northern Regional Research Center of the U. S. Dept. of Agriculture. The culture conditions were the same as those described by Hehre (25).

Crude Enzyme Mixture

A preparation of crude-culture supernatant-fluid was used that had been dialyzed, and concentrated, as previously described for *L. mesenteroides* B-512F (26) and B-1355 (1). This preparation contained 0.6 IU/mL of glucansucrase as measured by a radiochemical assay (26,27), and will be referred to as CSDC (culture supernatant, dialyzed, and concentrated). It also contained 25 mg of carbohydrate per mL.

Chromatography

Molecular-size exclusion-chromatography was conducted with Bio-Gel P-6 and P-10 and A-15m (Bio-Rad, Richmond, CA) in columns that were eluted with 0.02% sodium azide at room temperature.

Thin-layer chromatograms were obtained either on plates of glass-backed, Whatman K5 silica gel, or plastic-backed plates of E. Merck silica gel. Two different solvent-systems were employed: *A*, 4:1 (v/v) acetonitrile-water, and *B*, 3:1:1 (v/v/v) ethanol-nitroethane-water. Visualization was achieved by charring with sulfuric acid for unlabeled compounds, and by autoradiography for ¹⁴C-labeled material. (Phenoxyacetyl)-cellulose (PA-cellulose) chromatography was performed as previously described (1).

Hydrolysis by Dextranase

Penicillium funiculosum endodextranase was purchased from Sigma Chemical Co., St. Louis, MO. Hydrolyses of dextrans were conducted at room temperature in 20 mM acetate buffer, pH 5.5. The extent of hydrolysis of radioactive dextran was determined by measuring the amount of labeled material that was rendered soluble in methanol, similar to the filter-paper assay for formation of D-glucan (26,27). Hydrolysis was monitored in this way until no further decrease in methanol-insoluble, radioactive material was observed.

Synthesis of Samples of D-Glucan

Two different sets of conditions were employed for preparing various D-glucan samples. The first, the usual method, consisted in incubating a glucansucrase preparation with sucrose at an initial concentration of 0.2 M sucrose in 20 mM pyridine acetate buffer, pH 5.4, containing 2 mM calcium chloride and 0.02% of sodium azide. The reaction was allowed to proceed at room temperature until no sucrose remained, as detected by t.l.c. in solvent A. The D-glucans were then fractionally precipitated by slowly adding ethanol (4). The precipitated D-glucans were redissolved in water, refractionated, and dried *in vacuo* for ~12 h at 40°.

An alternative method for synthesizing glucan was to place the glucansucrase in dialysis tubing (~3-5 mL), which was then placed in a vessel containing stirred, buffered, 0.2 M sucrose (1.5 L). The material inside the tubing was removed when it reached a pasty, gel-like consistency. This material was diluted with water, and the D-glucans were fractionated by precipitation with ethanol (4) as just described. These two methods will be referred to as the dialysis-bag method and the usual method. As will be shown, the resulting D-glucans differ significantly. An authentic sample of NRRC B-742 fraction S dextran was kindly supplied by Dr. Morey E. Slodki of the Northern Regional Research Center (NRRC) of the U. S. D. A. (Peoria, IL).

^{13}C -Nuclear Magnetic Resonance Spectrometry

Polysaccharides were analyzed by ^{13}C -n.m.r. spectroscopy in order to determine the relative amounts of different linkage types, in a manner similar to that of Seymour *et al.* (17). Dry polysaccharide (~50-150 mg) was dissolved in deuterium oxide (2 mL) in a 10-mm, glass, n.m.r. tube. Spectra were recorded at 80° with a JEOL FX-90Q, F.t.-n.m.r. spectrometer (22.5 MHz) operated in the proton-decoupled, ^{13}C mode.

RESULTS

It was found that, on hydrolysis by endodextranase, fraction S dextran that had been produced under the usual conditions by our B-742 CSDC gave large quantities of oligosaccharide products, and the solution, initially opalescent, became clear. When a sample of B-742 S dextran supplied by the NRRC was subjected to hydrolysis with endodextranase under the same conditions, only slight traces of oligosaccharides were detected by t.l.c., and the solution remained opalescent, indicating that high-molecular-weight dextran was not broken down.

After exhaustive treatment with endodextranase, the NRRC sample of B-742 S dextran gave the chromatographic elution patterns in Fig. 1A; these showed that endodextranase hydrolyzes only a small proportion of the NRRC B-742 S dextran.

On the other hand, fraction S dextran produced by our B-742 CSDC under the usual conditions was hydrolyzed to a much greater degree by endodextranase. When a ^{14}C -labeled sample of our fraction S dextran was subjected to the action of endodextranase, 49% of the ^{14}C -labeled material was rendered methanol-soluble. A ^{14}C -labeled sample of our fraction L was also hydrolyzed by endodextranase, to give 42% of the labeled material in methanol-soluble products.

These results were somewhat surprising, considering the values obtained by Seymour *et al.* when these dextrans were analyzed by methylation and g.l.c.-m.s. (16). Their results (see Table I) showed that the NRRC sample of B-742 S contains at least 45-50% of $\alpha\text{-D-(1}\rightarrow\text{3)}$ branches, whereas fraction L contains only 12-15% of branched residues, although the branch linkages in L are $\alpha\text{-D-(1}\rightarrow\text{4)}$.

To further determine the nature of the dextranase breakdown-products of our ^{14}C -dextrans, they were separated by thin-layer chromatography on a plastic-backed, t.l.c. plate, using two ascents in solvent B. Spots containing radioactive material, located by autoradiography, were cut out, and counted for ^{14}C content by liquid scintillation spectrometry. The results, shown in Table II, revealed the presence of considerable amounts

Figure 1. Bio-Gel chromatography of dextranase-treated NRRL B-742 S dextran.

A: Bio-Gel P-6 elution-profile of endodextranase-treated NRRC fraction S dextran. [Column was 1 x 45 cm, fractions were 1.5 mL each. A_{470} was determined after phenol - sulfuric acid assays of fractions; each fraction (50 μ L) was mixed with H₂O (0.45 mL) + 5% phenol (0.5 mL), followed by the rapid addition of sulfuric acid (2.5 mL). The horizontal bar indicates the fractions that were pooled and concentrated for Bio-Gel P-10 chromatography.

B: Bio-Gel P-10 elution-profile of void-volume material from the P-6 column in Fig. 1A. [Column was 1 x 45 cm, fractions were 1.5 mL each. A_{470} was determined after phenol - sulfuric acid assays as in Fig. 1A

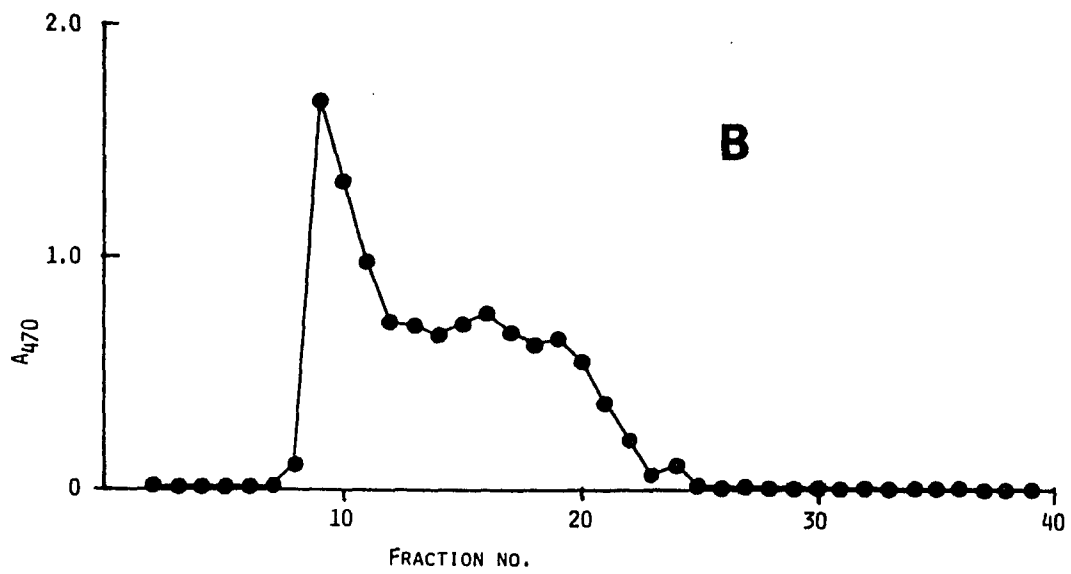
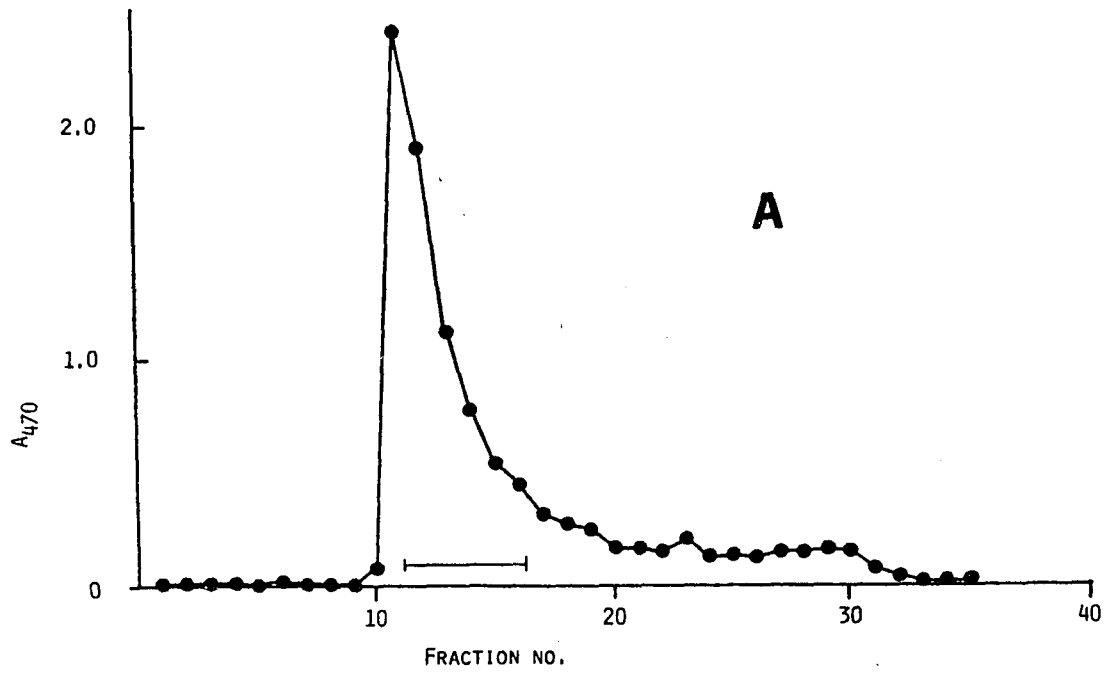


Table II. Product distribution of dextranase-hydrolyzed ^{14}C -dextrans

Product	Radioactivity (% of total)	
	B-742 L	B-742 S
D-Glucose	3.7	4.6
Isomaltose	23.2	19.8
Isomaltotriose	4.8	0.6
B ₄ ^a	2.4	7.8
B ₅	1.9	4.0
B ₆	6.4	6.0
B ₇ -B ₉	17.2	14.4
Origin	40.5	42.8

^aB_n compounds are isomalto-oligosaccharides having n D-glucosyl residues containing α -D-(1 \rightarrow 4) or α -D-(1 \rightarrow 3) branch linkages.

of oligosaccharides arising from both D-glucans. The two showed similar quantities of material at the origin, as well as similar amounts of glucose and isomaltose. The most notable differences appeared in the relative amounts of material in isomaltotriose and in the branched oligosaccharides having four and five D-glucosyl residues.

The products of dextranase hydrolysis were also studied by gel-permeation chromatography. After exhaustive hydrolysis with endodextranase, the product mixture was passed through a column of Bio-Gel P-6. The portion that was eluted in the void volume of the P-6 column was concentrated, and then chromatographed on Bio-Gel P-10. The results (see Fig. 2) showed that, besides the oligosaccharides identified by t.l.c., a high-molecular-weight portion remains after dextranase hydrolysis. This dextranase-resistant fragment is equivalent in size to a globular protein in the range of molecular weight of 6,000 to 20,000, and is polydisperse over this range. This high-molecular-weight fraction is still a good deal smaller than the original dextran (before dextranase treatment). Before hydrolysis, both the L and the S dextrans migrated in the void volume of a column of Bio-Gel A-15m.

^{13}C -N.m.r. spectra of these three dextrans (*i.e.*, the NRRC sample of B-742 S and our CSDC-synthesized B-742 fractions S and L) are shown in Figs. 3 and 4. The important features to note are the relative peak-intensity ratios of the two anomeric-carbon resonances in the spectra of the S fractions (peaks 1 and 2 in Figs. 3 and 4A). The peak-intensity ratio is a reliable measure (17) of the relative contributions due to anomeric carbon atoms linked to O-3 (peak 1) and O-6 (peak 2). In Fig. 3, the ratio is 0.8, whereas, in Fig. 4A, it is only 0.6. This indicates ~45% of branching at O-3 for the NRRC sample of B-742 S, as compared to ~30-35% of branching for our CSDC-prepared fraction S.

On the other hand, the spectrum of our CSDC-prepared B-742 L dextran (Fig. 4B) is almost identical to that obtained by Seymour *et al.* (17) for an NRRC sample of B-742 L, indicating that our B-742 fraction L does not differ significantly from that supplied by the NRRC and described by Seymour *et al.* (17). The ^{13}C -n.m.r. spectrum of the Bio-Gel P-6 void-

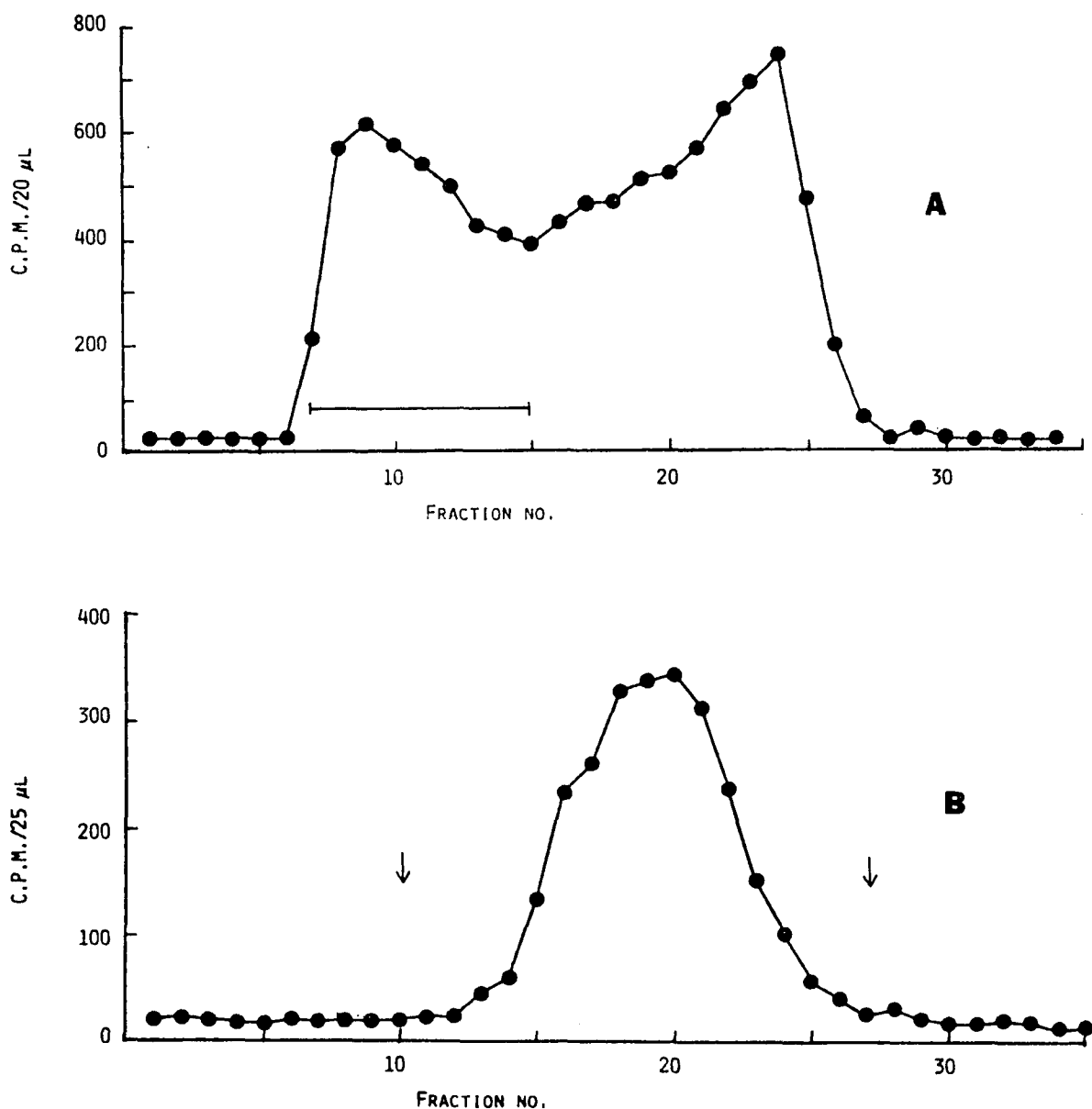


Figure 2. Bio-Gel chromatography of glucans produced by B-742 CSDC following dextranase treatment.

A: Bio-Gel P-6 elution-profile of endodextranase-hydrolyzed, ^{14}C -labeled, CSDC-produced, B-742 fraction S dextran. [Column was 1 x 45 cm, fractions were 1.5 mL each. The horizontal bar indicates those reactions pooled and concentrated for subsequent, Bio-Gel P-10 chromatography.]

B: Bio-Gel P-10 elution-profile of Bio-Gel P-6 void-volume material from column described for Fig. 2A. [Column was 1 x 45 cm, fractions were 1.5 mL each. Arrows indicate void volume and included volume.]

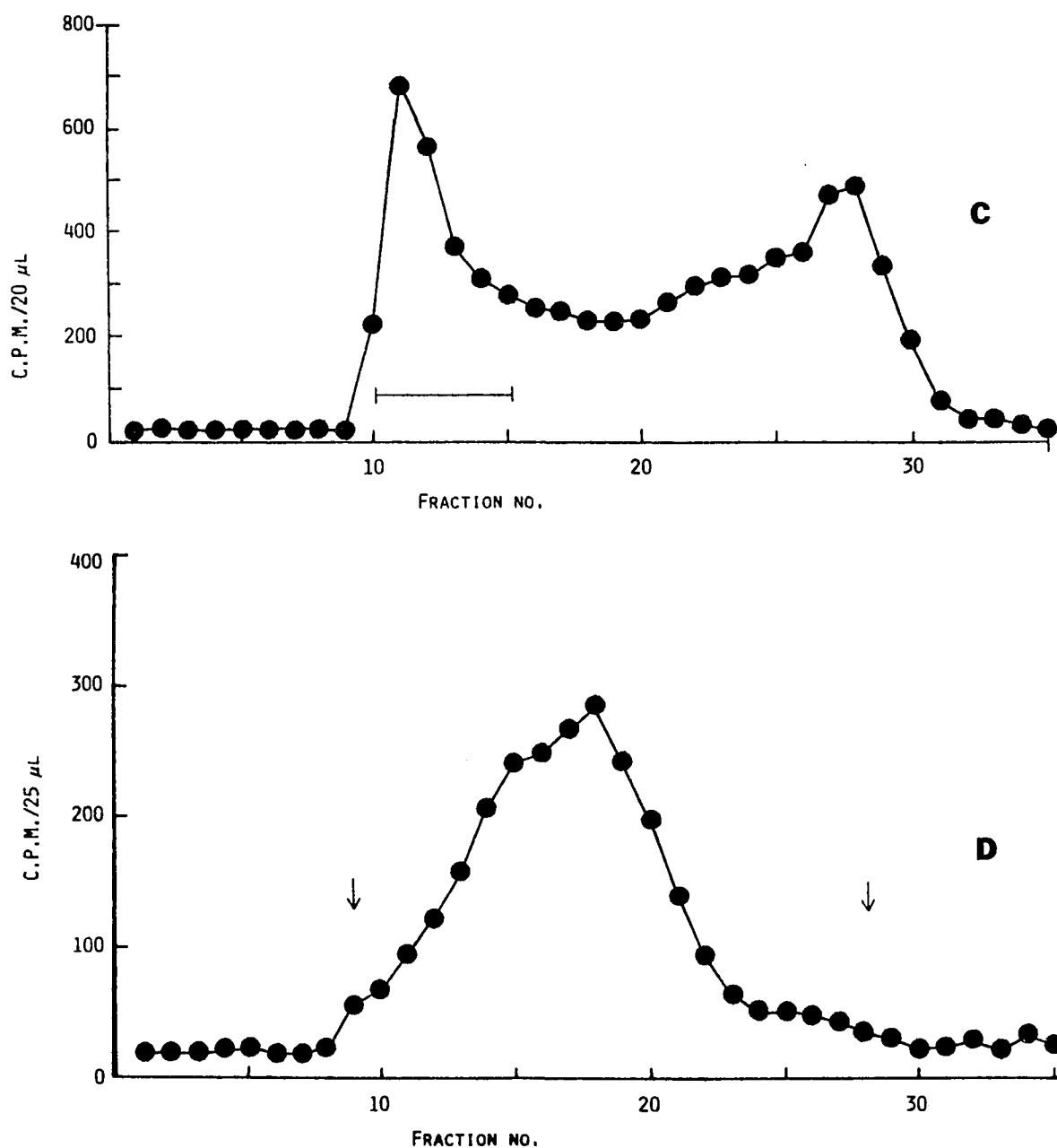


Figure 2 (continued)

C: Bio-Gel P-6 elution-profile of endodextranase-hydrolyzed, C-labeled, CSDC-produced, B-742 fraction L dextran. [Column was 1 x 45 cm, fractions were 1.5 mL each. Horizontal bar indicates those fractions pooled and concentrated for subsequent, Bio-Gel P-10 chromatography.]

D: Bio-Gel P-10 elution-profile of Bio-Gel P-6 void-volume material from column described for Fig. 2C. [Column was 1 x 45 cm and fractions were 1.5 mL each. Arrows indicate void volume and included volume.]

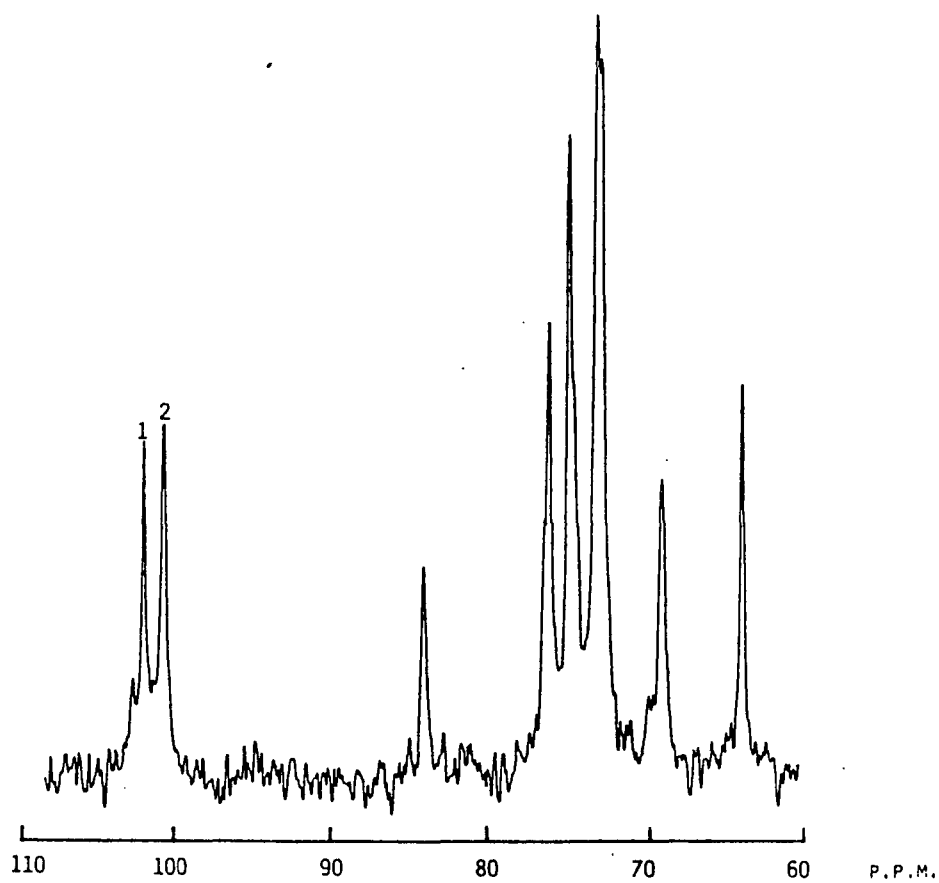


Figure 3. ^1H -Decoupled ^{13}C -n.m.r. spectrum of B-742 fraction S dextran obtained from NRRC. [Chemical shift in p.p.m. with respect to tetramethylsilane.]

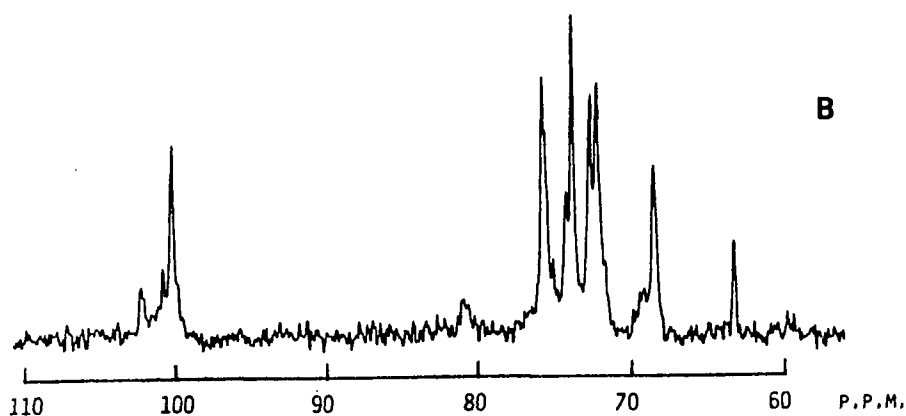
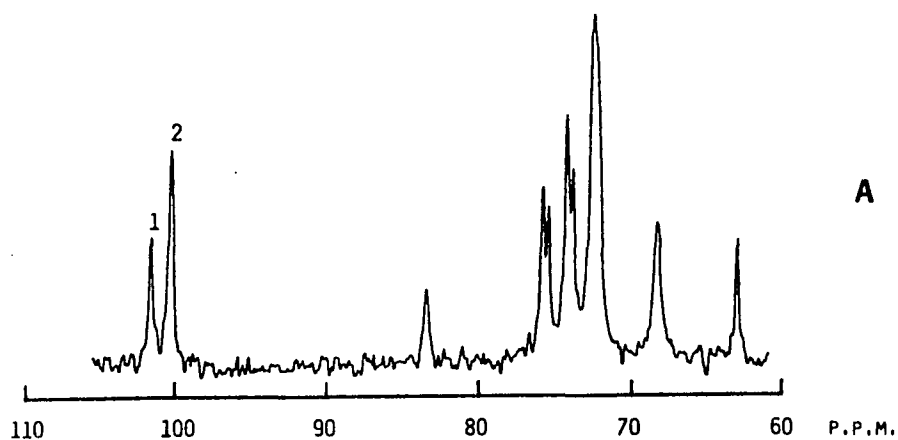


Figure 4. ^1H -Decoupled ^{13}C -n.m.r. spectra of glucans produced by B-742 CSDC.

A: ^1H -Decoupled ^{13}C -n.m.r. spectrum of our CSDC-produced fraction S dextran. [Chemical shift in p.p.m. with respect to tetramethylsilane.]

B: ^1H -Decoupled ^{13}C -n.m.r. spectrum of our CSDC-produced B-742 fraction L dextran. [Chemical shift in p.p.m. with respect to tetramethylsilane.]

volume portion of dextranase-hydrolyzed, CSDC-prepared B-742 S dextran was nearly identical to that depicted in Fig. 3, showing that the proportion of branch points in this dextranase-resistant fragment is higher than that in the original polysaccharide.

In contrast to the S glucan produced by the CSDC under the usual conditions, the D-glucan produced in the dialysis bag had a ^{13}C -n.m.r. spectrum identical to that shown in Fig. 3 for the NRRC sample, indicating that the fraction S glucan formed in the dialysis bag is considerably more branched than that formed under the more usual conditions.

To determine if the higher degree of branching produced by CSDC in the dialysis bag could be attributed to increased acceptor-reactions with less highly branched dextran, the following experiments were performed: ^{14}C -labeled B-742 S dextran (30 mg) produced under the usual conditions was mixed with B-742 CSDC (3 mL) and 2 mL of acetate buffer, pH 5.4, in a dialysis bag; the sealed dialysis bag was then placed in a vessel containing stirred 0.2 M sucrose in buffer (1.5 L); after 2.5 days, the gel that had formed inside the bag was removed, and diluted with water, and the L and S glucans were fractionated by precipitation with ethanol. The S fraction was then subjected to hydrolysis by endodextranase, whereupon it was found that 10% of the ^{14}C -labeled material had been rendered methanol-soluble. Chromatography of this dextranase-hydrolyzed material on Bio-Gel P-6 gave results similar to those shown in Fig. 1. The ^{13}C -n.m.r. spectrum of this S fraction was identical to that in Fig. 3.

An experiment similar to that just described was also performed in which the ^{14}C -labeled acceptor added was the L dextran produced by our B-742 CSDC under the usual conditions. When the products were removed from the dialysis bag, and fractionated by two successive, ethanol precipitations, it was found that 85% of the ^{14}C label was in the L fraction, and 15% in the S fraction. When the L fraction underwent hydrolysis by endodextranase, ~20% of the ^{14}C -labeled material became methanol-soluble. The ^{13}C -n.m.r. spectrum of fraction S was, as before, identical to that in Fig. 3. However, the ^{13}C -n.m.r. spectrum of the L fraction had, in addition to a peak arising from a C-4 atom in an α -linkage, an additional

resonance due to a C-3 atom in an α -linkage (see Fig. 5). The anomeric region also reflected the presence of both linkage types. Apparently, some of the L dextran can act as an acceptor in the formation of α -D-(1 \rightarrow 3) branch linkages under these conditions, and give rise to an S fraction (15%).

In light of these findings, further work was performed in order to determine whether such a D-glucan as B-512F dextran, which contains 95% of α -D-(1 \rightarrow 6) linkages and 5% of α -D-(1 \rightarrow 3) branch linkages, could undergo further branching by acceptor reactions catalyzed by B-742 S dextranase.

A reaction mixture consisting of 0.06 IU of our B-742 CSDC enzyme mixture, 14 C-labeled B-512F dextran (2 mg), and sucrose (30 μ mol) in 0.3 mL of 20 mM pyridine acetate buffer, pH 5.4, with 2 mM calcium chloride and 0.01% of sodium azide, was incubated at room temperature until all of the sucrose had been consumed. Ethanol (2 vol.) was then added, and the precipitated polysaccharide was redissolved in buffer. This mixture of D-glucans was treated with endodextranase, and the solubility of the 14 C-labeled product in methanol was monitored as previously described. It was found that 50% of the labeled material was hydrolyzed, to give methanol-soluble products. In contrast, the original, labeled B-512F dextran was hydrolyzed to give >90% of the label in methanol-insoluble products (see Table III). These results indicated that the B-742 CSDC is capable of so modifying the B-512F dextran as to make it much more resistant to hydrolysis by endodextranase. Bio-Gel P-6 chromatography of the dextranase products arising from the modified, and unmodified, 14 C-labeled B-512F dextrans also supported these findings.

Because the possibility existed that either of the two (or more) enzymes present in the B-742 CSDC could have been responsible for the modification of the B-512F dextran, it was necessary to isolate one, or both, of them free from contamination by the other. It was found that chromatography on (phenoxyacetyl)cellulose could be used to isolate the B-742 L dextranase in the same manner that it had previously been used (1) to isolate alternansucrase from a CSDC preparation from *Leuconostoc mesenter-*

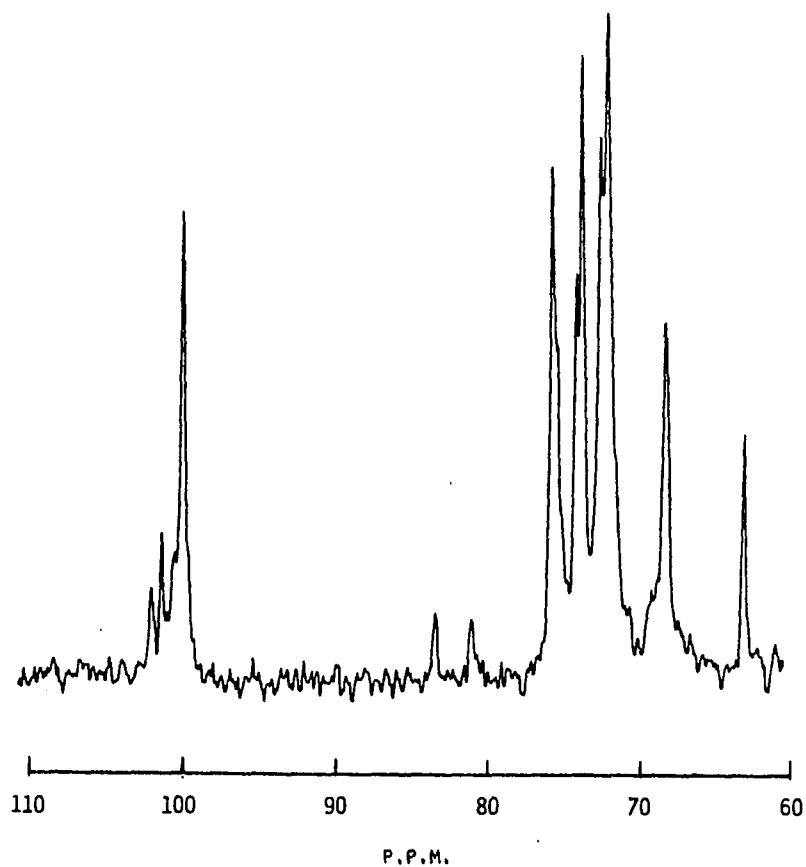


Figure 5. ^1H -Decoupled ^{13}C -n.m.r. spectrum of B-742 fraction L from dialysis-bag synthesis with CSDC and ^{14}C -labeled B-742 L acceptor dextran. [Chemical shift in p.p.m. with respect to tetramethylsilane.]

Table III. Modification of ^{14}C -labeled B-512F dextran
by B-742 acceptor reactions

Enzyme preparation used to modify [^{14}C]-B-512F dextran	% of ^{14}C remaining methanol- insoluble after dextranase hydrolysis
None	7 ± 2
B-742 CSDC	50 ± 3
B-742 L dextransucrase	10 ± 2

oides B-1355.

When a sample of B-742 CSDC was chromatographed on PA-cellulose, there was immediately eluted a nonbinding portion that contained all of the polysaccharide present in the CSDC, but the only glucansucrase activity present in this fraction was that which synthesized fraction L dextran; this was shown by ethanol fractionation (no new S glucan was synthesized) and by the ^{13}C -n.m.r. spectrum of the D-glucan produced by this fraction. The only new D-glucan produced from sucrose by this fraction had a ^{13}C -n.m.r. spectrum identical to that in Fig. 4B. Additional glucansucrase could be eluted from the PA-cellulose column by buffer containing Triton X-100, Tween 80, or sodium taurocholate (see Fig. 6). This detergent-eluted fraction produced both L and S dextrans, with L preponderating, although the ratio was variable. Additional glucansucrase remained bound to the PA-cellulose, even after washing with 2% Triton X-100. Urea (6 M) could be used to remove additional enzyme from the PA-cellulose, but a large proportion remained bound even under those conditions.

The L-producing enzyme was then used for determining whether it could modify B-512F dextran to make it more resistant to dextranase hydrolysis. The experimental procedure was the same as previously outlined, except that B-742 L dextransucrase (PA-cellulose fraction I; fig. 6) was substituted for the B-742 CSDC used in the previous experiment. As is shown

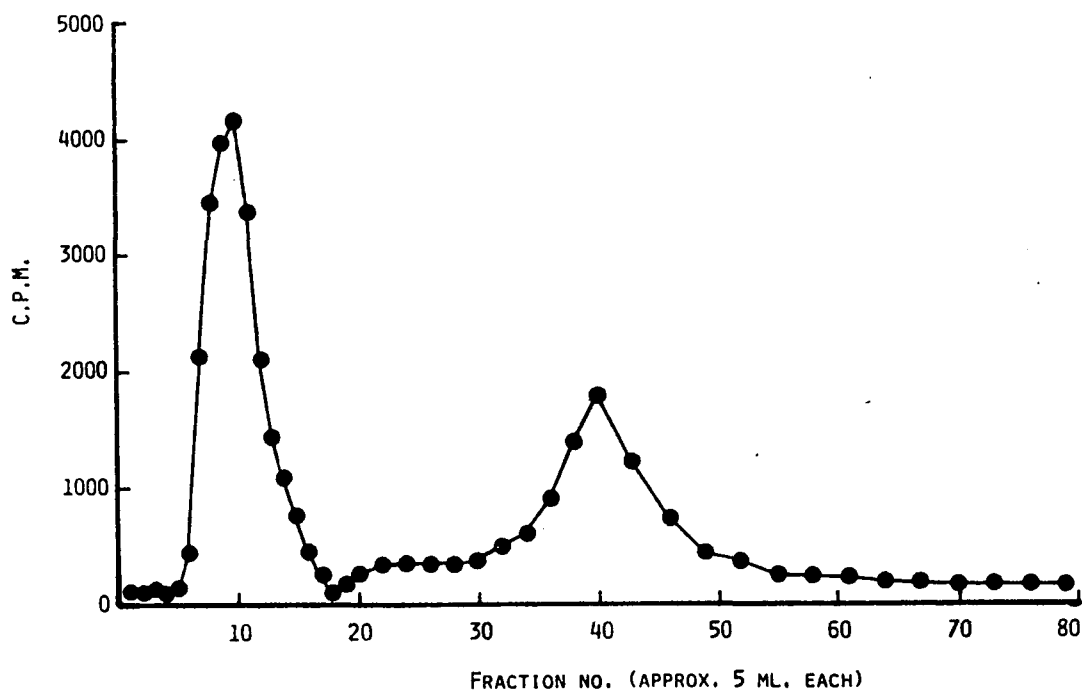


Figure 6. (Phenoxyacetyl)cellulose chromatography of B-742 CSDC. {CSDC (20 mL) was applied to a column (2.5 x 10 cm) of PA-cellulose, and first eluted with 20 mM pyridine acetate buffer, pH 5.3 (fractions 1-17) and then with 1% Triton X-100 in buffer (fractions 18-80). Value in c.p.m. is amount of D-[^{14}C]glucose incorporated into methanol-insoluble D-glucan in the following assay: each fraction (20 μL) was incubated at 24° with 10 μL of 0.3 M [U- ^{14}C]sucrose (108 c.p.m./ μg) in pH 5.3 buffer for 2.5 h. Each assay-mixture (20 μL) was absorbed onto filter-paper squares (1.5 x 1.5 cm), which were then washed with methanol, dried, and counted for ^{14}C in toluene cocktails.}

in Table III, B-512F dextran was not significantly modified by B-742 L dextranase.

Although the B-742 CSDC was shown to modify dextran by synthesizing α -D-(1 \rightarrow 3) branch linkages, only α -D-(1 \rightarrow 6) linkages formed in acceptor reactions with D-glucose, maltose, and isomaltose. This was shown by allowing B-742 CSDC to react with sucrose plus one of the three acceptors, already named, in the same manner as described for B-1355 alternansucrase (2). Thin-layer chromatography of the products showed that only α -D-(1 \rightarrow 6)-linked products were formed, and that these were identical to those formed by B-512F dextranase (28,29). The unusual saccharides characteristic of alternansucrase acceptor-reactions (2), compounds that contain α -D-(1 \rightarrow 3), as well as α -D-(1 \rightarrow 6) linkages, were not detected in the acceptor reactions catalyzed by B-742 CSDC.

DISCUSSION

The history of *Leuconostoc mesenteroides* NRRL strain B-742 can be traced back to 1930, when Hucker and Pederson (5) first isolated it from spoiled canned-tomatoes, and referred to it as *Leuconostoc mesenteroides*, strain 5. Shortly thereafter, Tarr and Hibbert (6) studied the conditions required for optimum production of dextran by this strain, which they referred to as culture 4. Early methylation studies (7,8) of the dextran produced by this strain showed the presence of (1→6) and (1→4) linkages, although it is not known whether the methods used would have distinguished the different types of branched D-glucose residues. It was not until the early 1950s that two separate dextran fractions were isolated from this strain (10,11), and their structures analyzed by periodate oxidation. These early studies indicated 20-26% of α -D-(1→3) branches in fraction S dextran, as well as the 17-24% of α -D-(1→4) branches also found therein. Fraction L was consistently found to contain 18-20% of α -D-(1→4) branches, with no α -D-(1→3) linkages (9-13).

In 1954, in a survey of the dextrans produced by 96 strains of bacteria, Jeanes *et al.* (13) found differences in results obtained from analyses of the B-742 dextrans as compared with earlier studies, and attributed these differences to changes in the organisms over time, and to different conditions of storage and culture. They also described *Leuconostoc mesenteroides* strain B-1142, which had originated from an early isolate of B-742, but had changed with storage in a different culture collection, so that it produced only an S dextran fraction (13). More detailed, structural analyses of the B-742 dextrans did not occur until 1979, when Seymour *et al.* (16) published the methylation data shown in Table I.

In light of this history of variability in the properties of the B-742 dextrans, then, it is not too surprising that the fraction S dextran produced by our enzyme preparation differs from that studied by others. This may be partially attributable to differences in culture conditions, but the conditions under which the dextran is synthesized also play an important role. This observation is not entirely without precedent. For

example, it has been reported that calcium, which is required by B-512F dextransucrase, may play a role in branch formation by another dextransucrase (26,30). It was found that both the pH and the concentration of divalent metal ions influenced the degree of branching in a dextran produced by the dextransucrase from *Leuconostoc mesenteroides* IAM 1046 (31).

Because branch formation by dextransucrase may occur by acceptor reactions with preformed dextran (3,28,32), anything that affects the relative rate of acceptor reactions with dextran should affect the degree of branching. It is for this reason that the dextrans formed in the dialysis-bag experiments are more highly branched. Under the usual circumstances, as dextran is formed from sucrose, D-fructose is released. Acceptor reactions with D-fructose will give rise to leucrose (33), whereas acceptor reactions with dextran give branched dextran (3). Under normal circumstances, the concentrations of D-fructose and dextran both increase as the reaction proceeds. However, in the dialysis bag, the concentration of D-fructose, and, therefore, of leucrose, remains relatively low, because they are able to diffuse out of the bag as soon as they are formed. The concentration of dextran, on the other hand, increases within the bag, and acceptor reactions with this dextran, to give more highly branched dextran, are considerably favored. This is apparently the reason why the B-742 S dextran formed in the dialysis bag is more highly branched. The presence of α -D-(1 \rightarrow 3) branches in the fraction L dextran formed in the dialysis bag can be explained in the same way, *i.e.*, B-742 L dextran, normally a poor acceptor for B-742 S dextransucrase, can act as an acceptor for α -D-(1 \rightarrow 3) branch formation if conditions favor such a reaction with the S dextransucrase. The transfer of α -D-(1 \rightarrow 3) branches to B-742 L dextran would also explain why some workers have observed the presence of small proportions of α -D-(1 \rightarrow 3) linkages in fraction L (15,16,34).

The results in Table III show that the dextransucrase that forms B-742 fraction S dextran is capable of transferring D-glucosyl groups from sucrose to an α -(1 \rightarrow 6)-linked D-glucan, to form α -D-(1 \rightarrow 3) branches. It is the presence of a high percentage of branched residues that makes branched

dextrans more resistant to hydrolysis by endodextranase.

These results indicate that branch formation by dextransucrase can be attributed to acceptor reactions, even for such a highly branched dextran as B-742 S. It is possible, then, that the only major difference between a dextransucrase that forms a dextran having few branches, such as B-512F, and one that forms a highly branched dextran, such as B-742 S, is the size, or the stereochemistry, of the site to which acceptors bind, or the affinity of such a site for polymeric acceptors. An increased affinity for unbranched dextran and an increased rate of acceptor reactions with dextrans, relative to the rate of chain elongation by D-glucosyl insertion (35), would result in more, as well as shorter, branches. Thus, the more highly branched is a dextran, the shorter would be the average branch length. For a dextran having a high degree of branching, such as B-742 S, all of the branches would be single D-glucosyl groups, whereas a dextran such as B-512F would have a considerable number of longer branch-chains.

The structure of B-742 fraction S dextran, then, is determined to some extent by the conditions of synthesis. It is probably a comb-like polymer, as suggested by Seymour *et al.* (16), but the "comb" may have "missing teeth", *viz.*, unbranched D-glucosyl residues within the main chain. The extent of branching may, thus, vary. The distribution of branches seems to be fairly random, as indicated by the distribution of hydrolysis products after dextranase treatment. This is what would be expected if the branching resulted from acceptor reactions.

The dextran fraction L, which we did not study in as much detail as fraction S, differs in some respects from the more commonly studied dextrans. These differences seem to arise from the presence of α -D-(1 \rightarrow 4) branch linkages. It is possible that an α -D-(1 \rightarrow 4) branch point imparts to the dextran chain a conformation different from that given by an α -D-(1 \rightarrow 3) branch point; this was first suggested by Torii *et al.* (36) to explain the "anomalous" behavior of B-742 fraction L with certain carbohydrate-binding proteins (36,37). They stated that inspection of molecular models showed that an α -D-(1 \rightarrow 4) branch causes a perturbation in the conformation of the

dextran chain compared to that of unbranched or α -D-(1 \rightarrow 3) branched dextrans. This perturbation may be responsible for rendering a greater portion of the dextran molecule inaccessible to dextran-binding proteins, whether they be immunoglobulins or endodextranases. This appears to be the most likely explanation for the fact that B-742 L dextran, with only 12-14% of α -D-(1 \rightarrow 4) branching, is hydrolyzed by endodextranase to the same extent as our CSDC-produced B-742 S dextran, having >30% of α -D-(1 \rightarrow 3) branching.

The lower affinity of B-742 L dextransucrase for (phenoxyacetyl)cellulose, compared to that of the S dextransucrase, is similar to that in the B-1355 system (1), where the enzyme which was bound more tightly to the PA-cellulose was the one that formed the α -D-(1 \rightarrow 6) glucan having α -D-(1 \rightarrow 3) branch linkages.

We have observed that the structure of our CSDC-produced, B-742 fraction S dextran bears a number of similarities to the soluble D-glucan formed by an extracellular glucansucrase (GTF-S) from *Streptococcus mutans* 6715. Preliminary results suggest that branch formation by the streptococcal enzyme may proceed in a manner similar to that of the *Leuconostoc mesenteroides* B-742 S dextransucrase.

ACKNOWLEDGMENTS

We thank Mr. Steve Eklund for his valuable technical assistance in recording the n.m.r. spectra, and Dr. Morey E. Slodki of the Northern Regional Research Center in Peoria, Illinois, for supplying us with dextran samples from *Leuconostoc mesenteroides* B-742 and B-1142.

REFERENCES

1. G. L. Côté and J. F. Robyt. Isolation and characterization of an extracellular glucansucrase from *Leuconostoc mesenteroides* NRRL B-1355 that synthesizes an alternating (1→6), (1→3)- α -D-glucan. Carbohydr. Res. 101, 57-74 (1982).
2. G. L. Côté and J. F. Robyt. Acceptor reactions of alternansucrase from *Leuconostoc mesenteroides* NRRL B-1355. Carbohydr. Res. 111, 127-142 (1982).
3. J. F. Robyt and H. Taniguchi. The mechanism of dextransucrase action. Biosynthesis of branch linkages by acceptor reactions with dextran. Arch. Biochem. Biophys. 174, 129-135 (1976).
4. C. A. Wilham, B. H. Alexander, and A. Jeanes. Heterogeneity in dextran preparations. Arch. Biochem. Biophys. 59, 61-75 (1955).
5. G. J. Hucker and C. S. Pederson. Studies on the coccaceae. XVI. The genus *Leuconostoc*. N. Y. State Agric. Expt. Station, Geneva, N. Y., Tech. Bull. 167, 3-80 (1930).
6. H. L. A. Tarr and H. Hibbert. Studies on reactions relating to carbohydrates and polysaccharides. XXXVII. The formation of dextran by *Leuconostoc mesenteroides*. Can. J. Res. 5, 414-427 (1931).
7. F. L. Fowler, I. K. Buckland, F. E. Brauns, and H. Hibbert. Studies on the reactions relating to carbohydrates and polysaccharides. LII. Structure of the dextran synthesized by the action of *Leuconostoc mesenteroides*. Can. J. Res. 15B, 486-497 (1937).
8. I. Levi, W. L. Hawkins, and H. Hibbert. Studies on reactions relating to carbohydrates and polysaccharides. LXVI. Structure of the dextran synthesized by the action of *Leuconostoc mesenteroides* on sucrose. J. Amer. Chem. Soc. 64, 1959-1962 (1942).
9. A. Jeanes and C. A. Wilham. Periodate oxidation of dextran. J. Amer. Chem. Soc. 72, 2655-2657 (1950).
10. A. Jeanes and C. A. Wilham. Dextran triacetates. J. Amer. Chem. Soc. 74, 5339-5341 (1952).
11. R. Lohmar. Evidence of new linkages in dextrans. J. Amer. Chem. Soc. 74, 4974 (1952).
12. J. W. Sloan, B. H. Alexander, R. L. Lohmar, I. A. Wolff, and C. E. Rist. Determination of dextran structure by periodate oxidation techniques. J. Amer. Chem. Soc. 76, 4429-4434 (1954).
13. A. Jeanes, W. C. Haynes, C. A. Wilham, J. C. Rankin, E. H. Melvin, M. J. Austin, J. E. Cluskey, B. E. Fisher, H. M. Tsuchiya, and C. E. Rist. Characterization and classification of dextrans from ninety-six strains of bacteria. J. Amer. Chem. Soc. 76, 5041-5052 (1954).

14. W. M. Pasika and L. H. Cragg. The detection and estimation of branching in dextran by proton magnetic resonance spectroscopy. *Can. J. Chem.* 41, 293-299 (1963).
15. H. Suzuki and E. J. Hehre. The differentiation of serotype A and B dextrans by partial acetolysis. *Arch. Biochem. Biophys.* 104, 305-313 (1964).
16. F. R. Seymour, E. C. M. Chen, and S. H. Bishop. Methylation structural analysis of unusual dextrans by combined gas-liquid chromatography - mass spectrometry. *Carbohydr. Res.* 68, 113-121 (1979).
17. F. R. Seymour, R. D. Knapp, S. H. Bishop, and A. Jeanes. High-temperature enhancement of ^{13}C -n.m.r. chemical shifts of unusual dextrans and correlation with methylation structural analysis. *Carbohydr. Res.* 68, 123-140 (1979).
18. F. R. Seymour, R. D. Knapp, and S. H. Bishop. ^{13}C -Spin-lattice relaxation studies for resonance assignment to specific, carbon positions of dextrans. *Carbohydr. Res.* 72, 229-234 (1979).
19. A. Jeanes and F. R. Seymour. The α -D-glucopyranosidic linkages of dextrans: comparison of percentages from structural analysis by periodate oxidation and by methylation. *Carbohydr. Res.* 74, 31-40 (1979).
20. F. R. Seymour, R. D. Knapp, and S. H. Bishop. Correlation of the structure of dextrans to their ^1H -n.m.r. spectra. *Carbohydr. Res.* 74, 77-92 (1979).
21. F. R. Seymour, R. D. Knapp, E. C. M. Chen, A. Jeanes, and S. H. Bishop. Structural analysis of dextrans containing 4-O- α -D-glucosylated α -D-glucopyranosyl residues at the branch points, by use of ^{13}C -nuclear magnetic resonance spectroscopy and gas-liquid chromatography-mass spectrometry. *Carbohydr. Res.* 75, 275-294 (1979).
22. F. R. Seymour and R. D. Knapp. Structural analysis of α -D-glucans by ^{13}C -nuclear magnetic resonance, spin-lattice relaxation studies. *Carbohydr. Res.* 81, 67-103 (1980).
23. F. R. Seymour and R. D. Knapp. Structural analysis of dextrans, from strains of *Leuconostoc* and related genera, that contain 3-O- α -D-glucosylated α -D-glucopyranosyl residues at the branch points, or in consecutive, linear positions. *Carbohydr. Res.* 81, 105-129 (1980).
24. F. R. Seymour, R. L. Julian, A. Jeanes, and B. L. Lamberts. Structural analysis of insoluble D-glucans by Fourier-transform-infrared difference spectrometry: correlation between structures of dextrans from strains of *Leuconostoc mesenteroides* and of D-glucans from strains of *Streptococcus mutans*. *Carbohydr. Res.* 86, 227-246 (1980).
25. E. J. Hehre. Polysaccharide synthesis from disaccharides. *Methods Enzymol.* 1, 178-180 (1955).

26. J. F. Robyt and T. F. Walseth. Production, purification, and properties of dextransucrase from *Leuconostoc mesenteroides* NRRL B-512F. Carbohydr. Res. 68, 95-111 (1979).
27. G. R. Germaine, C. F. Schachtele, and A. M. Chludzinski. Rapid filter paper assay for the dextransucrase activity from *Streptococcus mutans*. J. Dent. Res. 53, 1355-1360 (1974).
28. J. F. Robyt and T. F. Walseth. The mechanism of acceptor reactions of *Leuconostoc mesenteroides* NRRL B-512F dextransucrase. Carbohydr. Res. 61, 433-445 (1978).
29. H. J. Koepsell, H. M. Tsuchiya, N. N. Hellmann, A. Kazenko, C. A. Hoffman, E. S. Sharpe, and R. W. Jackson. Enzymatic synthesis of dextran. Acceptor specificity and chain initiation. J. Biol. Chem. 200, 793-801 (1953).
30. G. R. Lawford, A. Kligerman, and T. Williams. Dextran biosynthesis and dextransucrase production by continuous culture of *Leuconostoc mesenteroides*. Biotechnol. Bioeng. 21, 1121-1131 (1979).
31. Y. Tsumuraya, N. Nakamura, and T. Kobayashi. Dextransucrase, and the role of metallic ions in the formation of branch links in dextran. Agric. Biol. Chem. 40, 1471-1477 (1976).
32. K. H. Ebert and M. Brosche. Origin of branches in native dextran. Biopolymers 5, 423-430 (1967).
33. F. H. Stodola, E. S. Sharpe, and H. J. Koepsell. The preparation, properties, and structure of the disaccharide leucrose. J. Amer. Chem. Soc. 78, 2514-2518 (1956).
34. T. Sawai, T. Tohyama, and T. Natsume. Hydrolysis of fourteen native dextrans by *Arthrobacter* isomaltodextranase, and correlation with dextran structure. Carbohydr. Res. 66, 195-205 (1978).
35. J. F. Robyt, B. K. Kimble, and T. F. Walseth. The mechanism of dextransucrase action. Direction of dextran biosynthesis. Arch. Biochem. Biophys. 165, 634-640 (1974).
36. M. Torii, E. A. Kabat, and H. Weigel. Immunochemical studies on dextrans. IV. Further characterization of the determinant groups on various dextrans involved in their reactions with the homologous human antidextrans. J. Immunol. 96, 797-805 (1966).
37. A. M. Wu, E. A. Kabat, and M. G. Weigert. Immunochemical studies on dextran-specific and levan-specific myeloma proteins from NZB mice. Carbohydr. Res. 66, 113-124 (1978).

SECTION IV

DISPROPORTIONATION REACTIONS CATALYZED BY
Leuconostoc AND *Streptococcus* GLUCANSUCRASES

Thomas P. Binder, Gregory L. Côté, and John F. Robyt
Department of Biochemistry and Biophysics
Iowa State University, Ames, Iowa 50011

ABSTRACT

Glucansucrases from *Leuconostoc mesenteroides* NRRL B-512F and *Streptococcus mutans* 6715 were found to utilize a number of D-glucosyl oligosaccharides as D-glucosyl donors as well as acceptors. These donors included isomaltotriose and its homologues, as well as panose, maltotriose, and dextran. In each case, D-glucosyl units were transferred from the donor to an acceptor sugar. When the donor sugar also acted as an acceptor, disproportionation reactions occurred. Isomaltotriose, for example, gave rise to isomaltose and isomaltotetraose initially, and to a series of isomalto oligosaccharides eventually. In addition to forming α -(1 \rightarrow 6) linkages in these reactions, dextransucrase from *S. mutans* 6715 was also capable of forming α -(1 \rightarrow 3)-linked products.

INTRODUCTION

Glucansucrases are glucosyltransferases which catalyze the synthesis of D-glucans from sucrose. *Leuconostoc mesenteroides* NRRL B-512F dextran-sucrase synthesizes a dextran which has 95% α -(1 \rightarrow 6) linkages and 5% α -(1 \rightarrow 3) branch linkages (1). *Streptococcus mutans* 6715 produces two D-glucans from sucrose, a water-soluble dextran containing 73% α -(1 \rightarrow 6) linkages and 27% α -(1 \rightarrow 3) branch linkages (2), and a water-insoluble glucan ("mutan") which contains 93% α -(1 \rightarrow 3) linkages and 7% α -(1 \rightarrow 6) linkages (3). The enzymes which synthesize the soluble and insoluble glucans are usually referred to as GTF-S and GTF-I, respectively (4,5).

Although sucrose is the usual glucosyl donor for these enzymes, other substrates can also act as glucosyl donors. These include α -D-glucopyranosyl fluoride (6), α -D-glucopyranosyl- α -L-sorbofuranoside (7), and 4^F-O- α -D-galactopyranosylsucrose (8).

Reactions in which glycosyl units are transferred from one saccharide donor to identical or similar saccharide acceptors are known as disproportionation reactions (*e.g.*, 2 maltose \rightleftharpoons glucose + maltotriose), and have been observed for such enzymes as cellulase (9), amylase (10), glucoamylase (11), transglucosylase (12), glucodextranase (11), and isomaltodextranase (13). Disproportionation reactions catalyzed by glucansucrases, however, are not well known, and have been overlooked by most investigators and reviewers (14,15).

In control studies on acceptor specificities of purified *L. mesenteroides* NRRL B-512F dextran-sucrase and *S. mutans* 6715 GTF-S, we have observed that a number of saccharides can act as D-glucosyl donors as well as acceptors.

MATERIALS AND METHODS

Carbohydrates

Isomaltooligosaccharides were prepared by partial acid hydrolysis of commercial B-512F dextran. Fifteen grams of dextran (Sigma Chemical Co., St. Louis, MO) were dissolved in 500 mL of 0.3 M trifluoroacetic acid and heated on a steam bath for approximately two to three h. Individual oligosaccharides were isolated from the resulting mixture by charcoal column chromatography (16). Panose was prepared by the acceptor reaction of dextransucrase with sucrose and maltose (17). Maltotriose was prepared by the method of French *et al.* (18). Maltose was purchased from EM Laboratories, Elmsford, NY; T-10 dextran was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden; and methyl α -D-glucopyranoside was recrystallized from a preparation purchased from Eastman Kodak Co., Rochester, NY. Planteose (19) and isopanose (20) were obtained from the laboratories of the late Professor Dexter French. Turanose and melibiose were purchased from Sigma Chemical Co., and melezitose was purchased from Nutritional Biochemicals Corp., Cleveland, OH.

Enzymes

Streptococcus mutans 6715 was grown on the medium described by Ciardi *et al.* (21). After removal of cells and concentration of the filtrate using a Pellicon membrane cassette filtration system (Millipore Corp., Bedford, MA), the exocellular glucansucrases were separated and purified by the following, successive steps: Bio-Gel A-15m chromatography (2); DEAE-cellulose chromatography (22) in 20 mM pH 6.8 sodium phosphate buffer, using a 0 to 0.2 M gradient of sodium chloride; DEAE-Bio-Gel A chromatography (23), using the same buffer and gradient; and Sephadex G-50 affinity chromatography (23). The final enzyme preparation contained 45 IU of GTF-S per mg of protein when assayed in the presence of 3.3 mg/mL of T-10 dextran.

Leuconostoc mesenteroides B-512F dextransucrase was purified by the

method of Miller and Robyt (24); briefly, this entailed DEAE-cellulose chromatography, Sephadex G-200 affinity chromatography, and DEAE-Trisacryl chromatography. The specific activity was 76 IU/mg.

Chromatography

Oligosaccharides were separated and analyzed by thin-layer chromatography on Whatman K5F silica gel plates in one of the following solvents: A, 1-propanol - nitromethane - acetonitrile - water, 5:2:4:4 (v/v/v/v), or B, acetonitrile - water, 17:3 (v/v). The sugars were visualized by spraying the plates with 20% (vol.) sulfuric acid in methanol, followed by heating to 120° for 10 min.

Sephadex was purchased from Pharmacia Fine Chemicals, Bio-Gel was purchased from Bio-Rad Laboratories (Richmond, CA), and DEAE-cellulose was purchased from Sigma Chemical Co. DEAE-Trisacryl was purchased from LKB Instruments, Inc. (Gaithersburg, MD).

Reaction Conditions

All reactions were carried out at 30° in 50 mM, pH 5.5, sodium acetate buffer, containing 0.02% sodium azide, unless stated otherwise.

Oligosaccharide disproportionation reactions were carried out by reacting 0.1 mL of 0.1 M saccharide with 0.1 mL of enzyme, which contained approximately 5 IU/mL of *S. mutans* 6715 GTF-S, or 10 IU/mL of *L. mesenteroides* B-512F dextransucrase. Reactions in the presence of sucrose contained 0.1 mL of 0.1 M oligosaccharide, 90 µL of one of the enzymes described, and 10 µL of 1.0 M sucrose. The reactions between dextran and methyl α-D-glucoside contained 116 µL of enzyme, 17 µL of 0.6 M methyl α-D-glucoside, and 67 µL of a solution containing 150 mg/mL of T-10 dextran.

RESULTS

Both *Streptococcus mutans* 6715 GTF-S and *Leuconostoc mesenteroides* B-512F dextranucrase were found to disproportionate isomaltodextrins. Figures 1 and 2 show that the first products to appear from the reaction with isomaltotriose are isomaltose and isomaltotetraose. After further reaction, higher d.p. isomaltooligosaccharides appear which differ from one to the next by a single D-glucosyl unit. Eventually, after prolonged reaction times, D-glucose is also produced in small amounts. Figure 3 shows that the products formed in the presence of sucrose are the same as those formed in the absence of sucrose, but the rate of formation of these products is much faster when sucrose is the D-glucosyl donor. It should be noted that the two enzymes both form the same products (isomaltooligosaccharides), even though the glucans formed by each differ significantly in their structures. It was observed, however, that after much longer incubation times, the *S. mutans* GTF-S made small amounts of other products, which may contain linkages other than α -(1 \rightarrow 6).

On the other hand, the products formed by the disproportionation of panose (4-O- α -isomaltosyl-D-glucose) differ between the two enzymes. Figure 4 shows that *L. mesenteroides* B-512F dextranucrase forms a series of 4-O- α -isomaltodextrinyl-D-glucose oligosaccharides from panose, by transferring α -D-glucosyl units one at a time; maltose is also released as a result of the removal of the nonreducing terminal D-glucose residue from panose. The products formed in the presence of sucrose (Fig. 5) are the same as those formed in the absence of sucrose.

The products formed by *S. mutans* 6715 GTF-S, however, differ from those formed by B-512F dextranucrase. Figure 4 shows that, in addition to maltose and 4-O- α -isomaltodextrinyl-D-glucose oligosaccharides, smaller amounts of other oligosaccharides are also formed. These other products are not as prominent, however, when sucrose is present (Fig. 5). The identities of these oligosaccharides are not presently known, but we believe they contain α -(1 \rightarrow 3) as well as α -(1 \rightarrow 6) linkages.

The ability of dextranucrase to utilize panose as a D-glucosyl donor

Figure 1. Thin layer chromatogram of products arising from *Streptococcus mutans* 6715 GTF-S action on isomaltotriose. Two ascents in solvent A, at 37°. S indicates a series of isomalto-oligosaccharides, used as standards. D.P. refers to the degree of polymerization of each of the isomaltooligosaccharides. Reaction times, from left to right: t = 0, 5 min, 30 min, 3 h, 18 h, 2 days, 4 days, 8 days, and 11 days. Five μL of each reaction mixture was chromatographed

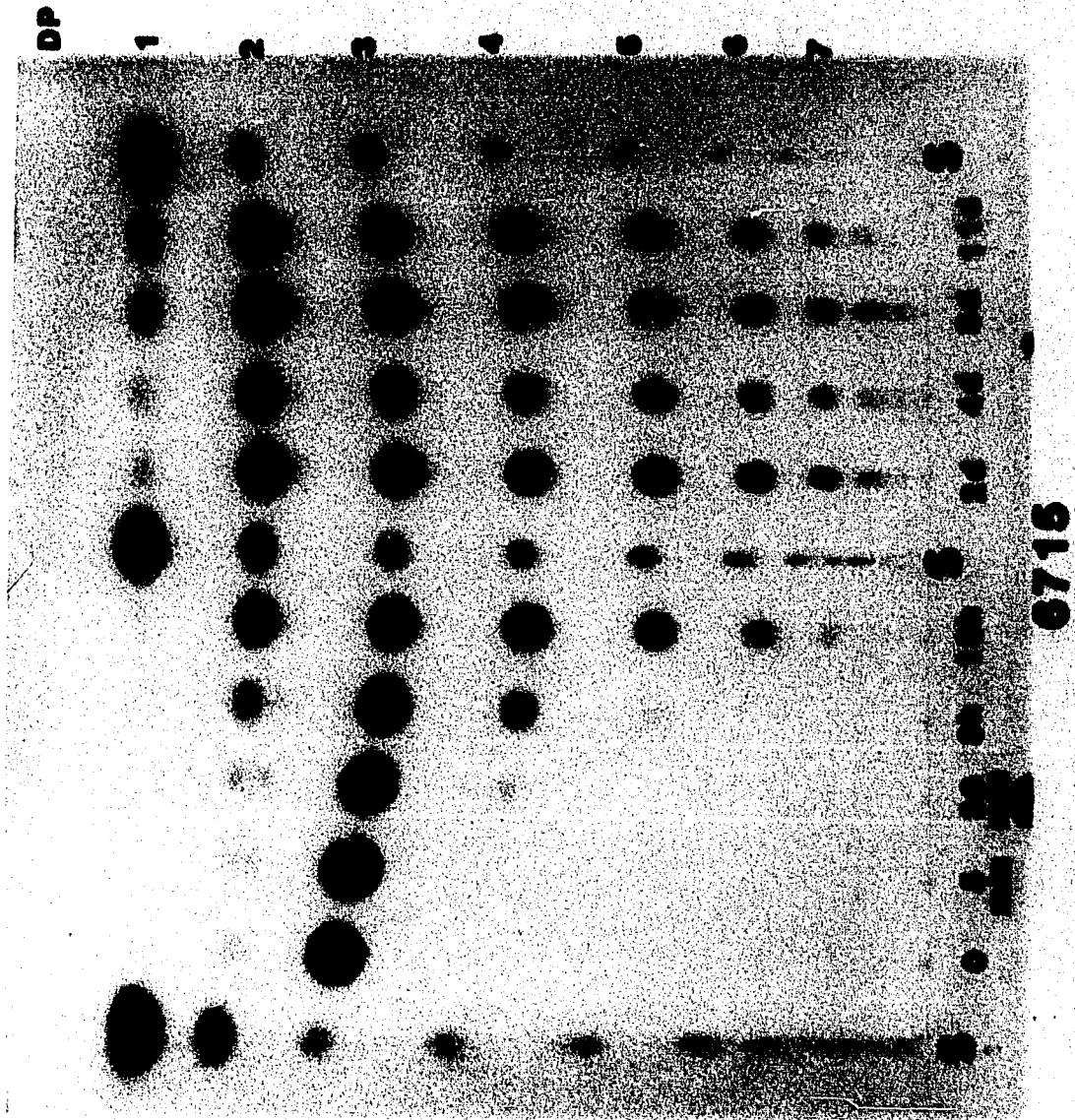


Figure 2. Thin layer chromatogram of products arising from *Leuconostoc mesenteroides* B-512F dextran-sucrase action on isomaltotriose. For details, see Fig. 1



Figure 3. Thin layer chromatogram of products arising from the action of dextransucrases on isomaltotriose in the presence of sucrose. S refers to isomaltooligosaccharide standards. Left side of plate: *Streptococcus mutans* 6715 GTF-S reaction mixture; time points taken at t = 0, 30 min, 2.5 h, and 24 h. Right side of plate: *Leuconostoc mesenteroides* B-512F dextransucrase action on isomaltotriose in the presence of sucrose. Time points same as for *S. mutans*. Five μ L of each mixture was chromatographed for two ascents at 37° in solvent A

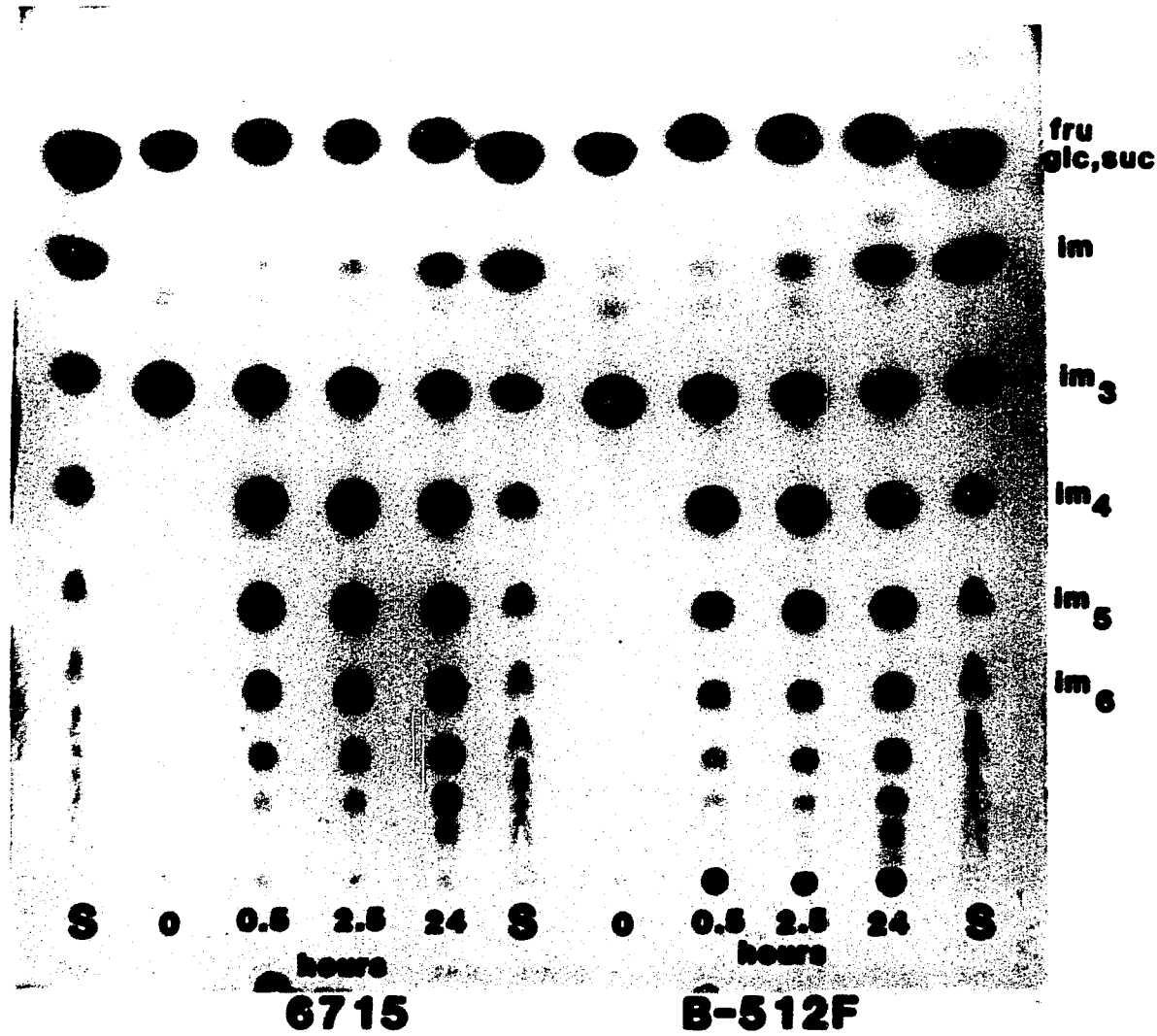


Figure 4. Thin layer chromatogram of products arising from the action of dextransucrases on panose. S refers to isomaltooligosaccharide standards. Left side of plate: *Streptococcus mutans* 6715 GTF-S reaction mixture; time points taken as $t = 0, 2.5 \text{ h}, 24 \text{ h}, \text{ and } 150 \text{ h}$. Right side of plate: *Leuconostoc mesenteroides* B-512F dextransucrase reaction mixture; same time points as for *S. mutans*. Five μL of each mixture was chromatographed for two ascents at 37° in solvent A

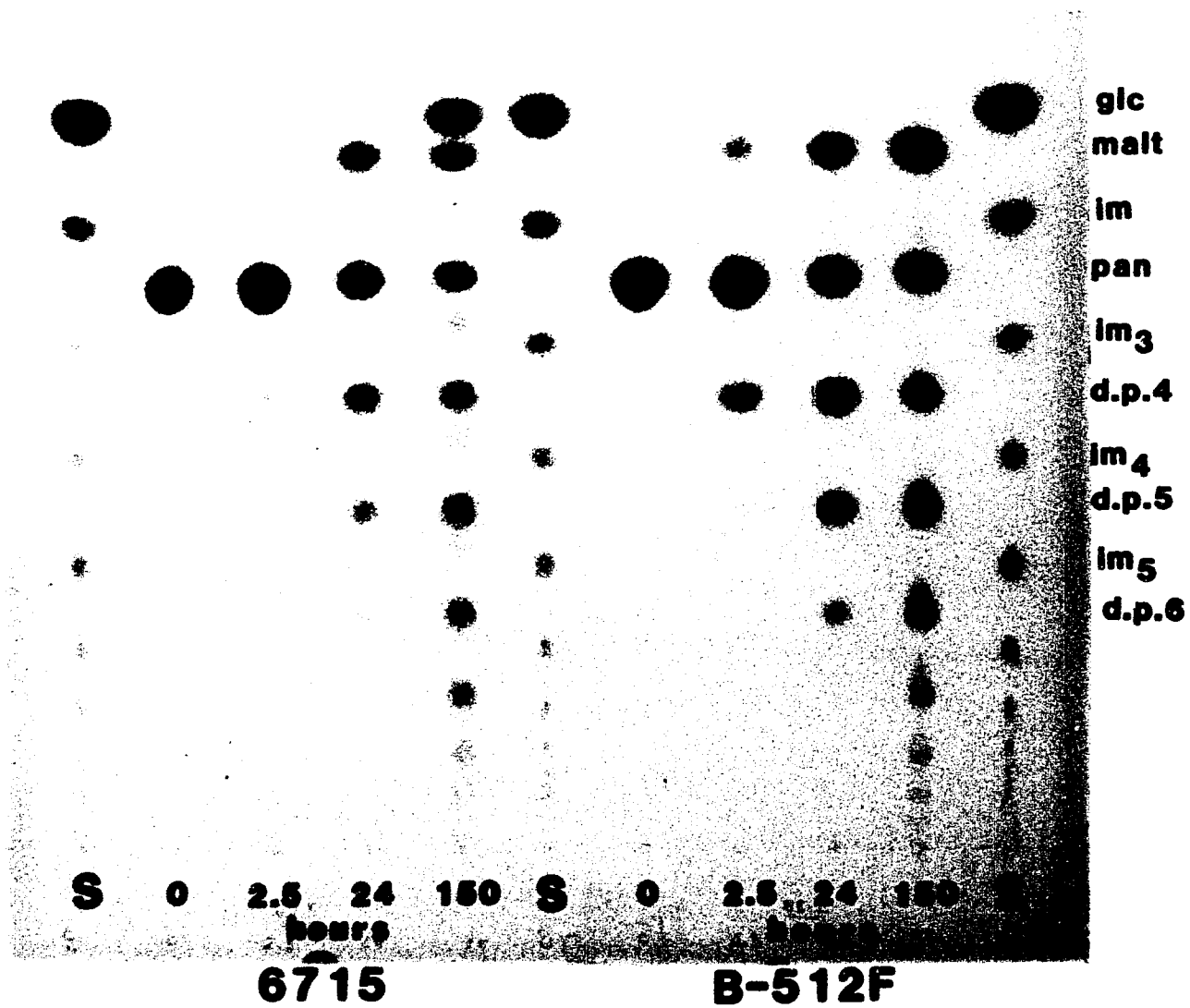
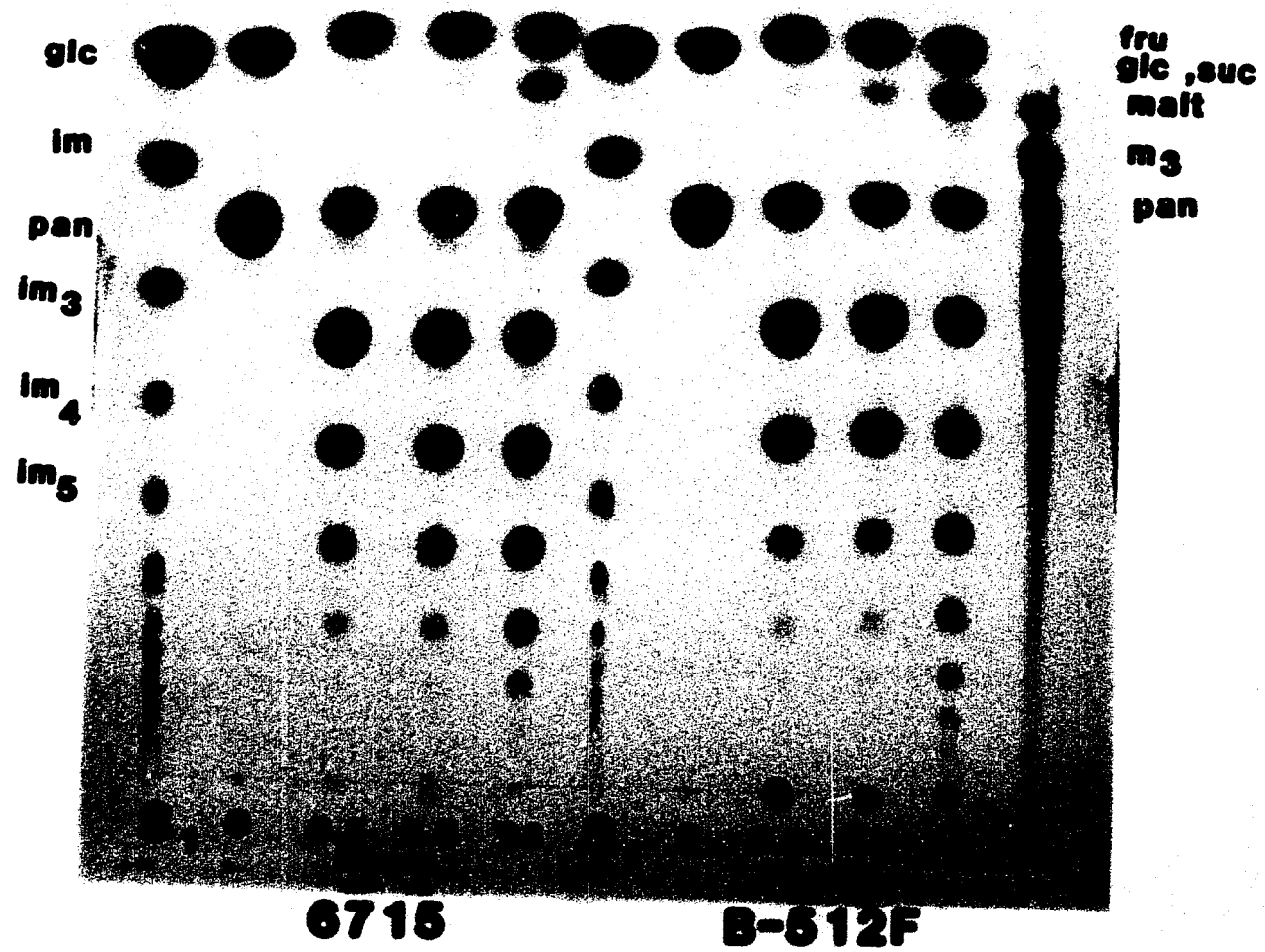


Figure 5. Thin layer chromatogram of products arising from the action of dextransucrases on panose in the presence of sucrose. S₁ refers to isomaltooligosaccharide standards, S₂ refers to maltooligosaccharide standards. Left side of plate: *Streptococcus mutans* 6715 GTF-S reaction mixture; time points taken at t = 0, 30 min, 2.5 h, and 24 h. Right side of plate: *Leuconostoc mesenteroides* B-512F reaction mixture; same time points as for *S. mutans*. Five µL of each mixture was chromatographed for two ascents at 37° in solvent A



suggests a certain amount of flexibility in the enzyme's substrate binding site. Further evidence for this is provided by the observation that both of the enzymes studied are capable of acting on maltotriose (Fig. 6). Figure 6 also shows that the two enzymes differ in their specificity of action on maltotriose. *S. mutans* 6715 GTF-S acts on this substrate to give D-glucose, maltose, and a higher d.p. oligosaccharide of unknown structure, while *L. mesenteroides* B-512F dextransucrase forms maltose and a higher d.p. oligosaccharide which differs from that produced by GTF-S. After a prolonged reaction time, a second, minor product also appears and migrates on t.l.c. slightly ahead of the major, higher d.p. product from B-512F dextransucrase. It is important to note that maltotriose alone does not give rise to a series of higher isomaltodextrinyl oligosaccharides.

The major products of *S. mutans* 6715 GTF-S action on maltotriose are D-glucose, maltose, and a tetrasaccharide (Fig. 6). The formation of D-glucose occurs when water acts as an acceptor instead of maltotriose to give hydrolysis rather than disproportionation. This is not the case when the enzymes are incubated with both sucrose and maltotriose. Figure 7 shows that different products are formed when sucrose acts as the D-glucosyl donor. In addition to the two saccharides that are formed when sucrose is absent, *L. mesenteroides* B-512F dextransucrase reacts with sucrose and maltotriose to give a homologous series, which ostensibly are isomaltodextrinyl saccharides with maltotriose at the reducing end. No maltose was detected.

The GTF-S reaction with maltotriose and sucrose gives, in addition to the products noted in the absence of sucrose, a series of oligosaccharides, which appear to be the same as those produced by B-512F dextransucrase. Maltose, however, is also released by GTF-S. It should be noted that the relative amounts of the higher saccharides differ between the two enzymes, with GTF-S giving more of the higher d.p. products just ahead of the origin, while B-512F dextransucrase gives lesser amounts of these, compared to greater amounts of those of d.p. approximately six to eight (Fig. 7).

Perhaps most notably, it was found that both of these glucosyltrans-

Figure 6. Thin layer chromatogram of products arising from the action of dextransucrases on maltotriose. S refers to isomaltooligosaccharide standards. Left side of plate: *Streptococcus mutans* 6715 GTF-S reaction mixture; time points taken at $t = 0$, 2.5 h, 24 h, and 150 h. Right side of plate: *Leuconostoc mesenteroides* B-512F dextransucrase reaction mixture; time points same as for *S. mutans*. Five μL of each mixture was chromatographed for two ascents at 37° in solvent A

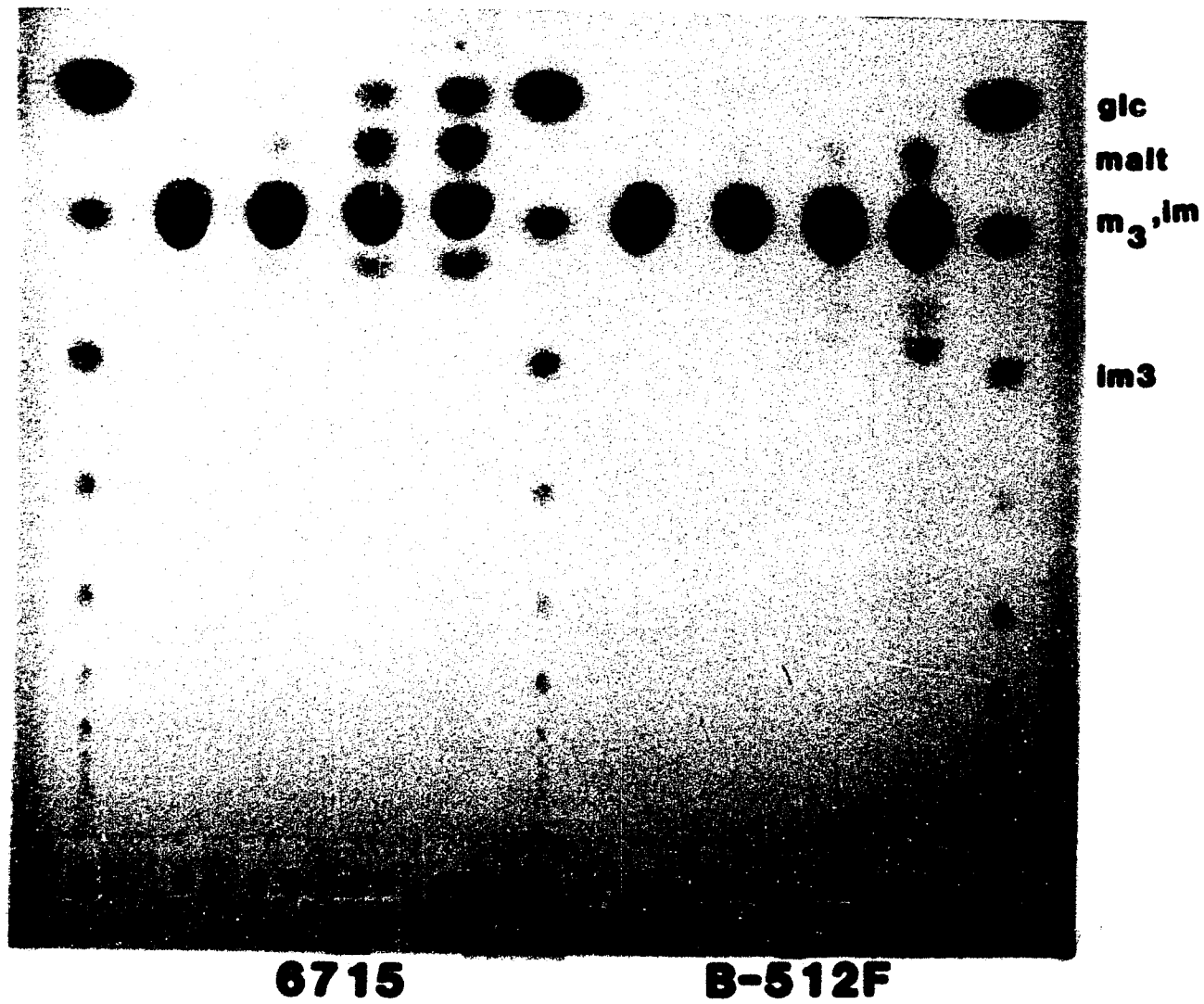
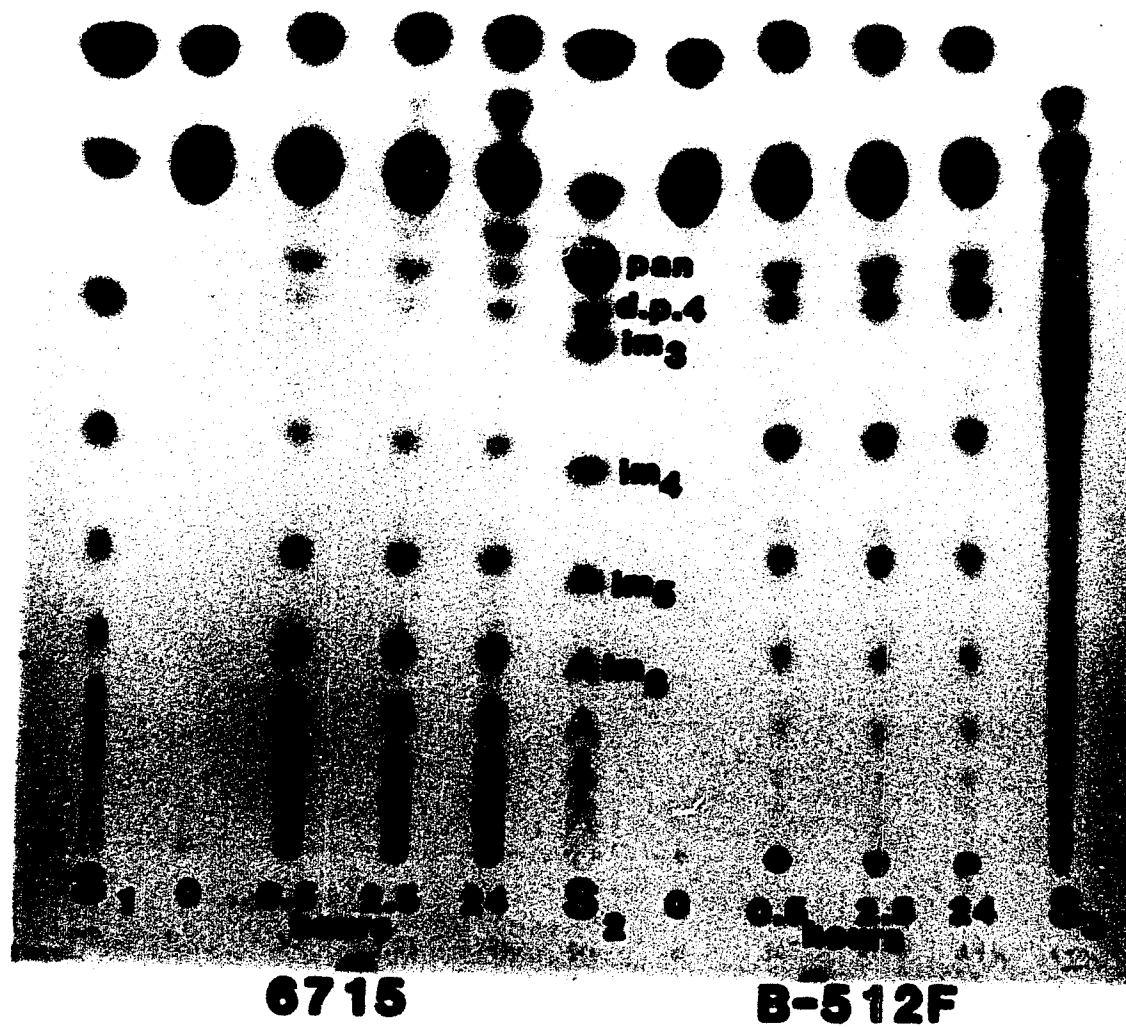


Figure 7. Thin layer chromatogram of products arising from the action of dextransucrases on maltotriose in the presence of sucrose. S₁ refers to isomaltooligosaccharide standards, S₃ refers to maltooligosaccharide standards, and S₂ refers to the following standards (top to bottom): D-glucose, isomaltose, panose, 4²-O- α -isomaltosyl maltose, isomaltotriose, and higher d.p. isomaltooligosaccharides. Left side of plate: *Streptococcus mutans* 6715 GTF-S reaction mixture; time points taken at t = 0, 30 min, 2.5 h, and 24 h. Right side of plate: *Leuconostoc mesenteroides* B-512F dextransucrase reaction mixture; time points same as for *S. mutans*. Five μ L of each reaction mixture was chromatographed for two ascents at 37° in solvent A



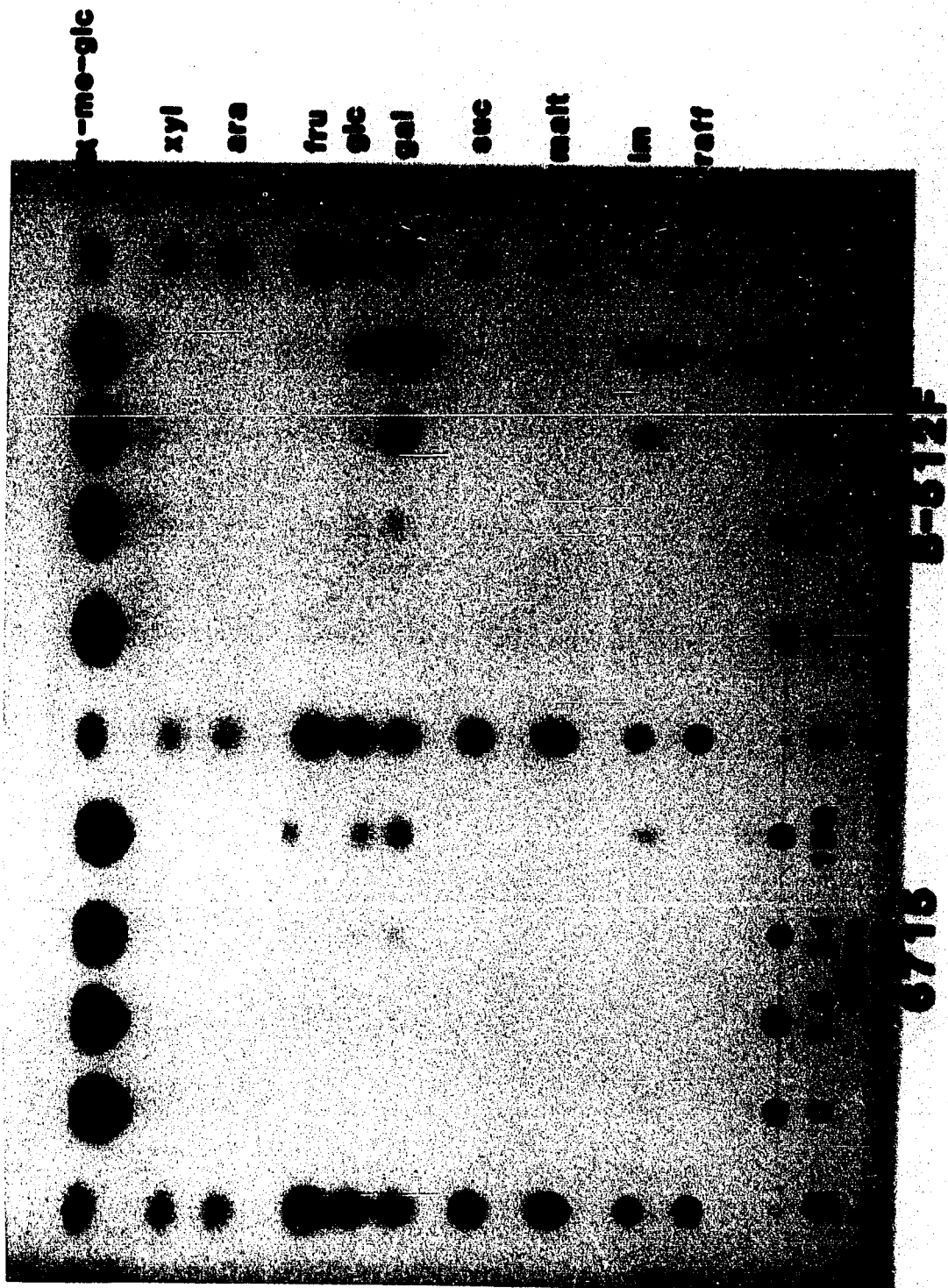
ferases were capable of transferring D-glucosyl units from a predominantly α -(1 \rightarrow 6)-linked dextran to an acceptor sugar. Figure 8 shows that both glucansucrases transfer single D-glucosyl residues from a B-512F clinical-sized dextran (average molecular weight about 10,000) to methyl α -D-glucopyranoside. B-512F dextransucrase gives rise to methyl α -isomaltoside in 24 and 150 h as judged by mobility on t.l.c. *S. mutans* 6715 GTF-S reacts more slowly to give the same products as well as another, faster-moving disaccharide, most probably methyl α -nigeroside (see Fig. 8). On prolonged incubation, D-glucose and higher d.p. products were also observed. D-Glucose was identified by its chromatographic mobility and by detection on t.l.c. by orthonilic acid (25), which did not reveal the methyl α -glycosides.

When D-fructose was present in enzyme digests where dextran or isomaltooligosaccharides served as D-glucosyl donors, leucrose was formed (data not shown), just as it is when sucrose acts as the D-glucosyl donor (26).

A number of other sugars were also tested as D-glucosyl donors, but were found to be relatively unreactive compared to the previously described substrates. Planteose, turanose, and melezitose gave only very slight traces of products after prolonged incubation with large amounts of enzyme, while melibiose, isopanose, and 6,6'-dideoxy-6,6'-difluorosucrose (27) gave no products at all.

We have also found that *S. mutans* 6715 GTF-I ("mutansucrase") is capable of catalyzing similar types of transfer reactions as described for GTF-S, but we have not investigated them in detail.

Figure 8. Thin layer chromatogram of products arising from the action of dextransucrases on B-512F clinical dextran in the presence of methyl α -D-glucoside. S refers to the following standards (top to bottom): methyl α -D-glucoside, D-xylose, L-arabinose, D-fructose, D-glucose, D-galactose, sucrose, maltose, isomaltose, and raffinose. Left side of plate: *Streptococcus mutans* 6715 GTF-S reaction mixture; time points taken at t = 0, 2.5 h, 24 h, and 150 h. Right side of plate: *Leuconostoc mesenteroides* B-512F dextransucrase reaction mixture; time points same as for *S. mutans*. Five μ L of each mixture was chromatographed at 25° for three ascents in solvent B



DISCUSSION

Disproportionation reactions catalyzed by dextransucrase were observed in our laboratory during a study of acceptor reactions with oligosaccharides of d.p. ≥ 3 . These reactions were observed with both glucansucrase preparations, regardless of purity. The fact that the reaction products were the same as those produced by acceptor reactions suggested that this phenomenon was due to glucansucrase itself.

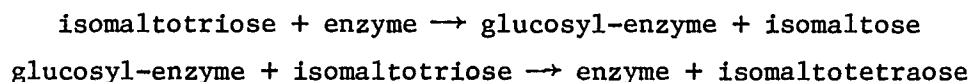
Over two decades ago, Tsuchiya (28) reported similar observations while working with a relatively crude preparation of dextransucrase from *Leuconostoc mesenteroides* B-512F. His results have been greeted with some degree of skepticism since then, and have been interpreted by some as evidence of the presence of a contaminating glycosidase activity. The issue was further clouded by Tsuchiya's own observation that his preparation also contained levansucrase activity (28). Since then, no other reports have appeared which confirm his findings. In fact, Kobayashi and Matsuda (29) reported that their highly purified dextransucrase from *L. mesenteroides* B-512F did not act on isomaltotetraose, although it did act on raffinose. These findings differ from ours, but this may be due to the fact that they examined a dextransucrase fraction which did not bind to Sephadex, while our enzyme binds tightly to Sephadex and could be released by 3 M urea (24). Walker (30) has described a dextransucrase preparation from *Streptococcus mutans* OMZ 176 which she reported as having no hydrolytic action on isomaltooligosaccharides, but she did not state how this was determined. If a reducing-value assay was employed, it should be realized that disproportionation itself would not give any increase in reducing value except after very long reaction times, during which a significant amount of D-glucose would eventually be produced.

It is unlikely that the type of D-glucosyl transfer that we have observed is due to an endodextranase. *S. mutans* endodextranase is incapable of transferring single D-glucosyl units (31-34). Instead, the initial products of *S. mutans* endodextranase action on dextran are isomaltotriose, isomaltotetraose, and isomaltopentaose (4,31-34). Streptococcal endodex-

tranase is also incapable of hydrolyzing maltotriose (35); compare this with Fig. 6. The patterns of products seen with our glucansucrase preparations are not consistent with those that are characteristic of endodextranase action.

It is also unlikely that these reactions are due to exodextranase or α -glucosidase activities, since these enzymes are predominantly endocellular in these particular organisms (33,36), while our enzyme preparations are derived from the exocellular culture fluid. In addition, had an exodextranase or α -glucosidase been present, isomaltose would have been hydrolyzed faster than dextran or isomaltodextrins (33,37), but just the opposite was found. Also, the endocellular glycosidases would have been expected to act on such saccharides as isopanose, turanose, melezitose, planteose, and melibiose, but only a trace of such activity was observed. Furthermore, *S. mutans* 6715 GTF-S prepared as described is reported to lack any detectable contaminating activity of this sort (2,22,23). Likewise, invertase action could not account for these reactions, since *S. mutans* invertase is a β -D-fructofuranosidase (38,39).

The proposed mechanism for the observed disproportionation reaction is the formation of an enzyme-glucosyl intermediate from the substrate and the subsequent displacement of the glucosyl group by an acceptor to give disproportionation of the substrate:



Although the disproportionation reactions are slow compared to D-glucosyl transfer from sucrose, they are sufficiently rapid so as to be of concern in many instances. For example, our results with isomaltotriose indicate that this saccharide can undergo disproportionation in the presence of sucrose to yield isomaltose, which is produced even before all of the sucrose has been consumed. Thus, the rate of formation and the distribution of certain products not only depend on the transfer of D-glucosyl units from sucrose, but also on the transfer of D-glucosyl residues to and from the products themselves. The transfer of D-glucosyl residues from one dextran chain to another, although not directly observed, may be of con-

siderable interest if it is found that this does occur. This is especially interesting in light of our findings that branching in dextrans can occur by acceptor reactions (40,41).

The findings described herein support those of Tsuchiya (28) that dextran and α -(1 \rightarrow 6)-linked D-glucose oligosaccharides can act as D-glucosyl donors for B-512F dextransucrase. We have also shown that these reactions can be catalyzed by a streptococcal glucansucrase, as well as by *L. mesenteroides* B-512F dextransucrase. These reactions show that, in addition to acting as acceptors, a rather wide variety of D-glucooligosaccharides can also act as D-glucosyl donors. This adds further evidence to support the idea that the active site of dextransucrase is flexible enough, or nonspecific enough, to bind to, and catalyze transfer to and from, a number of different substrates. This was first suggested for dextransucrase by Neely (42), who found that partially denatured *L. mesenteroides* B-512F dextransucrase was capable of disproportionating maltose into glucose and a trisaccharide, probably panose.

It is not known how these reactions would affect the findings of others (43-45) with respect to the specificity of dextransucrase action in the presence of isomaltooligosaccharides, but the question warrants consideration.

REFERENCES

1. J. W. Van Cleve, W. C. Schaefer, and C. E. Rist. The structure of NRRL B-512 dextran. Methylation studies. *J. Amer. Chem. Soc.* 78, 4435-4438 (1956).
2. A. Shimamura, H. Tsumori, and H. Mukasa. Purification and properties of *Streptococcus mutans* extracellular glucosyltransferase. *Biochim. Biophys. Acta* 702, 72-80 (1982).
3. K. Fukui, T. Moriyama, Y. Miyake, K. Mizutani, and O. Tanaka. Purification and properties of glucosyltransferase responsible for water-insoluble glucan synthesis from *Streptococcus mutans*. *Infect. Immun.* 37, 1-9 (1982).
4. M. D. Hare, S. Svensson, and G. J. Walker. Characterization of the extracellular water-insoluble α -D-glucans of oral streptococci by methylation analysis and by enzymic synthesis and degradation. *Carbohydr. Res.* 66, 245-264 (1978).
5. J. F. Robyt and P. J. Martin. Mechanism of synthesis of D-glucans by D-glucosyltransferases from *Streptococcus mutans* 6715. *Carbohydr. Res.* 113, 301-315 (1983).
6. D. S. Genghof and E. J. Hehre. *De novo* glycosidic synthesis by glycosylases: α -D-glucosyl fluoride polymerization by dextransucrase. *Proc. Soc. Exp. Biol. Med.* 140, 1298-1301 (1972).
7. J. C. Mazza, A. Akgerman, and J. R. Edwards. Synthesis of α -D-glucopyranosyl α -L-sorbofuranoside and its use as a D-glucosyl donor. *Carbohydr. Res.* 40, 402-406 (1975).
8. E. J. Hehre and H. Suzuki. New reactions of dextransucrase: α -D-glucosyl transfers to and from the anomeric sites of lactulose and fructose. *Arch. Biochem. Biophys.* 113, 675-683 (1966).
9. T. Kanda, I. Noda, K. Wakabayashi, and K. Nisizawa. Transglucosylation activities of exo- and endo-type cellulases from *Irpes lacteus* (*Polyporus tulipiferae*). *J. Biochem.* 93, 787-794 (1983).
10. D. French. Cyclodextrin transglycosylase (*Bacillus macerans* amylase). *Methods Enzymol.* 5, 148-155 (1962).
11. S. Kitihata, C. F. Brewer, D. S. Genghof, T. Sawai, and E. J. Hehre. Scope and mechanism of carbohydrase action. Stereocomplementary hydrolytic and glucosyl-transferring actions of glucoamylase and glucodextranase with α - and β -D-glucosyl fluoride. *J. Biol. Chem.* 256, 6017-6026 (1981).
12. J. H. Pazur, T. Budovich, and C. L. Tipton. The enzymatic synthesis and disproportionation of 3-O- α -D-glucopyranosyl-D-glucose. *J. Amer. Chem. Soc.* 79, 625-628 (1957).

13. T. Sawai and Y. Niwa. Transisomaltosylation activity of a bacterial isomaltodextranase. *Agric. Biol. Chem.* 39, 1077-1083 (1975).
14. R. L. Sidebotham. Dextrans. *Adv. Carbohydr. Chem. Biochem.* 30, 371-444 (1974).
15. T. J. Montville, C. L. Cooney, and A. J. Sinskey. *Streptococcus mutans* dextranase: a review. *Adv. Appl. Microbiol.* 24, 55-84 (1978).
16. W. J. Whelan. Isomaltodextrins. Partial acid hydrolysis of dextran. *Methods Carbohydr. Chem.* 1, 321-324 (1962).
17. M. Killey, R. J. Dimler, and J. E. Cluskey. Preparation of panose by the action of NRRL B-512 dextranase on a sucrose-maltose mixture. *J. Amer. Chem. Soc.* 77, 3315-3318 (1955).
18. D. French, J. F. Robyt, M. Weintraub, and P. Knock. Separation of maltodextrins by charcoal chromatography. *J. Chromatogr.* 24, 68-75 (1966).
19. D. French. The raffinose family of oligosaccharides. *Adv. Carbohydr. Chem.* 9, 149-184 (1954).
20. J. H. Pazur and T. Ando. The hydrolysis of glucosyl oligosaccharides with α -D-(1 \rightarrow 4) and α -D-(1 \rightarrow 6) bonds by fungal amyloglucosidase. *J. Biol. Chem.* 235, 297-302 (1960).
21. J. E. Ciardi, A. J. Beaman, and C. L. Wittenberger. Purification, resolution, and interaction of the glucosyltransferases of *Streptococcus mutans* 6715. *Infect. Immun.* 18, 237-246 (1977).
22. G. R. Germaine, S. K. Harlander, W.-L. S. Leung, and C. F. Schachtele. *Streptococcus mutans* dextranase: functioning of primer dextran and endogenous dextranase in water-soluble and water-insoluble glucan synthesis. *Infect. Immun.* 16, 637-648 (1977).
23. R. M. Hamelik and M. M. McCabe. An endodextranase inhibitor from batch cultures of *Streptococcus mutans*. *Biochem. Biophys. Res. Commun.* 106, 875-880 (1982).
24. A. W. Miller and J. F. Robyt, Iowa State University, unpublished.
25. F. Iinuma, Y. Hiraga, T. Kinoshita, and M. Watanabe. Simultaneous fluorimetric and colorimetric detection of carbohydrates on silica gel plates using *o*-aminobenzenesulfonic acid. *Chem. Pharm. Bull.* 27, 1268-1271 (1979).
26. F. H. Stodola, E. S. Sharpe, and H. J. Koepsell. The preparation, properties, and structure of the disaccharide leucrose. *J. Amer. Chem. Soc.* 78, 2514-2518 (1956).
27. J. N. Zikopoulos, S. H. Eklund, and J. F. Robyt. Synthesis of 6,6'-dideoxy-6,6'-difluorosucrose. *Carbohydr. Res.* 104, 245-251 (1982).
28. H. M. Tsuchiya. Dextranase. *Bull. Soc. Chim. Biol.* 42, 1777-1788 (1960).

29. M. Kobayashi and K. Matsuda. Characterization of the multiple forms and main component of dextransucrase from *Leuconostoc mesenteroides* NRRL B-512F. *Biochim. Biophys. Acta* 614, 46-62 (1980).
30. G. J. Walker. Preparation of isomalto-cligosaccharides labelled with ^{14}C in the non-reducing terminal unit, and their use in studies of dextranase activity. *Carbohydr. Res.* 30, 1-10 (1973).
31. D. W. Ellis and C. H. Miller. Extracellular dextran hydrolase from *Streptococcus mutans* strain 6715. *J. Dent. Res.* 56, 57-69 (1977).
32. A. Pulkownik and G. J. Walker. Purification and substrate specificity of an endo-dextranase of *Streptococcus mutans* K1R. *Carbohydr. Res.* 54, 237-251 (1977).
33. G. J. Walker, A. Pulkownik, and J. G. Morrey-Jones. Metabolism of the polysaccharides of human dental plaque: release of dextranase in batch cultures of *Streptococcus mutans*. *J. Gen. Microbiol.* 127, 201-208 (1981).
34. A. Pulkownik, J. A. Thoma, and G. J. Walker. The action pattern and subsite map of *Streptococcus mutans* K1R dextranase. *Carbohydr. Res.* 61, 493-497 (1978).
35. G. J. Walker and A. Pulkownik. Action of α -1,6-glucan glucohydrolase on oligosaccharides derived from dextran. *Carbohydr. Res.* 36, 53-66 (1974).
36. R. W. Bailey and E. J. Bourne. Intracellular glycosidases of dextran-producing bacteria. *Nature* 191, 277-278 (1961).
37. M. D. Dewar and G. J. Walker. Metabolism of the polysaccharides of human dental plaque. I. Dextranase activity of streptococci, and the extracellular polysaccharides synthesized from sucrose. *Caries Res.* 9, 21-35 (1975).
38. K. Fukui, Y. Fukui, and T. Moriyama. Purification and properties of dextransucrase and invertase from *Streptococcus mutans*. *J. Bacteriol.* 118, 796-804 (1973).
39. M. M. McCabe and E. E. Smith. Invertase activity in *Streptococcus mutans* and *Streptococcus sanguis*. *Arch. Oral Biol.* 18, 525-531 (1973).
40. G. L. Côté and J. F. Robyt. The formation of α -(1 \rightarrow 3) branch linkages by an exocellular glucansucrase from *Leuconostoc mesenteroides* NRRL B-742. *Carbohydr. Res.* 119, 141-156 (1983).
41. J. F. Robyt and H. Taniguchi. The mechanism of dextransucrase action. Biosynthesis of branch linkages by acceptor reactions with dextran. *Arch. Biochem. Biophys.* 174, 129-135 (1976).
42. W. B. Neely. Studies on the enzyme dextransucrase. IV. Altering the substrate specificity pattern of the enzyme. *J. Amer. Chem. Soc.* 81, 4416-4418 (1959).

43. G. R. Germaine, A. M. Chludzinski, and C. F. Schachtele. *Streptococcus mutans* dextransucrase: requirement for primer dextran. J. Bacteriol. 120, 287-294 (1974).
44. G. J. Walker. Branching-enzyme activity of an α -D-glucosyltransferase of *Streptococcus mutans*. Carbohydr. Res. 82, 404-410 (1980).
45. T. Koga, S. Sato, M. Inoue, K. Takeuchi, T. Furuta, and S. Hamada. Role of primers in glucan synthesis by glucosyltransferases from *Streptococcus mutans* strain OMZ 176. J. Gen. Microbiol. 129, 751-754 (1983).

SECTION V.

THE FORMATION OF α -(1 \rightarrow 3) BRANCH LINKAGES
BY A SOLUBLE-GLUCAN-PRODUCING GLUCANSUCRASE
FROM *Streptococcus mutans* 6715

Gregory L. Côté and John F. Robyt
Department of Biochemistry and Biophysics
Iowa State University, Ames, Iowa 50011

ABSTRACT

An exocellular glucansucrase which synthesizes a water-soluble α -(1 \rightarrow 6)-linked glucan with a high amount of α -(1 \rightarrow 3) branches was purified from the culture broth of *Streptococcus mutans* 6715. The rate of incorporation of ^{14}C -glucose from ^{14}C -sucrose into high-molecular-weight glucan by this enzyme was increased (stimulated) by the presence of exogenous *Leuconostoc mesenteroides* B-512F dextran, and it was found that this dextran could act as an acceptor. A highly branched dextran, containing 45-50% α -(1 \rightarrow 3) branch linkages, did not stimulate the enzyme nearly as much as B-512F dextran, which has a low degree (5%) of α -(1 \rightarrow 3) branches. We interpret this as evidence that the stimulating effects of dextran are not due to priming. If they were, the more highly branched dextran should have produced the greatest stimulation per unit weight, because a much greater number of nonreducing-end, priming sites would be available. We show that the glucansucrase was capable of transferring glucosyl units from sucrose to B-512F dextran to form α -(1 \rightarrow 3) branches, thereby rendering the dextran more resistant to hydrolysis by endodextranase. The presence of 1.6 M ammonium sulfate caused the enzyme to synthesize a glucan with a much higher percentage of α -(1 \rightarrow 3) linkages.

INTRODUCTION

Streptococcus mutans has been shown to be the primary organism responsible for dental caries (1,2). Its ability to form insoluble plaque on tooth surfaces is attributed to its production of exocellular polysaccharides, which mediate cell-cell and cell-tooth adherence (1,2). Most cariogenic strains of *S. mutans* produce two different exocellular polysaccharides, a water-insoluble glucan, linked predominantly α -(1 \rightarrow 3), and a water-soluble glucan, linked predominantly α -(1 \rightarrow 6) with varying amounts of α -(1 \rightarrow 3) branch linkages (1-17). Both glucans are synthesized from sucrose by exocellular enzymes known as glucansucrases (1), which are often referred to as GTF-I (glucosyltransferase-insoluble), which produces the water-insoluble glucan, and GTF-S (glucosyltransferase-soluble), which produces the water-soluble glucan (12,18). The two glucansucrases can interact with either or both of the two glucans, so that in a mixture of the two enzymes, a wide range of polysaccharide structures may be possible (1,5,6,10,12,18-33). In addition, *S. mutans* produces an exocellular dextranase which can further affect the composition and structure of the glucans produced (22,34-38).

This paper describes our studies on the mechanism of formation of α -(1 \rightarrow 3) branch linkages in the soluble glucan produced by GTF-S from *S. mutans* 6715, which is reported to contain 27% α -(1 \rightarrow 3) branches (39), and the interaction of this enzyme with so-called "primer" dextran.

MATERIALS AND METHODS

Enzymes

Streptococcus mutans 6715 was grown in the medium described by Ciardi *et al.* (24). Cells were removed using a Millipore (Bedford, MA) Pellicon membrane cassette system with an HVLP Durapore microfiltration membrane. The filtrate was then concentrated using a PTGC membrane cassette, which has a nominal molecular weight cutoff of 10,000. This material was further concentrated using an Amicon ultrafiltration cell with an XM-100 membrane. The concentrate was then chromatographed on a column of Bio-Gel A-15m (Bio-Rad, Richmond, CA) as described by Shimamura *et al.* (39). This was followed by separation of the GTF-I and GTF-S on DEAE-cellulose (Sigma Chemical Co., St. Louis, MO) in a manner similar to that of Germaine *et al.* (22), except that the chromatography was carried out at pH 6.8 in 20 mM potassium phosphate buffer, using a sodium chloride gradient of 0 to 0.2 M. The fraction which contained only GTF-S activity (see ref. 22) was re-chromatographed on a DEAE-Bio-Gel A column in the same phosphate buffer, with the same sodium chloride gradient. The final purification step consisted of an affinity procedure in which the enzyme, in 0.02% sodium azide, was bound to a column of Sephadex G-50 (Pharmacia Fine Chemicals, Uppsala, Sweden), and then eluted with 4 M guanidine hydrochloride, in a manner similar to that of Hamelik and McCabe (38). After buffer exchange by dialysis against 50 mM, pH 5.5, sodium acetate buffer containing 0.02% sodium azide, the purified GTF-S contained 50 ± 7 IU/mg of protein, when assayed in the presence of 3.3 mg/mL of B-512F dextran, and 57 μ g of carbohydrate per mg of protein.

The dextranase used in these experiments was an endodextranase produced by *Penicillium funiculosum*, and was purchased from Sigma Chemical Co. It had a specific activity of 55 IU/mg, and the solution used in these experiments contained 100 IU/mL. Unless stated otherwise, one volume of dextranase was used to hydrolyze ten volumes of glucan, so that the dextranase content of the digests was 9 IU/mL.

Carbohydrates

Dextran T-2000 was purchased from Pharmacia. [U- ^{14}C]Dextran was produced from [U- ^{14}C]sucrose (New England Nuclear, Boston, MA) by dextran-sucrase from *Leuconostoc mesenteroides* NRRL B-512F (40). Native, high-molecular-weight B-512F dextran was purchased from Sigma Chemical Co., and *L. mesenteroides* NRRL B-742 fraction S and B-1142 dextrans (41) were kindly supplied by Dr. Morey Slodki of the U.S. Department of Agriculture's Northern Regional Research Center, Peoria, IL. *L. mesenteroides* NRRL B-1355 fraction S glucan (41) (alternan) was prepared as previously described (42). ^{14}C -Labeled *S. mutans* 6715 water-soluble glucan was prepared from 0.1 M [U- ^{14}C]sucrose by purified GTF-S. Pullulan was obtained from the laboratories of the late Professor Dexter French, and rabbit liver glycogen was purchased from Sigma Chemical Co.

Analytical Methods

Total carbohydrate analysis was by the phenol-sulfuric acid method (43), and protein concentration was determined by the Coomassie Blue method of Bradford (44).

Glucanase activity was assayed by the procedure described by Germaine *et al.* (45). Susceptibility of ^{14}C -labeled glucans to dextranase hydrolysis was measured in an analogous manner: dextranase was added to a sample of [^{14}C]glucan, and the amount of ^{14}C -labeled material rendered methanol-soluble was determined by pipetting 40 μL aliquots onto 1.5 cm squares of Whatman (Clifton, NJ) 3MM filter paper. The paper squares were immediately dropped into a stirred beaker of methanol and washed in five changes of methanol, for ten minutes each time. After drying, the papers were counted for ^{14}C by liquid scintillation in toluene cocktails. Percentage of methanol-insoluble ^{14}C was calculated by comparing the amount of ^{14}C on methanol-washed papers to that on paper squares which had not been washed. The hydrolysis was judged to be complete when no further decrease in methanol-insoluble ^{14}C was observed, even after the addition of more dextranase to the digest.

Proton-decoupled ^{13}C -nuclear magnetic resonance spectra were obtained on approximately 100–150 mg of polysaccharide in deuterium oxide at 80° , using a JEOL FX-90Q Fourier-transform n.m.r. spectrometer at 22.5 MHz. Approximately 10,000 scans were obtained for each spectrum.

Production of Enzymatically Modified D-Glucans

^{14}C -Labeled *L. mesenteroides* B-512F dextran was enzymatically modified by methods similar to those previously described (46). Three different sets of conditions were employed: in the first, 1 mL of *S. mutans* 6715 GTF-S (3.2 IU/mL when assayed in the presence of 3.3 mg/mL of B-512F dextran) was incubated with 0.9 mL of $[^{14}\text{C}]$ B-512F dextran (22 mg/mL, 55 c.p.m./ μg) and 0.11 mL of 1.7 M sucrose in 50 mM, pH 5.5, sodium acetate buffer at 25° for approximately 36 h. The resulting polysaccharide was precipitated with 1.5 volumes of ethanol, and redissolved in 3 mL of sodium acetate buffer. In the second procedure, 2 mL of GTF-S was mixed with 2 mL of $[^{14}\text{C}]$ B-512F dextran solution, and the mixture was placed in a dialysis bag. The sealed bag was then placed in a stirred vessel containing 1.4 liters of 10 mM sucrose in sodium acetate buffer, and allowed to react at 25° for approximately 36 h. The polysaccharide inside the bag was precipitated with ethanol and redissolved in 10 mL of sodium acetate buffer. The third procedure was the same as the second, except that the sucrose concentration outside the dialysis bag was 0.1 M, and the precipitated polysaccharide was redissolved in 15 mL of buffer.

RESULTS

Table I gives the effects of a number of α -D-glucans on the relative rate of glucan synthesis by *S. mutans* 6715 GTF-S. Both T-2000 dextran and Sigma dextran, which are only 5% branched (41), activated the enzyme to the greatest extent. *L. mesenteroides* B-1142 dextran, with 28% α -(1 \rightarrow 3) branches (41), activated GTF-S to a lesser degree, while *L. mesenteroides* B-742 fraction S dextran, with nearly every glucose residue branched (45% α -(1 \rightarrow 3) branches) (41), activated GTF-S even less. The other α -D-glucans (pullulan, glycogen, and alternan), which differ from dextran in that they contain no consecutive sequences of α -(1 \rightarrow 6)-linked glucose residues (47), did not stimulate GTF-S to any significant extent.

Table II shows that *S. mutans* 6715 GTF-S is capable of modifying B-512F dextran to make it more resistant to hydrolysis by dextranase. It also shows that B-512F dextran, modified by GTF-S in the dialysis bag, was more resistant to dextranase action than was dextran which had been modified in the test-tube experiments, and that a higher concentration of sucrose outside the dialysis bag favored a greater degree of modification. It was, in fact, possible to modify B-512F dextran so that it was more resistant to dextranase hydrolysis than *S. mutans* 6715 soluble glucan was, although it must be noted that the *S. mutans* 6715 glucan in Table II was synthesized under a different set of conditions; *i.e.*, in a test tube, rather than in a dialysis bag.

The dextranase digests in Table II were each chromatographed on Bio-Gel P-2 in order to demonstrate that the degree of methanol-insolubility was directly related to the amount of higher-molecular-weight material remaining after dextranase hydrolysis. Figure 1 shows that nearly all of the B-512F dextran is reduced to low-molecular-weight material by dextranase hydrolysis. In contrast, *S. mutans* 6715 soluble glucan contains a large amount of higher-molecular-weight material which is resistant to dextranase hydrolysis, as shown in Figure 2. Figures 3-5 show that the modified B-512F glucans also contain this higher-molecular-weight, dextranase-resistant material, and that those which were more resistant to

Table I. Stimulation of *S. mutans* 6715 GTF-S activity by α -D-glucans

α -D-Glucan ^a	GTF-S activity ^b		Methylation data (41,47)					
	IU/mL	Relative %	Mole % of each methyl ether of D-glucose					
			2,3,4	2,3,4,6	2,4	2,4,6	2,3,6	2,3
T-2000	3.0	100	90	5	5	0	0	0
B-512F, native	2.9	97	90	5	5	0	0	0
B-1142	2.6	87	44	28	28	0	0	0
B-742 S	2.1	70	4	46	50	0	0	0
B-1355 S (alternan)	0.20	7	47	7	11	35	0	0
Pullulan	0.20	7	33	<1	0	0	66	0
Glycogen	0.15	5	0	6	0	0	89	5
None	0.10	3	--	--	--	--	--	--

^aAll glucans were at a concentration of 0.5 mg/mL.

^bAssayed at 37° with 0.1 M sucrose.

Table II. Endodextranase hydrolysis of modified and unmodified ^{14}C -labeled α -D-glucans

α -D-Glucan (^{14}C -labeled)	Modified by GTF-S ?	Sucrose concentration (M)	Method of modification or synthesis	Percent of ^{14}C remaining insol- uble in methanol after dextranase hydrolysis	
				mean \pm S.D.	No. of determinations
6715 S glucan	no	0.1 ^a	test tube	28.1 \pm 0.6	4
B-512F dextran	yes	0.09 ^b	test tube	12 \pm 1.0	3
B-512F dextran	yes	0.01 ^b	dialysis bag	19.7 \pm 0.4	3
B-512F dextran	yes	0.1 ^b	dialysis bag	34 \pm 1.6	3
B-512F dextran	no	0.1 ^a	test tube	5 \pm 1.5	7

^aSucrose concentration used to synthesize labeled glucan.

^bSucrose concentration used to modify labeled glucan.

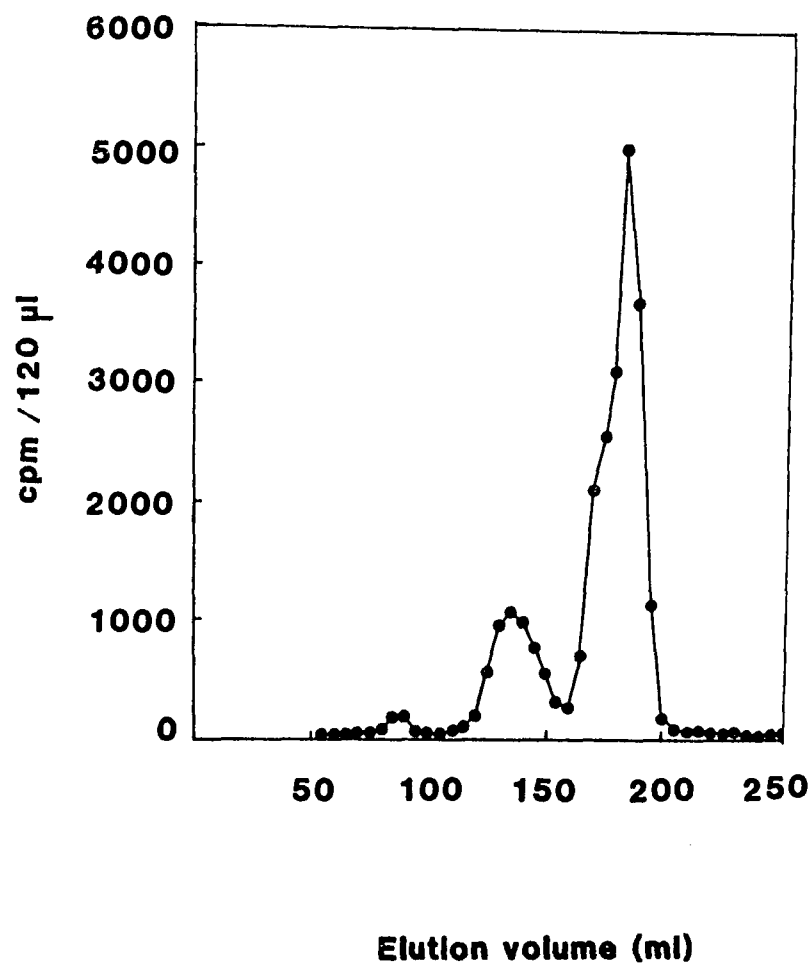


Figure 1. Bio-Gel P-2 chromatography of endodextranase hydrolyzate of ^{14}C -labeled *L. mesenteroides* B-512F dextran. Void volume = 85 mL; total included volume = 190 mL. Column was 2.5 x 53 cm, eluent was 0.02% NaN_3 , and temperature was 25°

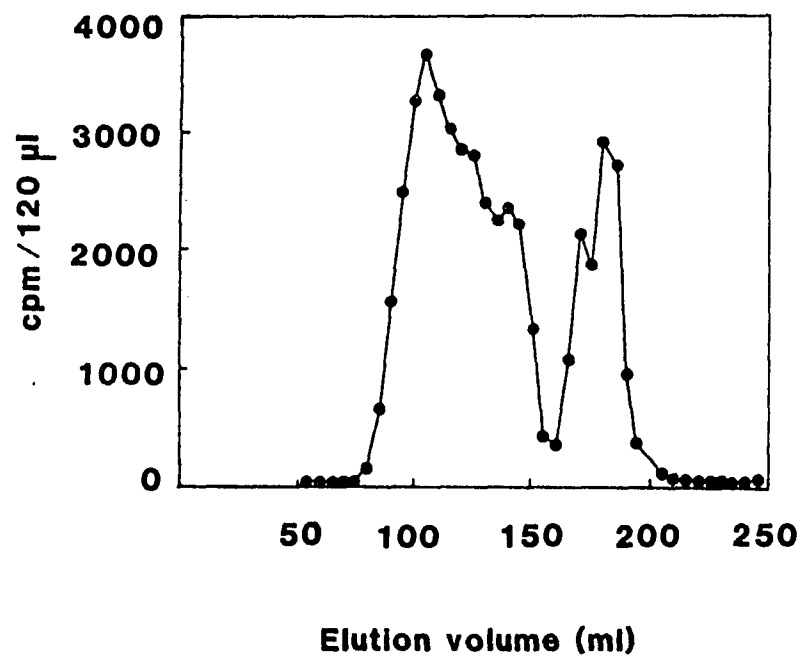


Figure 2. Bio-Gel P-2 chromatography of endodextranase hydrolyzate of ^{14}C -labeled *S. mutans* 6715 soluble glucan. See Fig. 1 for details

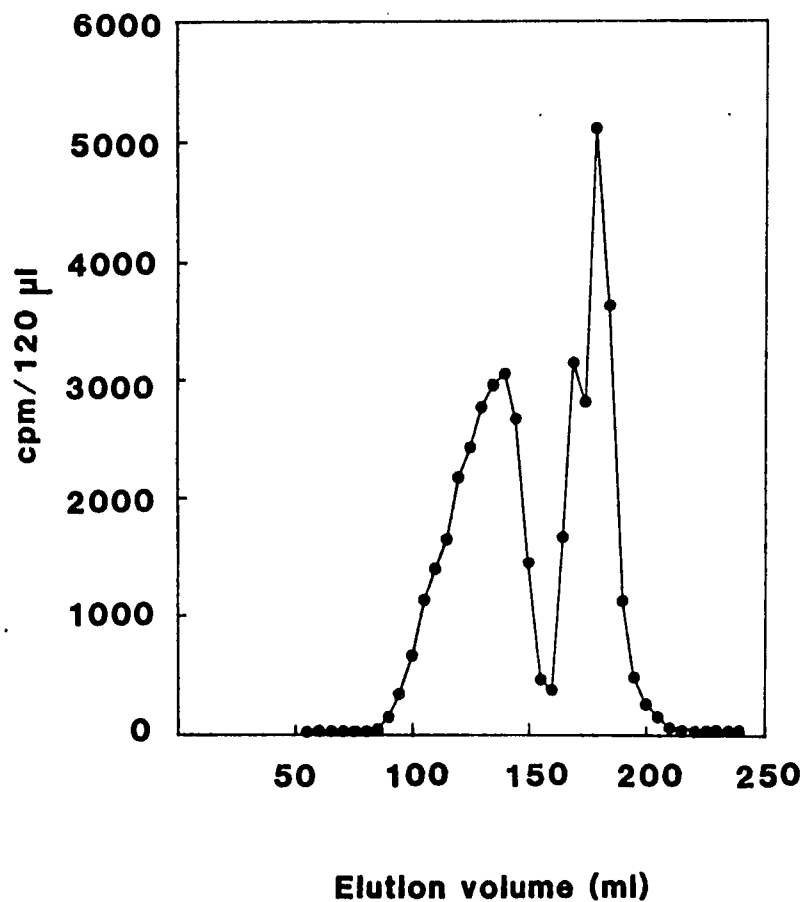


Figure 3. Bio-Gel P-2 chromatography of endodextranase hydrolyzate of ^{14}C -labeled B-512F dextran which had been modified by *S. mutans* 6715 GTF-S with 0.09M sucrose in a test tube. See Fig. 1 for details

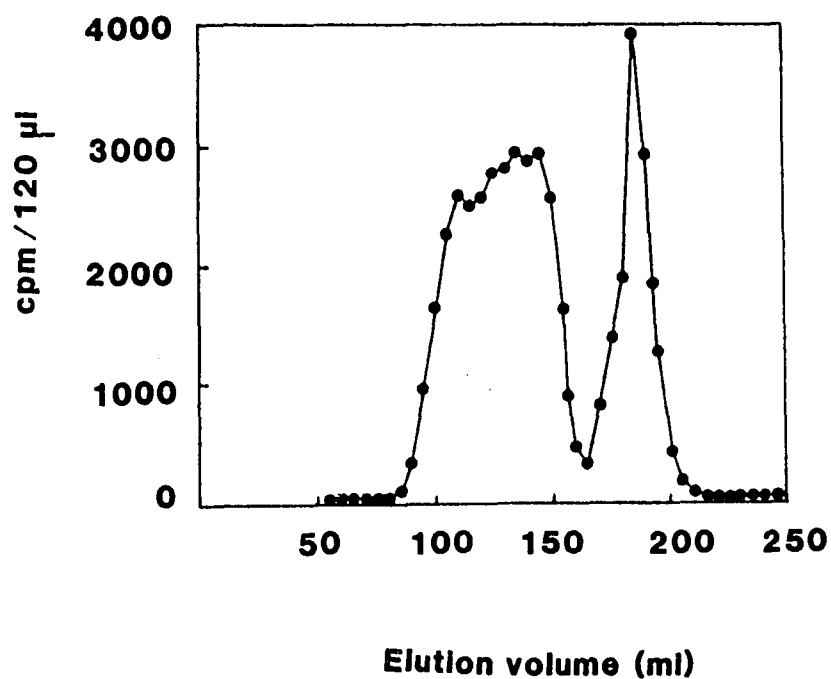


Figure 4. Bio-Gel P-2 chromatography of endodextranase hydrolyzate of ^{14}C -labeled B-512F dextran which had been modified by *S. mutans* 6715 GTF-S in a dialysis bag, which was placed in 0.01 M sucrose. See Fig. 1 for details

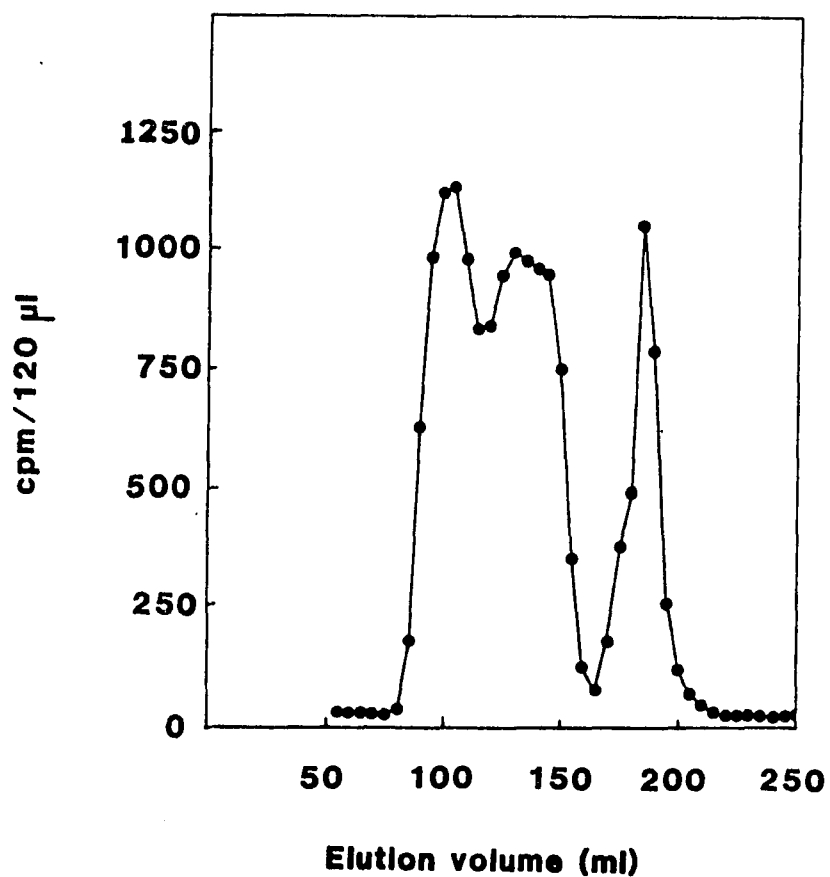


Figure 5. Bio-Gel P-2 chromatography of endodextranase hydrolyzate of ^{14}C -labeled B-512F dextran which had been modified by *S. mutans* 6715 GTF-S in a dialysis bag, which was placed in 0.1 M sucrose. See Fig. 1 for details

dextranase contain more of the higher-molecular-weight compounds.

These results suggest that the conditions of synthesis play a role in determining the amount of α -(1 \rightarrow 3) branching in the glucan formed by GTF-S. However, a ^{13}C -spectrum of the glucan formed from 0.1 M sucrose by GTF-S in a test tube (Fig. 6) appeared nearly identical to a spectrum of that which was formed in a dialysis bag which had been placed in 0.1 M sucrose. Small differences, though, may not be observable by n.m.r. alone. One variable which did significantly affect the amount of α -(1 \rightarrow 3) linkages formed was the presence of a high concentration of ammonium sulfate. Figure 7 shows a ^{13}C -n.m.r. spectrum of the glucan formed from 0.1 M sucrose by GTF-S in the presence of 1.6 M ammonium sulfate. It is clearly evident that the glucan formed in the presence of such a high concentration of ammonium sulfate contains a much greater percentage of α -(1 \rightarrow 3) linkages, as indicated by the relative intensity of peak 1 compared to peak 2. In Figs. 6 and 7, peak 1 arises from an anomeric carbon (C-1) of glucose in an α -(1 \rightarrow 3) linkage, while peak 2 is due to an anomeric carbon (C-1) in an α -(1 \rightarrow 6) linkage. Note that in Fig. 6 the intensity of peak 1 is about 60% that of peak 2, while in Fig. 7, peak 1 is of approximately the same intensity as peak 2, indicating a relatively greater proportion of α -(1 \rightarrow 3) linkages.

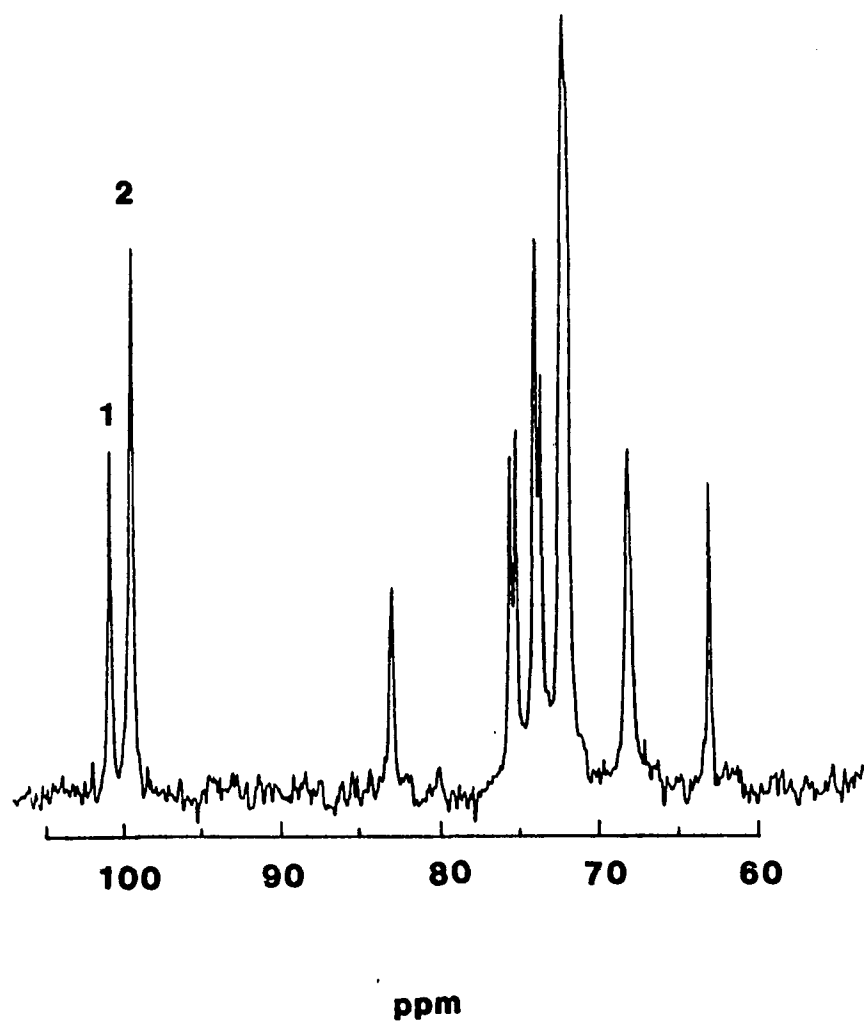


Figure 6. ^1H -Decoupled ^{13}C -n.m.r. spectrum of D-glucan formed by *S. mutans* 6715 GTF-S from 0.1 M sucrose in a test tube. Peak 1 is due to C-1 linked to C-3, and peak 2 is due to C-1 linked to C-6

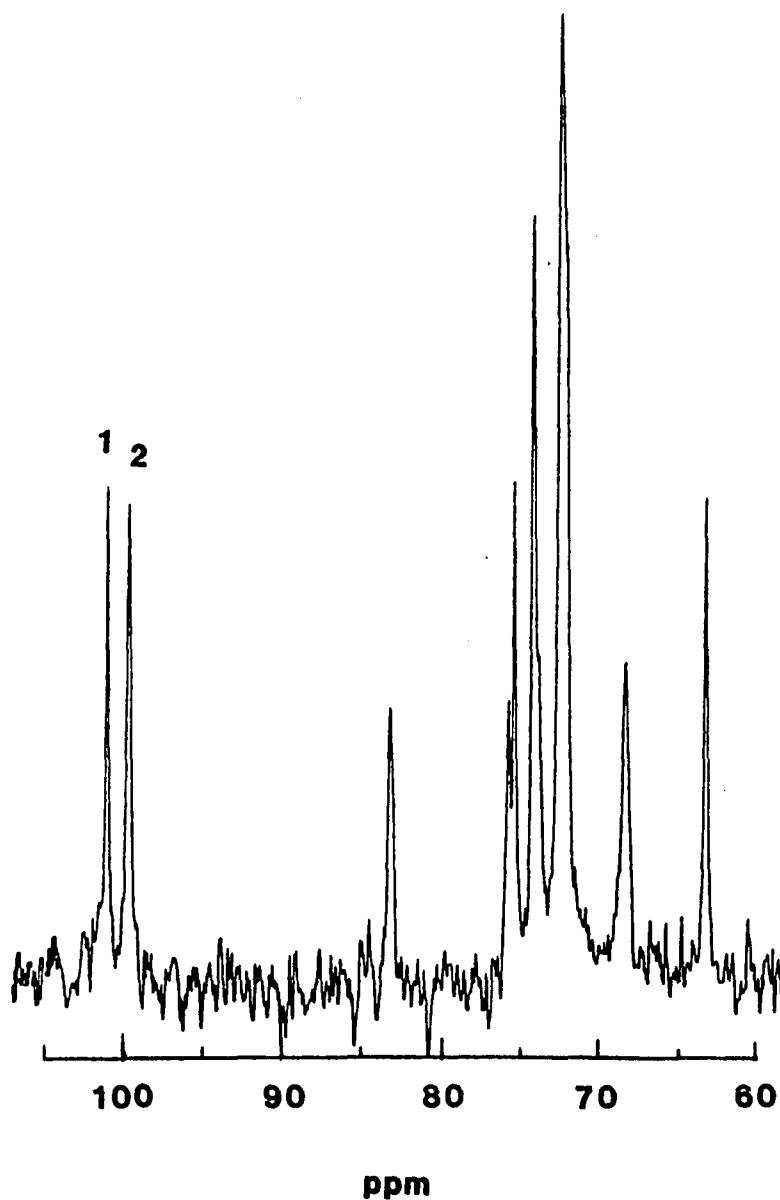


Figure 7. ^1H -Decoupled ^{13}C -n.m.r. spectrum of D-glucan formed by *S. mutans* 6715 GTF-S from 0.1 M sucrose in a test tube, in the presence of 1.6 M ammonium sulfate. Peak 1 is due to C-1 linked to C-3, and peak 2 is due to C-1 linked to C-6

DISCUSSION

We have found, as have others (1,20,22,24,39), that glucan synthesis by *S. mutans* 6715 GTF-S is stimulated by exogenous dextran. Dextran from *L. mesenteroides* B-512F were the most effective activators, while the more branched dextrans from strains B-1142 and B-742 (fraction S) were less effective. Alternan, the more soluble glucan from *L. mesenteroides* B-1355, did not stimulate GTF-S to form glucan, nor did pullulan or glycogen. These results demonstrate that a glucan must contain linear, consecutive sequences of α -(1 \rightarrow 6)-linked glucosyl units in order to activate GTF-S, and that the more highly branched dextrans are less effective in their ability to activate GTF-S, indicating that this activation is not due to their acting as "primers", as some workers have suggested (20,22,33). If this activation were due to the dextrans acting as primers, *i.e.*, having glucosyl residues transferred to the nonreducing ends, then the more highly branched dextrans, which contain more nonreducing ends per unit of weight, would be expected to be more effective in stimulating glucan synthesis. Just the opposite, however, is what is actually observed, suggesting that it is the presence in these dextrans of unbranched sequences of α -(1 \rightarrow 6)-linked glucosyl residues that is one of the main causes of GTF-S activation.

In an earlier paper, Robyt and Corrigan (21) showed that the activation of *S. mutans* OMZ 176 dextransucrase by dextran and chemically modified dextran was not due to its acting as a "primer". They proposed that the stimulation could be due to its acting as an acceptor for the formation of α -(1 \rightarrow 3) branch linkages. It should be pointed out that while primers are a special type of acceptor, not all acceptors are primers. When dextran acts as an acceptor to give rise to branch linkages, it is not acting as a primer in the generally understood sense, *viz.*, a primer is a necessary oligomeric or polymeric molecule whose end units (usually, if not always, the nonreducing ends) are required for the addition of new monomer units to give chain elongation. This was the definition of the term as originally used for phosphorylase (48,49). Much confusion has arisen when

the terms "acceptor" and "primer" are used interchangeably (20, 22, 28, 50, 51). It should be understood that, although dextran can act as an acceptor and activator for GTF-S, this is not the same as "priming" (52).

Robyt and Taniguchi (53) have shown that α -(1 \rightarrow 3) branch formation by dextransucrase from *L. mesenteroides* B-512F occurs by acceptor reactions with dextran. It was not known, however, whether this type of reaction could also occur with streptococcal glucansucrases, which produce soluble glucans with much higher amounts of α -(1 \rightarrow 3) branches. Our work with *L. mesenteroides* B-742 S dextransucrase (46) has shown that even in a very highly branched dextran, these branch linkages could be formed by acceptor reactions with dextran.

The experimental results shown in Table II indicate that *S. mutans* 6715 GTF-S may be operating by a similar mechanism. Others have shown that exogenous dextrans can act as acceptors for, and are modified by, streptococcal GTF-S from a number of strains (12,20,22,24,25), but the exact nature of this modification has not been shown.

Our results here show that this modification is at least partially due to the formation of α -(1 \rightarrow 3) branches close enough to one another to cause segments of the dextran chain to become resistant to endodextranase hydrolysis. The methanol-insolubility and P-2 chromatography indicate that these dextransucrase-resistant fragments contain at least 8-12 glucosyl residues. The ability of GTF-S to form such highly branched segments solely by acceptor reactions means that the mechanism put forth by Robyt *et al.* (18,21,52-54) can explain the synthesis of even the most highly branched dextrans, regardless of whether they are produced by *L. mesenteroides* or by *S. mutans*.

L. mesenteroides B-742 S dextran contains an extremely high number of single glucosyl residues attached to the main chain by α -(1 \rightarrow 3) branch linkages, and it is possible that glucosyl residues from sucrose could be transferred to them to form either α -(1 \rightarrow 6) or α -(1 \rightarrow 3) linkages. Previous work has shown, however, that this is not the primary mechanism by which the glucan chains are elongated (18). There is substantial evidence to support an insertion mechanism for dextran formation, in which glucosyl

units are added to the reducing end of a growing enzyme-dextranosyl chain (18,54). In such a mechanism, acceptor reactions would play two roles: they could account for the formation of branch linkages, and they could serve to terminate chain elongation. Although we have demonstrated that GTF-S can form α -(1 \rightarrow 3)-linked single glucosyl branches by acceptor reactions, it is possible that as GTF-S produces the α -(1 \rightarrow 6)-linked main chain, glucosyl units are transferred to this chain from a separate enzyme-glucosyl site to form single α -(1 \rightarrow 3)-linked glucosyl branches (52). This site could also transfer glucosyl units to exogenous dextran, to form α -(1 \rightarrow 3) branches by acceptor reactions.

There still remains a good deal to be learned about the mechanism of formation of α -(1 \rightarrow 3) linkages by *S. mutans* 6715 GTF-S. As stated by Shimamura *et al.* (39), it is not known whether the formation of α -(1 \rightarrow 6) and α -(1 \rightarrow 3) linkages both occur at the same active site, at different active sites on the same enzyme molecule, or on different subunits of an enzyme consisting of two or more protein chains. There is some evidence that factors which may influence protein conformation and the aggregation state of the enzyme also affect the relative amounts of α -(1 \rightarrow 3) linkages formed by other, similar glucansucrases from strains of *S. mutans*. Newman *et al.* (55) have found that a high concentration (1.55 M) of ammonium sulfate induced GTF-S from strain 3209 to produce an insoluble glucan, which contained a significant proportion of consecutive α -(1 \rightarrow 3) linkages. In addition, Mukasa *et al.* (56) have described the effects of various salts on the relative amounts of soluble and insoluble glucans formed by GTF-I from strain 6715, and hypothesized that salts may affect the interactions between the enzyme and acceptor glucans. Our observation that 1.6 M ammonium sulfate induces GTF-S from *S. mutans* 6715 to form a greater amount of α -(1 \rightarrow 3) linkages supports these findings, although the resulting glucan was not insoluble. We have not yet determined whether this increase is due to increased branching, or to linear sequences of α -(1 \rightarrow 3)-linked glucosyl residues. Linear sequences of α -(1 \rightarrow 3)-linked glucosyl residues tend to render a glucan insoluble, but glucans containing small proportions of α -(1 \rightarrow 3) linear sequences may not necessarily be insoluble (12,57).

In summary, we have shown that the stimulation of GTF-S activity by dextrans is due at least in part to dextran acting as an acceptor for the formation of α -(1 \rightarrow 3) branch linkages. Branch formation by acceptor reactions can account for the high degree of branching in the glucan formed by *S. mutans* 6715 GTF-S, but the details of such reactions are not yet known. These findings are consistent with the insertion mechanism proposed by Robyt *et al.* (18,52,54), and may account for the stimulation by dextran observed by others (20,22,24,39,50,51).

ACKNOWLEDGMENTS

We thank Dr. Morey Slodki for supplying the samples of *L. mesenteroides* B-742 and B-1142 dextrans, and Mr. Steve Eklund for his assistance in obtaining ^{13}C -n.m.r. spectra and in typing the manuscript.

REFERENCES

1. T. J. Montville, C. L. Cooney, and A. J. Sinskey. *Streptococcus mutans* dextranucrase: a review. *Adv. Appl. Microbiol.* 24, 55-84 (1978).
2. S. Hamada and H. D. Slade. Biology, immunology, and cariogenicity of *Streptococcus mutans*. *Microbiol. Rev.* 44, 331-384 (1980).
3. W. J. Lewicki, L. W. Long, and J. R. Edwards. Determination of the structure of a broth dextran produced by a cariogenic streptococcus. *Carbohydr. Res.* 17, 175-182 (1971).
4. L. W. Long and J. R. Edwards. Detailed structure of a dextran from a cariogenic bacterium. *Carbohydr. Res.* 24, 216-217 (1972).
5. M. Ceska, K. Granath, B. Norrman, and B. Guggenheim. Structural and enzymatic studies on glucans synthesized with glucosyltransferases of some strains of oral streptococci. *Acta Chem. Scand.* 26, 2223-2230 (1972).
6. J. K. Baird, V. M. C. Longyear, and D. C. Ellwood. Water insoluble and soluble glucans produced by extracellular glycosyltransferases from *Streptococcus mutans*. *Microbios* 8, 143-150 (1973).
7. T. Usui, H. Sugiyama, S. Seto, S. Araya, T. Nisizawa, S. Imai, and K. Kosaka. Structural determination of glucans from *Streptococcus mutans* JC2 (dental caries bacterium) by carbon-13 nuclear magnetic resonance. *J. Biochem.* 78, 225-227 (1975).
8. T. Nisizawa, S. Imai, H. Akada, M. Hinoide, and S. Araya. Extracellular glucans produced by oral streptococci. *Arch. Oral Biol.* 21, 207-213 (1976).
9. T. Nisizawa, S. Imai, and S. Araya. Methylation analysis of extracellular glucans produced by *Streptococcus mutans* strain JC2. *Arch. Oral Biol.* 22, 281-285 (1977).
10. M. Freedman, D. Birked, and K. Granath. Analyses of glucans from cariogenic and mutant *Streptococcus mutans*. *Infect. Immun.* 21, 17-27 (1978).
11. T. S. Meyer, B. L. Lamberts, and R. S. Egan. Structural studies of extracellular glucans of *Streptococcus mutans* by proton magnetic resonance. *Carbohydr. Res.* 66, 33-42 (1978).
12. M. D. Hare, S. Svensson, and G. J. Walker. Characterization of the extracellular, water-insoluble α -D-glucans of oral streptococci by methylation analysis, and by enzymic synthesis and degradation. *Carbohydr. Res.* 66, 245-264 (1978).
13. P. Colson, H. C. Jarrell, B. L. Lamberts, and I. C. P. Smith. Determination, by carbon-13 nuclear magnetic resonance spectroscopy, of the composition of glucans synthesized by enzymes of the cariogenic organism *Streptococcus mutans*. *Carbohydr. Res.* 71, 265-272 (1979).

14. M. Inoue and T. Koga. Fractionation and properties of glucans produced by *Streptococcus mutans*. *Infect. Immun.* 25, 922-931 (1979).
15. F. R. Seymour, R. D. Knapp, and B. L. Lamberts. Structural analysis of soluble D-glucans of *Streptococcus mutans* by ^{13}C -nuclear magnetic resonance spectrometry. *Carbohydr. Res.* 84, 187-195 (1980).
16. F. R. Seymour, R. L. Julian, A. Jeanes, and B. L. Lamberts. Structural analysis of insoluble D-glucans by Fourier-transform, infrared difference spectrometry: correlation between structures of dextrans from strains of *Leuconostoc mesenteroides* and of D-glucans from strains of *Streptococcus mutans*. *Carbohydr. Res.* 86, 227-246 (1980).
17. F. R. Higon and H. Weigel. The structure of an extracellular, water-soluble polysaccharide elaborated by the cariogenic *Streptococcus mutans* GS-5. *Carbohydr. Res.* 86, 105-112 (1980).
18. J. F. Robyt and P. J. Martin. Mechanism of synthesis of D-glucans by D-glucosyltransferases from *Streptococcus mutans* 6715. *Carbohydr. Res.* 113, 301-315 (1983).
19. B. Guggenheim and E. Newbrun. Extracellular glucosyltransferase activity of an HS strain of *Streptococcus mutans*. *Helv. Odont. Acta* 13, 84-97 (1969).
20. A. M. Chludzinski, G. R. Germaine, and C. F. Schachte. *Streptococcus mutans* dextransucrase: purification, properties, and requirement for primer dextran. *J. Dent. Res.* 55, C75-C86 (1976).
21. J. F. Robyt and A. J. Corrigan. The mechanism of dextransucrase action. Activation of dextransucrase from *Streptococcus mutans* OMZ 176 by dextran and modified dextran, and the nonexistence of the primer requirement for the synthesis of dextran. *Arch. Biochem. Biophys.* 183, 726-731 (1977).
22. G. R. Germaine, S. K. Harlander, W.-L. S. Leung, and C. F. Schachte. *Streptococcus mutans* dextransucrase: functioning of primer dextran and endogenous dextransucrase in water-soluble and water-insoluble glucan synthesis. *Infect. Immun.* 16, 637-648 (1977).
23. M. M. McCabe and E. E. Smith. Specific method for the purification of *Streptococcus mutans* dextransucrase. *Infect. Immun.* 16, 760-765 (1977).
24. J. E. Ciardi, A. J. Beaman, and C. L. Wittenberger. Purification, resolution, and interaction of the glucosyltransferases of *Streptococcus mutans* 6715. *Infect. Immun.* 18, 237-246 (1977).
25. M. M. McCabe and E. E. Smith. The dextran acceptor reaction of dextransucrase from *Streptococcus mutans* K1R. *Carbohydr. Res.* 63, 223-239 (1978).
26. D. J. Smith, M. A. Taubman, and J. L. Ebersole. Preparation of glucosyltransferase from *Streptococcus mutans* by elution from water-insoluble polysaccharide with a dissociating solvent. *Infect. Immun.* 23, 446-452 (1979).

27. W. R. Figures and J. R. Edwards. Improved purification procedure for the extracellular D-glucosyltransferase from *Streptococcus mutans* 6715. Carbohydr. Res. 73, 245-253 (1979).
28. W. R. Figures and J. R. Edwards. D-Glucosyltransferase of *Streptococcus mutans*: isolation of two forms of the enzyme that bind to insoluble dextran. Carbohydr. Res. 88, 107-117 (1981).
29. K. Fukushima, R. Motoda, K. Takada, and T. Ikeda. Resolution of *Streptococcus mutans* glucosyltransferases into two components essential to water-insoluble glucan synthesis. FEBS Lett. 128, 213-216 (1981).
30. K. Fukushima, R. Motoda, and T. Ikeda. Effects of exogenous insoluble glucan primer on insoluble glucan synthesis by *Streptococcus mutans*. J. Dent. Res. 60, 1707-1712 (1981).
31. K. Fukui, T. Moriyama, Y. Miyaki, K. Mizutani, and O. Tanaka. Purification and properties of glucosyltransferase responsible for water-insoluble glucan synthesis from *Streptococcus mutans*. Infect. Immun. 37, 1-9 (1981).
32. S. Sato, T. Koga, T. Yakushiji, S. Nagasawa, and M. Inoue. Effects of exogenous soluble dextrans on insoluble glucan synthesis by *Streptococcus mutans* glucosyltransferase. Microbios 34, 99-112 (1982).
33. T. Koga, S. Sato, M. Inoue, K. Takeuchi, T. Furuta, and S. Hamada. Role of primers in glucan synthesis by glucosyltransferases from *Streptococcus mutans* strain OMZ 176. J. Gen. Microbiol. 129, 751-754 (1983).
34. D. W. Ellis and C. H. Miller. Extracellular dextran hydrolase from *Streptococcus mutans* strain 6715. J. Dent. Res. 56, 57-69 (1983).
35. A. Pulkownik and G. J. Walder. Purification and substrate specificity of an endo-dextranase of *Streptococcus mutans* K1R. Carbohydr. Res. 54, 237-251 (1977).
36. G. J. Walker, A. Pulkownik, and J. G. Morrey-Jones. Metabolism of the polysaccharides of human dental plaque: release of dextranase in batch cultures of *Streptococcus mutans*. J. Gen. Microbiol. 127, 201-208 (1981).
37. G. J. Walker, M. D. Hare, and J. G. Morrey-Jones. Effect of variation in growth conditions on endo-dextranase production by *Streptococcus mutans*. Carbohydr. Res. 107, 111-122 (1982).
38. R. M. Hamelik and M. M. McCabe. An endodextranase inhibitor from batch cultures of *Streptococcus mutans*. Biochem. Biophys. Res. Commun. 106, 875-880 (1982).
39. A. Shimamura, H. Tsumori, and H. Mukasa. Purification and properties of *Streptococcus mutans* extracellular glucosyltransferase. Biochim. Biophys. Acta 702, 72-80 (1982).

40. J. F. Robyt and T. F. Walseth. Production, purification, and properties of dextransucrase from *Leuconostoc mesenteroides* NRRL B-512F. Carbohydr. Res. 68, 95-111 (1979).
41. A. Jeanes and F. R. Seymour. The α -D-glucopyranosidic linkages of dextrans: comparison of percentages from structural analysis by periodate oxidation and by methylation. Carbohydr. Res. 74, 31-40 (1979).
42. G. L. Côté and J. F. Robyt. Isolation and partial characterization of an extracellular glucansucrase from *Leuconostoc mesenteroides* that synthesizes an alternating (1 \rightarrow 6), (1 \rightarrow 3)- α -D-glucan. Carbohydr. Res. 101, 57-74 (1982).
43. M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith. Colorimetric method for determination of sugars and related substances. Anal. Chem. 28, 350-356 (1956).
44. M. M. Bradford. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248-250 (1976).
45. G. R. Germaine, C. F. Schachtele, and A. M. Chludzinski. Rapid filter paper assay for the dextransucrase activity from *Streptococcus mutans*. J. Dent. Res. 53, 1355-1360 (1974).
46. G. L. Côté and J. F. Robyt. The formation of α -(1 \rightarrow 3) branch linkages by an exocellular glucansucrase from *Leuconostoc mesenteroides* NRRL B-742. Carbohydr. Res. 119, 141-156 (1983).
47. F. R. Seymour and R. D. Knapp. Structural analysis of α -D-glucans by ^{13}C -nuclear magnetic resonance, spin-lattice relaxation studies. Carbohydr. Res. 81, 67-103 (1980).
48. G. T. Cori and C. F. Cori. The activating effect of glycogen on the enzymatic synthesis of glycogen from glucose-1-phosphate. J. Biol. Chem. 131, 397-398 (1939).
49. M. A. Swanson and C. F. Cori. Studies on the structure of polysaccharides. III. Relation of structure to activation of phosphorylases. J. Biol. Chem. 172, 815-824 (1948).
50. G. R. Germaine, A. M. Chludzinski, and C. F. Schachtele. *Streptococcus mutans* dextransucrase: requirement for primer dextran. J. Bacteriol. 120, 287-294 (1974).
51. G. R. Germaine and C. F. Schachtele. *Streptococcus mutans* dextransucrase: mode of interaction with high-molecular weight dextran and role in cellular aggregation. Infect. Immun. 13, 365-372 (1976).
52. J. F. Robyt. Synthesis of glucans from the reducing end by insertion mechanisms. In "Glucosyltransferases, Glucans, and Dental Caries" (R. Doyle and J. E. Ciardi, eds.) Information Retrieval, Inc. Washington, D. C. (1983).

53. J. F. Robyt and H. Taniguchi. The mechanism of dextransucrase action. Biosynthesis of branch linkages by acceptor reactions with dextran. Arch. Biochem. Biophys. 174, 129-135 (1976).
54. J. F. Robyt, B. K. Kimble, and T. F. Walseth. The mechanism of dextransucrase action. Direction of dextran biosynthesis. Arch. Biochem. Biophys. 165, 634-640 (1974).
55. B. M. Newman, P. White, S. B. Mohan, and J. A. Cole. Effect of dextran and ammonium sulphate on the reaction catalyzed by a glucosyltransferase complex from *Streptococcus mutans*. J. Gen. Microbiol. 118, 353-366 (1980).
56. H. Mukasa, A. Shimamura, and H. Tsumori. Effect of salts on water-insoluble glucan formation by glucosyltransferase of *Streptococcus mutans*. Infect. Immun. 23, 564-570 (1979).
57. F. R. Seymour and R. D. Knapp. Structural analysis of dextrans, from strains of *Leuconostoc* and related genera, that contain 3-O- α -D-glucosylated α -D-glucopyranosyl residues at the branch points, or in consecutive, linear positions. Carbohydr. Res. 81, 105-129 (1980).

GENERAL DISCUSSION

The purpose of this discussion is to relate the five preceding Sections to one another and to what is presently known about the general topic as described in the Literature Review. A detailed discussion appears in each individual section, so an attempt will be made to minimize unnecessary repetition.

In order to study the properties of the enzyme alternansucrase, which produces an unusual glucan consisting of alternating α -(1 \rightarrow 3)- and α -(1 \rightarrow 6)-D-glucosidic linkages (48), it was first necessary to separate it from the dextransucrase also produced by *Leuconostoc mesenteroides* NRRL B-1355. The first method that accomplished this took advantage of two facts: the fact that these exocellular enzymes exist as very large aggregates associated with the polysaccharides present, and the fact that dextran can be degraded by endodextranase, while alternan cannot. Because of this, we were able to enzymatically degrade the dextran present in the culture supernatant, which allowed the dextransucrase to dissociate, leaving the alternan-alternansucrase complex intact as a high-molecular-weight aggregate. The alternansucrase could then be separated by gel-filtration chromatography on Bio-Gel A-5m (Section I, Fig. 2). A serious drawback of this method is that the hydrolysis of the dextran by dextransucrase must be complete. Otherwise, there will remain some high-molecular-weight aggregates of dextran and dextransucrase, which will elute in the void volume of the A-5m column along with the alternansucrase. If the dextransucrase hydrolysis is allowed to go on too long, however, there is an increased possibility that proteinase contaminants, often found in the dextransucrase preparations, could degrade the enzymes present in the culture supernatant, leading to reduced yields. The preferred method for the isolation of alternansucrase is the second method described in Section I, namely, chromatography of the culture supernatant on phenoxyacetyl cellulose. Although the initial cost of preparing the chromatographic medium is somewhat high, this is far outweighed by the simplicity and effectiveness of the method. The cost factor

can be minimized by reusing the PA-cellulose (128).

Alternansucrase prepared by PA-cellulose chromatography was used for the acceptor reactions studied in Section II. Unlike *L. mesenteroides* NRRL B-512F dextransucrase, alternansucrase was found to synthesize both α -(1 \rightarrow 3) and α -(1 \rightarrow 6) glucosidic linkages by acceptor reactions with low-molecular-weight sugars. The most interesting aspect of this is that the α -(1 \rightarrow 3) linkages are formed in these acceptor reactions only when an α -(1 \rightarrow 6) linkage is present at the nonreducing end of the acceptor. This specificity may play a role in determining what types of structures can be formed by alternansucrase, but its exact significance is not yet known. If, as some have suggested, dextran and alternan are synthesized by stepwise addition of glucosyl units to the nonreducing end (102), then alternansucrase would first have to synthesize an α -(1 \rightarrow 6) linkage before it could make an α -(1 \rightarrow 3) linkage by these acceptor-type reactions. This means that the first glucosyl-glucose bond at the reducing end of alternan would have to be an α -(1 \rightarrow 6) isomaltose-like linkage. Thus, if an α -(1 \rightarrow 3) nigerose-like linkage were found at the reducing end of alternan, this would be evidence that alternan biosynthesis does not occur by addition of glucosyl units to the nonreducing end. One would probably have difficulty in trying to find such a linkage, though, owing to the extremely high molecular weight of alternan. A possible method of looking for it might entail reduction of alternan with NaB^3H_4 , followed by partial hydrolysis of the glucan and subsequent chromatographic identification of the radioactively labeled products.

The PA-cellulose method used to isolate alternansucrase also proved to be useful in isolating the fraction L dextransucrase from *L. mesenteroides* NRRL B-742 (Section III). It is interesting to note that in both the B-1355 system and the B-742 system, the enzyme which was bound more strongly to the PA-cellulose was the one which formed an α -(1 \rightarrow 6) glucan with α -(1 \rightarrow 3) branch points, *i.e.*, B-742 S and B-1355 L. It is difficult to generalize from two examples, but it may be of interest to see if one could predict the behavior of other two-enzyme systems on PA-cellulose, such as *L. mesenteroides* NRRL strains B-1299, B-1399, B-1498, or B-1501,

or *Streptobacterium dextranicum* NRRL B-1254 (38).

Unfortunately, while it was *L. mesenteroides* NRRL B-742 fraction L dextranase which we were able to isolate by PA-cellulose chromatography, the enzyme we were especially interested in was the fraction S dextranase, which synthesizes an α -(1 \rightarrow 6) glucan with a high percentage of α -(1 \rightarrow 3) branches (34). Thus, our observations on branch formation by this enzyme were made on a mixed-enzyme system, containing both L and S enzymes. We were still able to show, however, that the degree of branching in B-742 fraction S dextran is dependent on the reaction conditions under which the dextran is synthesized. Conditions which were chosen to favor increased acceptor reactions were also found to favor the synthesis of a dextran with a higher proportion of α -(1 \rightarrow 3) branches. Furthermore, we found that B-742 S dextranase is capable of transferring glucosyl units from sucrose to B-512F dextran, which contains only 5% α -(1 \rightarrow 3) branches, to make a modified dextran containing a much higher percentage of α -(1 \rightarrow 3) branch points. Both of these findings show that *L. mesenteroides* NRRL B-742 S dextranase is capable of forming α -(1 \rightarrow 3) branch linkages in the same general manner as *L. mesenteroides* NRRL B-512F dextranase (12), *i.e.*, by acceptor reactions with dextran, even though B-742 S dextran is much more highly branched. The dependence of branching on reaction conditions helps explain the widely varying values obtained over the years for the proportion of α -(1 \rightarrow 3) linkages in B-742 fraction S dextran (see Section III for discussion).

Another way in which *L. mesenteroides* NRRL B-742 S dextranase is similar to B-512F dextranase is in the nature of the acceptor products formed from low-molecular-weight acceptor sugars. Both of these enzymes gave identical products from sucrose in the presence of fructose, glucose, maltose, or isomaltose. As stated in Section III, none of the unusual oligosaccharides containing α -(1 \rightarrow 3) linkages, which were formed by *Alteromonas*, were formed by B-742 S dextranase.

All of the findings described suggest a fundamental similarity between *L. mesenteroides* B-512F dextranase and B-742 S dextranase. It may be possible that the only real difference between these two enzymes

is the stereochemistry adjacent to the site where glucosyl units are transferred to acceptors, so that B-742 S dextranase is capable of putting α -(1 \rightarrow 3) branches directly adjacent to one another, while B-512F dextranase is sterically prohibited from forming branches this close together.

While working with *L. mesenteroides* NRRL B-742 S dextran, it became apparent that there were close similarities between it and the soluble glucan produced by *Streptococcus mutans* 6715. Note the close resemblance of the ^{13}C -n.m.r. spectrum shown in Section V, Fig. 6, to that shown in Section III, Fig. 4A, suggesting that these two dextrans must have nearly the same structure, *i.e.*, an α -(1 \rightarrow 6) chain with a high percentage of α -(1 \rightarrow 3) branches. This similarity raises the question, "Are the two glucans formed in the same way?". Robyt and Martin (10) have already shown that chain elongation by *S. mutans* GTF-S proceeds by the same reducing-end mechanism as *L. mesenteroides* NRRL B-512F (9), but the mechanism of formation of the α -(1 \rightarrow 3) branch linkages by *S. mutans* GTF-S was not known. Section V shows that the branches in the *S. mutans* 6715 soluble glucan can be formed in the same way as those formed by the dextranases from *L. mesenteroides* NRRL B-512F and B-742 (fraction S), *i.e.*, by acceptor reactions, in which glucosyl units are transferred from sucrose to dextran, to form α -(1 \rightarrow 3) branch points. It should be stated again, as in Section V, that the exact details of branch formation are not known. It seems unlikely that the enzymes studied here (*S. mutans* 6715 GTF-S and *L. mesenteroides* B-742 S dextranase) first synthesize unbranched dextran, which then dissociates and binds to another active enzyme site, which then catalyzes acceptor reactions to form branches. If this were the case, then one might expect to find unbranched dextran at some level at some point during synthesis of the glucans. This appears not to be the case. A more likely mechanism might involve an enzyme complex with two or more subsites, either on the same protein molecule or on different subunits. As the α -(1 \rightarrow 6) chain is formed at one site *via* the insertion mechanism, the extruded dextran may then act as an acceptor at the second, adjacent site, where glucosyl units are transferred to the 3-hydroxyl groups to form branch linkages. This second site may also be capable of reacting with exogenous dextran, such

as B-512F dextran, to form more branches, as the author has observed with both *L. mesenteroides* NRRL B-742 S dextransucrase and *S. mutans* 6715 GTF-S. The fact that these enzymes are capable of reacting with exogenous dextran, as well as the fact that they all have a fairly strong affinity for cross-linked dextran, seem to suggest that there is a specific binding site for α -(1 \rightarrow 6)-linked polymers of glucose. The activation of GTF-S and the stabilization of B-512F dextransucrase by dextran also support this notion, as does the fact that these enzymes are capable of reacting with isomaltodextrins, to carry out disproportionation reactions (Section IV). A study of the disproportionation reactions may help to answer the question, "If there is such a binding site, how many glucose residues will it bind?". This could probably be determined by studying the relative rates of disproportionation reactions with isomaltodextrins of various sizes, in a manner similar to that used to map the subsites of α -amylase (129) and endodextranase (130). Other investigators have reported the effect of different-sized isomaltodextrins on branch formation (123) and activation (73,80) of glucansucrases from *S. mutans*, but disproportionation reactions were not studied.

During our studies on disproportionation reactions, it was found that, in the presence of methyl α -D-glucoside or maltose, *S. mutans* 6715 GTF-S was capable of forming either an α -(1 \rightarrow 3) or α -(1 \rightarrow 6) linkage *via* acceptor reactions, but unlike alternansucrase, the presence of an α -(1 \rightarrow 6) linkage is not required for the formation of an α -(1 \rightarrow 3) linkage. This is quite different from the acceptor specificity of the S-dextran-forming dextransucrase from *L. mesenteroides* B-742, which forms only α -(1 \rightarrow 6) linkages by acceptor reactions with these and other low-molecular-weight sugars. Thus, although the glucans formed by B-742 S dextransucrase and 6715 GTF-S are similar in their basic structures, the enzymes which form them do not have the same product specificity in these acceptor reactions.

On the other hand, one way in which *S. mutans* 6715 GTF-S and *L. mesenteroides* B-742 dextransucrase are alike is in the way they can form branch linkages. As demonstrated in Sections III and V, both enzymes can form branch linkages by transferring glucosyl units from sucrose to dextran.

This modification of relatively linear dextran to form highly branched dextran is yet another way in which *S. mutans* can form a complex mixture of polysaccharides *in vivo*. It has been shown that *S. mutans* GTF-I can transfer chains of α -(1 \rightarrow 3)-linked glucan to soluble α -(1 \rightarrow 6)-linked glucan, to form a graft copolymer whose solubility and dextranase susceptibility depend on the relative proportions of the two types of chains (10,59,79, 107,109). It is also believed that endogenously produced dextranase is capable of modifying the polysaccharides produced *in vivo* by hydrolyzing susceptible α -(1 \rightarrow 6) sequences, to give a relative increase in the amount of α -(1 \rightarrow 3) linkages present (131). The modification of dextran to give branched dextran, which we have described, may fit into the overall picture by increasing the amount of α -(1 \rightarrow 3) linkages present, thus decreasing the amount of dextranase-susceptible glucan present in the medium or in dental plaque. The formation of a high percentage of α -(1 \rightarrow 3) branch points also results in a greater number of nonreducing ends, which may then be available to bind to cell-surface proteins of *S. mutans*, aiding in the formation of large aggregates of cells on tooth surfaces (6).

SUMMARY

1. Alternansucrase from *Leuconostoc mesenteroides* NRRL B-1355 was isolated and partially characterized.
2. Alternansucrase was found to form both α -(1 \rightarrow 6) and α -(1 \rightarrow 3) glucosidic linkages by acceptor reactions with low-molecular-weight sugars, but an α -(1 \rightarrow 3) bond can only be formed when an α -(1 \rightarrow 6) glucosidic linkage is present at the nonreducing end of the acceptor. The general mechanism of acceptor reactions appears to be the same as that described for *L. mesenteroides* NRRL B-512F dextransucrase acceptor reactions (14).
3. The glucansucrase which forms the L fraction dextran from *L. mesenteroides* NRRL B-742 was isolated by phenoxyacetyl cellulose chromatography.
4. The glucansucrase which forms the highly branched fraction S dextran from *L. mesenteroides* NRRL B-742 was shown to be capable of forming α -(1 \rightarrow 3) branch linkages by acceptor reactions with relatively unbranched B-512F dextran. The general mechanism appears to be the same as that described for branch formation by *L. mesenteroides* NRRL B-512F dextransucrase (12).
5. The GTF-S from *Streptococcus mutans* 6715 was shown to be capable of forming α -(1 \rightarrow 3) branch linkages in the same manner as the enzymes which form *L. mesenteroides* B-742 S and B-512F dextrans, *i.e.*, by acceptor reactions with dextran.
6. *S. mutans* 6715 GTF-S, as well as related glucansucrases, was shown to utilize such alternate glucosyl donors as dextran, panose, maltotriose, isomaltose and its higher isomaltodextrin homologues, in what can be considered to be the reverse of acceptor reactions.

7. Various dextrans were found to stimulate *S. mutans* 6715 GTF-S activity. Those with the lowest percentage of α -(1 \rightarrow 3) branches were the most effective activators, while those with higher percentages of α -(1 \rightarrow 3) branches were less effective. Other, non-dextran α -D-glucans, such as glycogen, pullulan, and alternan, did not activate GTF-S.

ADDITIONAL REFERENCES

1. T. H. Evans and H. Hibbert. Bacterial polysaccharides. *Adv. Carbohydr. Chem.* 2, 204-233 (1946).
2. W. B. Neely. Dextran: structure and synthesis. *Adv. Carbohydr. Chem.* 15, 341-369 (1960).
3. R. L. Sidebotham. Dextrans. *Adv. Carbohydr. Chem. Biochem.* 30, 371-444 (1974).
4. F. R. Seymour, R. D. Knapp, and B. L. Lamberts. Structural analysis of soluble D-glucans from strains of *Streptococcus mutans* by ^{13}C -nuclear magnetic resonance spectrometry. *Carbohydr. Res.* 84, 187-195 (1980).
5. A. Jeanes. Dextrans and pullulans: industrially significant α -D-glucans. Pp. 284-298 in P. A. Sandford and A. Laskin, eds., ACS Symp. Ser. No. 45, "Extracellular Microbial Polysaccharides". American Chemical Society, Washington, DC (1977).
6. T. J. Montville, C. L. Cooney, and A. J. Sinskey. *Streptococcus mutans* dextranase: a review. *Adv. Appl. Microbiol.* 24, 55-84 (1978).
7. S. Hamada and H. D. Slade. Biology, immunology, and cariogenicity of *Streptococcus mutans*. *Microbiol. Rev.* 44, 331-384 (1980).
8. J. Sharon, E. A. Kabat, and S. L. Morrison. Immunochemical characterization of binding sites of hybridoma antibodies specific for α -(1 \rightarrow 6) linked dextran. *Molecular Immunol.* 19, 375-388 (1982).
9. J. F. Robyt, B. K. Kimble, and T. F. Walseth. The mechanism of dextranase action. Direction of dextran biosynthesis. *Arch. Biochem. Biophys.* 165, 634-640 (1974).
10. J. F. Robyt and P. J. Martin. Mechanism of synthesis of D-glucans by D-glucosyltransferases from *Streptococcus mutans* 6715. *Carbohydr. Res.* 113, 301-315 (1983).
11. J. F. Robyt and S. H. Eklund. Stereochemistry involved in the mechanism of action of dextranase in the synthesis of dextran and the formation of acceptor products. *Bioorg. Chem.* 11, 115-132 (1982).
12. J. F. Robyt and H. Taniguchi. The mechanism of dextranase action. Biosynthesis of branch linkages by acceptor reactions with dextran. *Arch. Biochem. Biophys.* 174, 129-135 (1976).
13. H. J. Koepsell, H. M. Tsuchiya, N. N. Hellman, A. Kazenko, C. A. Hoffman, E. S. Sharpe, and R. W. Jackson. Enzymatic synthesis of dextran. Acceptor specificity and chain initiation. *J. Biol. Chem.* 200, 793-801 (1953).

14. J. F. Robyt and T. F. Walseth. The mechanism of acceptor reactions of *Leuconostoc mesenteroides* B-512F dextran sucrose. Carbohydr. Res. 61, 433-445 (1978).
15. A. Jeanes, W. C. Haynes, C. A. Wilham, J. C. Rankin, E. H. Melvin, M. J. Austin, J. E. Cluskey, B. E. Fisher, H. M. Tsuchiya, and C. E. Rist. Characterization and classification of dextrans from ninety-six strains of bacteria. J. Amer. Chem. Soc. 76, 5041-5052 (1954).
16. M. E. Durin. De la fermentation cellulosique de sucre de canne. Compt. Rend. 83, 128-131 (1876).
17. A. Grönwall and B. Ingelman. Dextran as a substitute for plasma. Nature 155, 45 (1945).
18. A. Jeanes. "Dextran Bibliography: Extensive Coverage of Research Literature (Exclusive of Clinical) and Patents 1861-1976." U. S. Dept. of Agriculture Misc. Pub. No. 1355, Washington, DC (1978).
19. A. Jeanes, C. A. Wilham, and J. C. Miers. Preparation and characterization of dextran from *Leuconostoc mesenteroides*. J. Biol. Chem. 176, 603-615 (1948).
20. C. A. Wilham, B. H. Alexander, and A. Jeanes. Heterogeneity in dextran preparations. Arch. Biochem. Biophys. 59, 61-75 (1955).
21. A. Jeanes, C. A. Wilham, H. M. Tsuchiya, and W. C. Haynes. Properties of dextrans isolated from whole cultures at various stages of incubation. Arch. Biochem. Biophys. 71, 293-302 (1957).
22. A. Jeanes. Dextrans. Preparation of dextrans from growing *Leuconostoc* cultures. Methods Carbohydr. Chem. 5, 118-127 (1965).
23. W. M. Pasika and L. H. Cragg. The detection and estimation of branching in dextran by proton magnetic resonance spectroscopy. Can. J. Chem. 41, 293-299 (1963).
24. I. J. Goldstein and W. J. Whelan. Structural studies of dextrans. Part I. A dextran containing α -1,3-glucosidic linkages. J. Chem. Soc. 170-175 (1962).
25. H. Suzuki and E. J. Hehre. The differentiation of serotype A and B dextrans by means of partial acetolysis. Arch. Biochem. Biophys. 104, 305-313 (1964).
26. D. Abbott and H. Weigel. Studies on dextrans and dextranases. Part VI. Type and degree of branching in two dextrans. J. Chem. Soc. 816-820 (1966).
27. O. Larm, B. Lindberg, and S. Svensson. Studies on the length of the side chains of the dextran elaborated by *Leuconostoc mesenteroides* NRRL B-512. Carbohydr. Res. 20, 39-48 (1971).
28. R. W. Bailey, D. H. Hutson, and H. Weigel. The action of a *Lactobacillus bifidus* dextranase on a branched dextran. Biochem. J. 80, 514-519 (1961).

29. E. J. Bourne, D. H. Hutson, and H. Weigel. Studies on dextrans and dextranases. 2. The action of mould dextranases on modified isomaltodextrins and the effect of anomalous linkages on dextran hydrolysis. *Biochem. J.* 85, 158-163 (1962).
30. E. J. Bourne, D. H. Hutson, and H. Weigel. Studies on dextrans and dextranases. 3. Structures of oligosaccharides from *Leuconostoc mesenteroides* (Birmingham) dextran. *Biochem. J.* 86, 555-562 (1963).
31. D. Abbott and H. Weigel. Studies on dextrans and dextranases. Part VII. Structures of oligosaccharides from an α -1 \rightarrow 4-branched dextran. *J. Chem. Soc.* 821-831 (1966).
32. M. T. Covacevich and G. N. Richards. Frequency and distribution of branching in a dextran: an enzymic method. *Carbohydr. Res.* 54, 311-315 (1977).
33. F. R. Seymour, R. D. Knapp, and S. H. Bishop. Determination of the structure of dextran by ^{13}C -nuclear magnetic resonance spectroscopy. *Carbohydr. Res.* 51, 179-194 (1976).
34. F. R. Seymour, M. E. Slodki, R. D. Plattner, and A. Jeanes. Six unusual dextrans: methylation structural analysis by combined g.l.c.-m.s. of per-*O*-acetyl-aldononitriles. *Carbohydr. Res.* 53, 153-166 (1977).
35. F. R. Seymour, E. C. M. Chen, and S. H. Bishop. Methylation structural analysis of unusual dextrans by combined gas-liquid chromatography - mass spectrometry. *Carbohydr. Res.* 68, 113-121 (1979).
36. F. R. Seymour, R. D. Knapp, S. H. Bishop, and A. Jeanes. High-temperature enhancement of ^{13}C -n.m.r. chemical-shifts of unusual dextrans, and correlation with methylation structural analysis. *Carbohydr. Res.* 68, 123-140 (1979).
37. F. R. Seymour, R. D. Knapp, E. C. M. Chen, A. Jeanes, and S. H. Bishop. Structural analysis of dextrans containing 2-*O*- α -D-glucosylated α -D-glucopyranosyl residues at the branch points, by use of ^{13}C -nuclear magnetic resonance spectroscopy and gas-liquid chromatography - mass spectrometry. *Carbohydr. Res.* 71, 231-250 (1979).
38. F. R. Seymour, R. D. Knapp, and S. H. Bishop. Carbon-13 spin-lattice relaxation studies for resonance assignment to specific, carbon positions of dextrans. *Carbohydr. Res.* 72, 229-234 (1979).
39. A. Jeanes and F. R. Seymour. The α -D-glucopyranosidic linkages of dextrans: comparison of percentages from structural analysis by periodate oxidation and by methylation. *Carbohydr. Res.* 74, 31-40 (1979).
40. F. R. Seymour, R. D. Knapp, E. C. M. Chen, S. H. Bishop, and A. Jeanes. Structural analysis of *Leuconostoc* dextrans containing 3-*O*- α -D-glucosylated α -D-glucosyl residues in both linear-chain and branch-point positions, by methylation and by ^{13}C -n.m.r. spectroscopy. *Carbohydr. Res.* 74, 41-62 (1979).

41. F. R. Seymour and R. L. Julian. Fourier-transform, infrared difference-spectrometry for structural analysis of dextrans. Carbohydr. Res. 74, 63-75 (1979).
42. F. R. Seymour, R. D. Knapp, and S. H. Bishop. Correlation of the structure of dextrans to their ^1H -n.m.r. spectra. Carbohydr. Res. 74, 77-92 (1979).
43. F. R. Seymour, R. D. Knapp, E. C. M. Chen, A. Jeanes, and S. H. Bishop. Structural analysis of dextrans containing 4-O- α -D-glucosylated α -D-glucopyranosyl residues at the branch points by use of ^{13}C -nuclear magnetic resonance spectroscopy and gas-liquid chromatography-mass spectrometry. Carbohydr. Res. 75, 275-294 (1979).
44. F. R. Seymour and R. D. Knapp. Structural analysis of α -D-glucans by ^{13}C -nuclear magnetic resonance, spin-lattice relaxation studies. Carbohydr. Res. 81, 67-103 (1980).
45. F. R. Seymour and R. D. Knapp. Structural analysis of dextrans, from strains of *Leuconostoc* and related genera, that contain 3-O- α -D-glucosylated α -D-glucopyranosyl residues at the branch points, or in consecutive, linear positions. Carbohydr. Res. 81, 105-129 (1980).
46. F. R. Seymour, R. L. Julian, A. Jeanes, and B. L. Lamberts. Structural analysis of insoluble D-glucans by Fourier-transform, infrared difference-spectrometry: correlation between structures of dextrans from strains of *Leuconostoc mesenteroides* and of D-glucans from strains of *Streptococcus mutans*. Carbohydr. Res. 86, 227-246 (1980).
47. G. L. Côté and J. F. Robyt. Isolation and characterization of an extracellular glucansucrase from *Leuconostoc mesenteroides* NRRL B-1355 that synthesizes an alternating (1 \rightarrow 6), (1 \rightarrow 3)- α -D-glucan. Carbohydr. Res. 101, 57-74 (1982).
48. A. Misaki, M. Torii, T. Sawai, and I. J. Goldstein. Structure of the dextran of *Leuconostoc mesenteroides* B-1355. Carbohydr. Res. 84, 273-285 (1980).
49. G. L. Côté and J. F. Robyt. The formation of α -(1 \rightarrow 3) branch linkages by an exocellular glucansucrase from *Leuconostoc mesenteroides* NRRL B-742. Carbohydr. Res. 119, 141-156 (1983).
50. W. J. Lewicki, L. W. Long, and J. R. Edwards. Determination of the structure of a broth dextran produced by a cariogenic streptococcus. Carbohydr. Res. 17, 175-182 (1971).
51. L. W. Long and J. R. Edwards. Detailed structure of a dextran from a cariogenic bacterium. Carbohydr. Res. 24, 216-217 (1972).
52. M. Ceska, K. Granath, B. Norrman, and B. Guggenheim. Structural and enzymatic studies on glucans synthesized with glucosyltransferases of some strains of oral streptococci. Acta Chem. Scand. 26, 2223-2230 (1972).

53. J. K. Baird, V. M. C. Longyear, and D. C. Ellwood. Water insoluble and soluble glucans produced by extracellular glycosyltransferases from *Streptococcus mutans*. *Microbios* 8, 143-150 (1973).
54. T. Usui, H. Sugiyama, S. Seto, T. Nisizawa, S. Imai, and K. Kosaka. Structural determination of glucans from *Streptococcus mutans* JC2 (dental caries bacterium) by carbon-13 nuclear magnetic resonance. *J. Biochem.* 78, 225-227 (1975).
55. T. Nisizawa, S. Imai, H. Akada, M. Hinoide, and S. Araya. Extracellular glucans produced by oral streptococci. *Arch. Oral Biol.* 21, 207-213 (1976).
56. T. Nisizawa, S. Imai, and S. Araya. Methylation analysis of extracellular glucans produced by *Streptococcus mutans* strain JC2. *Arch. Oral Biol.* 22, 281-285 (1977).
57. M. Freedman, D. Birked, and K. Granath. Analyses of glucans from cariogenic and mutant *Streptococcus mutans*. *Infect. Immun.* 21, 17-27 (1978).
58. T. S. Meyer, B. L. Lamberts, and R. S. Egan. Structural studies of extracellular glucans of *Streptococcus mutans* by proton magnetic resonance. *Carbohydr. Res.* 66, 33-42 (1978).
59. M. D. Hare, S. Svensson, and G. J. Walker. Characterization of the extracellular, water-insoluble α -D-glucans of oral streptococci by methylation analysis and by enzymic synthesis and degradation. *Carbohydr. Res.* 66, 245-264 (1978).
60. P. Colson, H. C. Jarrell, B. L. Lamberts, and I. C. P. Smith. Determination, by carbon-13 nuclear magnetic resonance spectroscopy, of the composition of glucans synthesized by enzymes of the cariogenic organism *Streptococcus mutans*. *Carbohydr. Res.* 71, 265-272 (1979).
61. F. R. Highton and H. Weigel. The structure of an extracellular, water-soluble polysaccharide elaborated by the cariogenic *Streptococcus mutans* GS-5. *Carbohydr. Res.* 86, 105-112 (1980).
62. B. Guggenheim and E. Newbrun. Extracellular glucosyltransferase activity of an HS strain of *Streptococcus mutans*. *Helv. Odont. Acta* 13, 84-97 (1969).
63. W. B. Neely and J. Nott. Dextranucrase, an induced enzyme from *Leuconostoc mesenteroides*. *Biochem.* 1, 1136-1140 (1962).
64. M. Kobayashi and K. Matsuda. The dextranucrase isoenzymes of *Leuconostoc mesenteroides* NRRL B-1299. *Biochim. Biophys. Acta* 370, 441-449 (1974).
65. M. Kobayashi and K. Matsuda. Conversion of the extracellular dextranucrase isoenzymes from *Leuconostoc mesenteroides* NRRL B-1299. *Agric. Biol. Chem.* 39, 2087-2088 (1975).

66. M. Kobayashi and K. Matsuda. Purification and characterization of two activities of the intracellular dextranucrase from *Leuconostoc mesenteroides* NRRL B-1299. *Biochim. Biophys. Acta* 397, 69-79 (1975).
67. M. Kobayashi and K. Matsuda. Purification and properties of the extracellular dextranucrase from *Leuconostoc mesenteroides* NRRL B-1299. *J. Biochem.* 79, 1301-1308 (1976).
68. D. Suzuki and T. Kobayashi. Purification of dextranucrase and branching factor in dextran synthesis. *Agric. Biol. Chem.* 39, 557-558 (1975).
69. Y. Tsumuraya, N. Nakamura, and T. Kobayashi. Dextranucrase, and the role of metallic ions in the formation of branch links in dextran synthesis. *Agric. Biol. Chem.* 40, 1471-1477 (1976).
70. J. F. Robyt and T. F. Walseth. Production, purification, and properties of dextranucrase from *Leuconostoc mesenteroides* NRRL B-512F. *Carbohydr. Res.* 68, 95-111 (1979).
71. M. Kobayashi and K. Matsuda. Characterization of the multiple forms and main components of dextranucrase from *Leuconostoc mesenteroides* NRRL B-512F. *Biochim. Biophys. Acta* 614, 46-62 (1980).
72. A. M. Chludzinski, G. R. Germaine, and C. F. Schachtele. Purification and properties of dextranucrase from *Streptococcus mutans*. *J. Bacteriol.* 118, 1-7 (1974).
73. G. R. Germaine, A. M. Chludzinski, and C. F. Schachtele. *Streptococcus mutans* dextranucrase: requirement for primer dextran. *J. Bacteriol.* 120, 287-294 (1974).
74. H. Mukasa and H. D. Slade. Mechanism of the adherence of *Streptococcus mutans* to smooth surfaces. III. Purification and properties of the enzyme complex responsible for adherence. *Infect. Immun.* 10, 1135-1145 (1974).
75. H. K. Kuramitsu. Characterization of extracellular glucosyltransferase activity of *Streptococcus mutans*. *Infect. Immun.* 12, 738-749 (1975).
76. G. J. Walker and M. D. Hare. Metabolism of the polysaccharides of human dental plaque. Part II. Purification and properties of *Cladosporium resinae* (1→3)- α -D-glucanase, and the enzymic hydrolysis of glucans synthesized by extracellular D-glucosyltransferases of oral streptococci. *Carbohydr. Res.* 58, 415-432 (1977).
77. K. Asem, A. C. Kenney, A. Cornish-Bowden, and J. A. Cole. Purification of dextranucrase from *Streptococcus mutans* and its conversion to multiple lower molecular weight forms. *Soc. Gen. Microbiol. Quart.* 8, 252 (1981).
78. K. Fukushima, R. Motoda, K. Takada, and T. Ikeda. Resolution of *Streptococcus mutans* glucosyltransferase into two components essential to water-insoluble glucan synthesis. *FEBS Lett.* 128, 213-216 (1981).

79. K. Fukui, T. Moriyama, Y. Miyuke, K. Muzutani, and O. Tanaka. Purification and properties of glucosyltransferase responsible for water-insoluble glucan synthesis from *Streptococcus mutans*. *Infect. Immun.* 37, 1-9 (1982).
80. T. Koga, S. Sato, M. Inoue, K. Takeuchi, T. Furuta, and S. Hamada. Role of primers in glucan synthesis by glucosyltransferases from *Streptococcus mutans* strain OMZ 176. *J. Gen. Microbiol.* 129, 751-754 (1983).
81. K. Fukui, Y. Fukui, and T. Moriyama. Purification and properties of dextransucrase and invertase from *Streptococcus mutans*. *J. Bacteriol.* 118, 796-804 (1974).
82. W. R. Scales, L. W. Long, and J. R. Edwards. Purification and characterization of a glycosyltransferase complex from the culture broth of *Streptococcus mutans* FA1. *Carbohydr. Res.* 42, 325-338 (1975).
83. G. R. Germaine, S. K. Harlander, W.-L. S. Leung, and C. F. Schachte. *Streptococcus mutans* dextransucrase: functioning of primer dextran and endogenous dextransucrase in water-soluble and water-insoluble glucan synthesis. *Infect. Immun.* 16, 637-648 (1977).
84. A. Shimamura, H. Tsumori, and H. Mukasa. Purification and properties of *Streptococcus mutans* extracellular glucosyltransferase. *Biochim. Biophys. Acta* 702, 72-80 (1982).
85. M. M. McCabe and E. E. Smith. Specific method for the purification of *Streptococcus mutans* dextransucrase. *Infect. Immun.* 16, 760-765 (1977).
86. R. R. B. Russell. Glycosyltransferases of *Streptococcus mutans* strain Ingbritt. *Microbios* 23, 135-146 (1979).
87. R. R. B. Russell. Glucan-binding proteins of *Streptococcus mutans* serotype c. *J. Gen. Microbiol.* 112, 197-201 (1979).
88. D. J. Smith, M. A. Taubman, and J. L. Ebersole. Preparation of glucosyltransferase from *Streptococcus mutans* by elution from water-insoluble polysaccharide with a dissociating solvent. *Infect. Immun.* 23, 446-452 (1979).
89. W. R. Figures and J. R. Edwards. Improved purification procedure for the extracellular D-glucosyltransferase from *Streptococcus mutans* 6715. *Carbohydr. Res.* 73, 245-253 (1979).
90. W. R. Figures and J. R. Edwards. D-Glucosyltransferase of *Streptococcus mutans*: isolation of two forms of the enzyme that bind to insoluble dextran. *Carbohydr. Res.* 88, 107-117 (1981).
91. S. B. Mohan, B. M. Newman, and J. A. Cole. The interconversion of dextransucrase and mutansucrase activities from cariogenic strains of *Streptococcus mutans*. *FEMS Microbiol. Lett.* 5, 69-72 (1979).

92. B. M. Newman, P. White, S. B. Mohan, and J. A. Cole. Effect of dextran and ammonium sulphate on the reaction catalysed by a glucosyltransferase complex from *Streptococcus mutans*. J. Gen. Microbiol. 118, 353-366 (1980).
93. S. Thaniyavarn, K. G. Taylor, S. Singh, and R. J. Doyle. Pyridine analogs inhibit the glucosyltransferase of *Streptococcus mutans*. Infect. Immun. 37, 1101-1111 (1982).
94. T. Koga, S. Sato, T. Yakushiji, and M. Inoue. Separation of insoluble and soluble glucan-synthesizing glucosyltransferases of *Streptococcus mutans* OMZ 176 (serotype d). FEMS Microbiol. Lett. 16, 127-130 (1983).
95. W. R. Figures and J. R. Edwards. Resolution of the glycosyltransferase activities from two strains of *Streptococcus mutans* by polyacrylamide gel electrophoresis in the presence of Tween 80. Biochim. Biophys. Acta 577, 142-146 (1979).
96. J. E. Ciardi, G. J. Hageage, Jr., and C. L. Wittenberger. Multicomponent nature of the glucosyltransferase system of *Streptococcus mutans*. J. Dent. Res. 55, C87-C96 (1976).
97. J. E. Ciardi, A. J. Beaman, and C. L. Wittenberger. Purification, resolution, and interaction of the glucosyltransferases of *Streptococcus mutans* 6715. Infect. Immun. 18, 237-246 (1977).
98. K. Fukushima, R. Motoda, and T. Ikeda. Effects of exogenous insoluble glucan primer on insoluble glucan synthesis by *Streptococcus mutans*. J. Dent. Res. 60, 1707-1712 (1981).
99. R. R. B. Russell. Purification of *Streptococcus mutans* glucosyltransferase by polyethylene glycol precipitation. FEMS Microbiol. Lett. 6, 197-199 (1979).
100. F. Eisenberg, Jr. and S. Hestrin. Glycosyl transfer by levansucrase and dextransucrase. Bull. Res. Council of Israel. 11A4, 269-274 (1963).
101. D. S. Genghof and E. J. Hehre. *De novo* glycosidic linkage synthesis by glycosylases: α -D-glucosyl fluoride polymerization by dextransucrase. Proc. Soc. Exper. Biol. Med. 140, 1298-1301 (1972).
102. E. J. Hehre. The direction of propagation in carbohydrase-catalyzed polymerizations. J. Polym. Sci. C 23, 239-244 (1968).
103. K. H. Ebert and E. Schenk. Mechanism of biopolymer growth: the formation of dextran and levan. Adv. Enzymol. 30, 179-221 (1968).
104. K. H. Ebert and G. Schenk. Fermentpolymerization. IX. Kinetik und Mechanismus der enzymatischen Dextransynthese und Wirkung der Akzeptoren auf diese Reaktion. [Enzymatic polymerization. IX. Kinetics and mechanism of enzymatic dextran syntheses and the effect of acceptors on the reaction.] Z. Naturforsch. 23B, 788-797 (1968).
105. K. H. Ebert and M. Brosche. Origin of branches in native dextrans. Biopolymers 5, 423-430 (1967).

106. G. L. Côté and J. F. Robyt. The formation of α -(1 \rightarrow 3) branch linkages by a soluble-glucan-producing glucansucrase from *Streptococcus mutans* 6715. Carbohydr. Res.(submitted).
107. J. F. Robyt and A. J. Corrigan. The mechanism of dextransucrase action. Activation of dextransucrase from *Streptococcus mutans* OMZ 176 by dextran and modified dextran and the nonexistence of the primer requirement for the synthesis of dextran. Arch. Biochem. Biophys. 183, 726-731 (1977).
108. M. M. McCabe and E. E. Smith. The dextran acceptor reaction of dextransucrase from *Streptococcus mutans* K1R. Carbohydr. Res. 63, 223-239 (1978).
109. S. Sato, T. Koga, T. Yakushiji, S. Nagasawa, and M. Inoue. Effects of exogenous soluble dextrans on insoluble glucan synthesis by *Streptococcus mutans* glucosyltransferase. Microbios 34, 99-112 (1982).
110. M. Killey, R. J. Dimler, and J. E. Cluskey. Preparation of panose by the action of NRRL B-512 dextransucrase on a sucrose-maltose mixture. J. Amer. Chem. Soc. 77, 3315-3318 (1955).
111. R. W. Jones, A. Jeanes, C. S. Stringer, and H. M. Tsuchiya. Crystalline methyl α -isomaltoside and its homologs obtained by synthetic action of dextransucrase. J. Amer. Chem. Soc. 78, 2499-2502 (1956).
112. F. H. Stodola, E. S. Sharpe, and H. J. Koepsell. The preparation, properties and structure of the disaccharide leucrose. J. Amer. Chem. Soc. 78, 2514-2518 (1956).
113. R. W. Bailey, S. A. Barker, E. J. Bourne, P. M. Grant, and M. Stacey. Immunopolysaccharides. Part IX. The enzymic synthesis of trisaccharides containing the α -1:2-glucosidic linkage. J. Chem. Soc. 1895-1902 (1958).
114. E. J. Bourne, J. Hartigan, and H. Weigel. Mechanism of the enzymic synthesis of a branched trisaccharide containing the α -1:2-glucosidic linkage. J. Chem. Soc. 2332-2337 (1959).
115. W. B. Neely. Studies on the enzyme dextransucrase. II. The role of raffinose as an acceptor. Arch. Biochem. Biophys. 79, 154-161 (1959).
116. E. S. Sharpe, F. H. Stodola, and H. J. Koepsell. Formation of isomaltulose in enzymatic dextran synthesis. J. Org. Chem. 25, 1062-1063 (1960).
117. E. J. Bourne, J. Hartigan, and H. Weigel. Biosynthesis of α -D-glucopyranosyl D-galactofuranoside and other D-galactose-containing saccharides by *Betacoccus arabinosaceus*. J. Chem. Soc. 1088-1092 (1961).
118. E. J. Bourne, D. H. Hutson, and H. Weigel. Oligosaccharides in dextran-producing cultures of *Streptococcus bovis*. Biochem. J. 79, 549-553 (1961).

119. H. Suzuki and E. J. Hehre. Lactulosucrose (4^F - β -galactosylsucrose), a new trisaccharide synthesized by cultures of *Leuconostoc mesenteroides* strain K (NRRL B-1299). Arch. Biochem. Biophys. 105, 339-348 (1964).
120. E. J. Hehre and H. Suzuki. New reactions of dextransucrase: α -D-glucosyl transfers to and from the anomeric sites of lactulose and fructose. Arch. Biochem. Biophys. 113, 675-683 (1966).
121. Y. Iriki and E. J. Hehre. The scope of interanomeric glycosyl transfer reactions: heterodialdoside synthesis by enzymic glucosylation of D-galactose and D-mannose. Arch. Biochem. Biophys. 134, 130-138 (1969).
122. F. Yamauchi and Y. Ohwada. Synthesis of oligosaccharides by growing culture of *Leuconostoc mesenteroides*. Part IV. Oligosaccharide formation in the presence of various types of glucobioses as acceptors. Agric. Biol. Chem. 33, 1295-1300 (1969).
123. G. J. Walker. Branching-enzyme activity of an α -D-glucosyltransferase of *Streptococcus mutans*. Carbohydr. Res. 82, 404-410 (1980).
124. R. M. Mayer, M. M. Matthews, C. L. Futerman, V. K. Parnaik, and S. M. Jung. Dextransucrase: acceptor substrate reactions. Arch. Biochem. Biophys. 208, 278-287 (1981).
125. Y. Tsumuraya and A. Misaki. Purification and properties of glucosyltransferase produced by *Streptococcus salivarius* HHT. Agric. Biol. Chem. 46, 333-343 (1982).
126. V. K. Parnaik and R. M. Mayer. Mechanism of chain initiation by dextransucrase. Absence of terminal "sucrose" linkage in newly synthesized dextran chains. FEBS Lett. 150, 482-484 (1982).
127. G. L. Côté and J. F. Robyt. Acceptor reactions of alternansucrase from *Leuconostoc mesenteroides* NRRL B-1355. Carbohydr. Res. 111, 127-142 (1982).
128. L. G. Butler. Enzyme immobilization by adsorption on hydrophobic derivatives of cellulose and other hydrophilic materials. Arch. Biochem. Biophys. 171, 645-650 (1975).
129. J. F. Robyt and D. French. The action pattern of porcine pancreatic α -amylase in relationship to the substrate binding site of the enzyme. J. Biol. Chem. 245, 3917-3927 (1970).
130. A. Pulkownik, J. A. Thoma, and G. J. Walker. The action pattern and subsite map of *Streptococcus mutans* K1R dextranase. Carbohydr. Res. 61, 493-497 (1978).
131. G. J. Walker, A. Pulkownik, and J. G. Morrey-Jones. Metabolism of the polysaccharides of human dental plaque: release of dextranase in batch cultures of *Streptococcus mutans*. J. Gen. Microbiol. 127, 201-208 (1981).

ACKNOWLEDGMENTS

I would like to express my gratitude to Dr. John F. Robyt for his advice, support, and guidance during my stay here at Iowa State.

I would like to thank Dr. Bernard J. White, Dr. Carl L. Tipton, Dr. Paul A. Hartman, and Dr. Tommy J. Barton for serving on my committee.

I would like to express my sincere appreciation to the members of Dr. Robyt's research group for their advice, assistance, and friendship during the past few years, especially Dr. John N. Zikopoulos, Art Miller, Steve Eklund, Tom Binder, and Paula Martin. I would like to thank Dr. George Rottinghaus for his advice, encouragement, and friendship.

Finally, I would like to express my deepest appreciation to my family, and especially to my wife Melinda, for their love, support, and encouragement throughout my stay here at Iowa State.