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
Luzio, Gary A.

THE STUDY OF THE CATALYTIC PATHWAYS OF DEXTRANSUCRASE

The Ohio State University

Ph.D. 1982

University Microfilms International 300 N. Zeeb Road, Ann Arbor, MI 48106
THE STUDY OF THE CATALYTIC PATHWAYS OF DEXTRANSUCRASE

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By

Gary A. Luzio, B.S.

***

The Ohio State University
1982

Reading Committee:
Prof. R. M. Mayer
Prof. R. Pless
Prof. M-D. Tsai

Approved by

Adviser
Department of Chemistry
DEDICATION

To my parents
ACKNOWLEDGMENTS

I would like to express my deepest gratitude to my adviser, Dr. Robert M. Mayer, for his understanding and guidance given during this research work. His help given in preparation of this dissertation is also greatly appreciated. I also wish to thank the colleagues in my research group for their help and support.

I wish to acknowledge the financial support given to me by the Department of Chemistry, and National Institutes of Health for this research project.
VITA

July 12, 1950 . . . . . . . . . . . . Born - Newark, Ohio

1973 . . . . . . . . . . . . . . . . B.S., The Ohio State University

1973-1976 . . . . . . . . . . . . Analytical Chemist
Nestle Company, Marysville, Ohio

1976-1980 . . . . . . . . . . . . Teaching Assistant, Chemistry
Department, The Ohio State
University, Columbus, Ohio

1980-1982 . . . . . . . . . . . . Research Associate, Chemistry
Department, The Ohio State
University, Columbus, Ohio

PUBLICATIONS

"The Dissociation of Aggregate Forms of Dextranucrase", Luzio, G. A.,
Grahame, D. A., Mayer, R. M., Arch. of Biochem. and Biophys.,

"The Hydrolysis of Sucrose by Dextranucrase", Luzio, G. A.,
Mayer, R. M., Carbohydr. Res., accepted for publication,
August 1982.

FIELD OF STUDY

Major Field: Biochemistry
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>TABLE OF CONTENTS</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEDICATION</td>
<td>ii</td>
</tr>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>iii</td>
</tr>
<tr>
<td>VITA</td>
<td>iv</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>vii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>viii</td>
</tr>
<tr>
<td>CHAPTERS</td>
<td></td>
</tr>
<tr>
<td>I. INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>A. Preface</td>
<td>1</td>
</tr>
<tr>
<td>B. Structure of Dextran</td>
<td>3</td>
</tr>
<tr>
<td>C. Purification and Properties of Dextranucrase</td>
<td>7</td>
</tr>
<tr>
<td>D. Mechanisms of Catalysis</td>
<td>8</td>
</tr>
<tr>
<td>1. Group Transfer</td>
<td></td>
</tr>
<tr>
<td>2. Carbonium Ion Mechanism</td>
<td></td>
</tr>
<tr>
<td>3. Covalent Mechanism</td>
<td></td>
</tr>
<tr>
<td>E. Reactions Catalyzed by Dextranucrase</td>
<td>15</td>
</tr>
<tr>
<td>1. Donor Substrate Specificity</td>
<td></td>
</tr>
<tr>
<td>2. Transfer to Acceptor</td>
<td></td>
</tr>
<tr>
<td>3. De Novo Polymerization</td>
<td></td>
</tr>
<tr>
<td>4. Hydrolysis Reaction</td>
<td></td>
</tr>
<tr>
<td>5. The Formation of Branches</td>
<td></td>
</tr>
<tr>
<td>6. Isotope Exchange Reaction</td>
<td></td>
</tr>
<tr>
<td>F. Purpose of Investigation</td>
<td>29</td>
</tr>
<tr>
<td>II. MATERIALS AND METHODS</td>
<td>34</td>
</tr>
<tr>
<td>A. Materials</td>
<td>34</td>
</tr>
<tr>
<td>1. Bacteria</td>
<td></td>
</tr>
<tr>
<td>2. Enzymes and Proteins</td>
<td></td>
</tr>
<tr>
<td>3. Saccharides and Derivatives</td>
<td></td>
</tr>
<tr>
<td>4. Chromatography Materials</td>
<td></td>
</tr>
<tr>
<td>5. Chemicals</td>
<td></td>
</tr>
<tr>
<td>B. Methods</td>
<td>35</td>
</tr>
<tr>
<td>1. Assay for Dextranucrase</td>
<td></td>
</tr>
<tr>
<td>2. Assay for Sucrose Hydrolysis</td>
<td></td>
</tr>
<tr>
<td>3. Purification of Dextranucrase</td>
<td></td>
</tr>
<tr>
<td>4. Paper Chromatography</td>
<td></td>
</tr>
</tbody>
</table>
CONTENTS (Continued)

5. Radiological Procedures
6. Electrophoresis
7. Preparation of Immobilized Enzyme
8. Protein Analysis
9. Reducing End Analysis

III. RESULTS ........................................................................... 41

A. Properties of Dextranucrase in the Presence of
   Denaturants ......................................................................... 41
   1. Activity of Dextranucrase in Detergents
   2. Demonstration of Disaggregation by Detergents
   3. Behavior of Disaggregated Dextranucrase after
      Reaction with Sucrase
   4. Denaturation of Sucrose Treated Enzyme

B. Partial Reactions of Dextranucrase ................................. 56
   1. Hydrolysis of Sucrose by Dextranucrase
   2. Charged Dextranucrase Chased with Fructose
   3. Charged Dextranucrase Chased with Sucrose
   4. Charged Dextranucrase Chased with an Acceptor

C. Analysis of Non-Mobile Material ................................. 101

D. Competition Between Transfer to Acceptor and
   De Novo Synthesis ......................................................... 107

IV. DISCUSSION ...................................................................... 112

V. BIBLIOGRAPHY ............................................................... 128
<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Charged Dextranucrase Chased with Maltose</td>
<td>97</td>
</tr>
<tr>
<td>2. Reducing End Analysis of Products Released with Heat</td>
<td>103</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

Figure | Page
-------|------
1. Postulated Structure of Dextran from *S. Sanguis* | 6
2. Model for Chain Growth at the Reducing End for Dextranucrase | 24
3. Possible Catalytic Pathways for Reactions Catalyzed by Dextranucrase | 32
4. The Effect of Detergents on Dextranucrase Activity | 45
5. Gel Permeation of Dextranucrase in the Presence of Detergents | 48
6. Gel Permeation in the Presence of Detergents of Dextranucrase Reacted with Sucrose | 52
7. Gel Permeation in the Presence of SDS and 8M Urea of Dextranucrase Reacted with Sucrose | 55
8. Formation of Glucose by Dextranucrase | 59
9. Examination of Dextranucrase Activities Through the Use of Electrophoresis | 62
10. Examination of Dextranucrase Activities Through the Use of Electrophoresis | 64
11. Hydrolysis of Sucrose by Immobilized Enzyme | 67
12. (A) The Hydrolysis of Sucrose as a Function of Time (B) The Hydrolysis of Sucrose as a Function of Concentration | 70
13. Binding of Radioactivity to Immobilized Enzyme as a Function of Time | 74
14. Binding of Radioactivity to Immobilized Enzyme as a Function of Sucrose Concentration | 76
15. Chromatographic Analysis of Heat Releasable Sugars | 78
FIGURES (CONTINUED)

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>16. The Release of Glucose from Immobilized Enzyme as a Function of Reaction Time with Sucrose</td>
<td>81</td>
</tr>
<tr>
<td>17. The Release of Glucose from Immobilized Enzyme as a Function of Sucrose Concentration</td>
<td>83</td>
</tr>
<tr>
<td>18. Charged Dextranase Chased with Fructose</td>
<td>86</td>
</tr>
<tr>
<td>19. Exposure of the Product of the Fructose Chase Experiment to Invertase</td>
<td>89</td>
</tr>
<tr>
<td>20. Charged Dextranase Chased with Sucrose</td>
<td>92</td>
</tr>
<tr>
<td>21. Charged Dextranase Chased with Maltose</td>
<td>95</td>
</tr>
<tr>
<td>23. Gel Permeation of Heat-Released Material</td>
<td>106</td>
</tr>
<tr>
<td>24. Dextran Formation by De Nova Synthesis as a Function of Acceptor Concentration</td>
<td>110</td>
</tr>
<tr>
<td>25. Proposed Pathway for Reactions Catalyzed by Dextranase</td>
<td>125</td>
</tr>
</tbody>
</table>
I. INTRODUCTION

A. Preface

Dextran, utilizing sucrose as the glucosyl donor substrate (1). This polymerase is produced by a wide variety of microorganisms, which include Leuconostoc, Streptococcus, and Lactobacillus (1-6). Many of these organisms are cariogenic and the formation of dextran represents a key step in the production of dental caries (7, 8). Hence, a study of dextransucrase catalyzing the formation of dextran is central toward our understanding the cariogenic process.

The production of dextran by dextransucrase is classified as a group transfer reaction and can be represented by the following equation:

\[ \text{n sucrose} + (G)_m \xrightarrow{\text{Dextranucrase}} (G)_{m+n} + \text{n fructose} \]

In one cycle of this reaction sucrose serves as the donor substrate and a glucosyl residue is transferred to an acceptor dextran molecule, \((G)_m\), which contains \(m\) glucose residues. The product is one glucosyl unit longer than the acceptor. Repeated transfers of \(n\) molecules of glucose to each newly formed product results in polymerization to form a dextran of \((G)_{m+n}\) glucosyl units. Each glucosyl transfer also results in the release of one molecule of fructose; the other product in this reaction. The reaction as illustrated is apparently irreversible since the enzyme does not produce detectable amounts of sucrose from dextran plus D-fructose (9).
The reaction catalyzed by dextran-sucrase represents the first step in the metabolism of sucrose by oral bacteria contained in dental plaque. Dental plaque is thought to be a key element in the cariogenic process (10). A major component of the dental plaque are the insoluble dextrans and these oral bacteria have been shown to agglutinate in the presence of dextrans (11). The dental plaque may anchor the bacteria close to the tooth surface and could serve as a stable matrix for trapping nutrients needed by the oral bacteria for their metabolism. The plaque is also thought to restrict diffusion of metabolic end products such as lactic, pyruvic, and acetic acids, which result from the catabolism of the fructose formed in the dextran-sucrase reaction. The increase in the local concentrations of these organic acids results in a low pH at the tooth surface promoting demineralization of the dental apatite. Thus, it is readily apparent that both products, fructose and dextran, produced by dextran-sucrase serve a basic role in promoting dental caries and hence, dextran-sucrase itself becomes a key element in this process. The following discussion will give details concerning the structure of dextran and the purification and properties of dextran-sucrase. Group transfer mechanisms of catalysis will also be discussed. The reaction illustrated above for dextran-sucrase represents only one of the reactions catalyzed by this enzyme. Evidence for other reactions catalyzed by dextran-sucrase will be presented. Proposed mechanisms and substrate specificities for each of these reactions will be given accordingly.
B. Structure of Dextran

Dextran is a polysaccharide whose backbone structure consists of \( \alpha-1\rightarrow6 \) linkages. This structural feature was first determined by Hibbert in 1937 (12). Later studies (13) on dextrans had shown the presence of other secondary linkages such as \( \alpha-1\rightarrow3 \) linkages. Jeanes and coworkers in 1954 (14) characterized the dextrans produced by a large number of bacterial strains. The number and types of secondary linkages varied among the species and were chiefly comprised of \( \alpha-1\rightarrow2, \alpha-1\rightarrow3 \) and/or \( \alpha-1\rightarrow4 \) links, however \( \alpha-1\rightarrow6 \) was always a predominant linkage. As will be shown, these secondary linkages can also form branch points in the molecule. These highly branched molecules may have molecular weights of greater than \( 10^7 \) (15).

The high molecular weight, relative insolubility and ability to adhere to smooth surfaces provides dextrans with the capacity to adhere to tooth enamel. The structure of dextran has been reviewed in several articles (12, 13, 16), with the most recent one being published by Sidebothom in 1974 (16).

The dextran produced by \textit{S. sanguis} ATCC 10558 has been studied extensively in this laboratory. Previous studies on this dextran by Sidebotham (17) using periodate oxidation, showed 68% \( \alpha-1\rightarrow6 \) linkages, 9% \( \alpha-1\rightarrow3 \) linkages, and 23% \( \alpha-1\rightarrow2 \) linkages. More recently, Arnett and Mayer (18) showed that 54% of the glucose residues were \( \alpha-1\rightarrow6 \) linked, 13.6% were \( \alpha-1\rightarrow3 \) linked, 16.2% were terminal residues and 16.2% were branch points. No \( \alpha-1\rightarrow2 \) linkages were detected. The method of analysis was by gas liquid chromatography and mass spectrophotometry of the methylated alditol acetates derived from the polymer. Their data is consistent with the dextran structure
illustrated in Figure 1.

The data obtained by Arnett and Mayer showed average linkage composition and do not indicate if more than one type of dextran is being produced by *S. sanguis*. Other researchers have observed at least 2 different dextrans being produced by a single organism. For example, this observation was made by Tung and coworkers (19) studying *S. mutans*, and Kobayashi and Matsuda (20) working with *L. mesenteroides* NRRLB-1299. In this laboratory, Chen (21) examined this issue by treating *S. sanguis* dextran with dextranase to produce oligosaccharides. The oligosaccharides were methylated by the Hakamori (22) method and analyzed by gas-liquid chromatography and mass spectrophotometry. The methylated alditol acetate derivative of a tetrasaccharide showed the presence of α-1→6 and α-1→3 linkages at internal positions in the molecule. These data indicate that a single type of dextran was being produced by *S. sanguis*.

The production of more than one dextran by a single organism raises an important issue. This suggests that two or more enzymes are involved in the dextran synthesis. In addition, Bovey (23) has proposed that a branching enzyme is responsible for the formation of branch points. In this laboratory, it has not been possible to separate an enzymic activity capable of forming α-1→6 linkages from one responsible for producing α-1→3 branch points (24). Neither has any evidence been found for a branching enzyme which may be present in relatively small quantities. The mechanistic possibilities for producing branch points will be discussed later. Nevertheless, the method utilized for purifying enzyme preparations becomes vitally important if one is attempting to separate closely related enzymes or
Figure 1. Postulated Structure of Dextran from Streptococcus sanguis ATCC 10558.

A = Terminal glucosyl residue
B = (1, 6) linkage
C = (1, 3) linkage
D = (1, 3, 6) branch
Figure 1
attempting to find enzymes which may be present in relatively small quantities. Improving methods for purifying dextranucrase produced by *S. sanguis* has been an ongoing project in this laboratory for many years, and will be discussed next.

C. Purification and Properties of Dextranucrase

Dextranucrase from a variety of microorganisms has been isolated and purified by several different procedures. A procedure which uses dextranase-treatment to remove associated dextran was used by Robyt to purify the enzyme from *Leuconostoc mesenteroides* (25). Two protein bands were observed on disc-gel electrophoresis and the enzyme had a high specific activity. Two or more forms of dextranucrase isolated from other bacteria have also been reported (20, 26, 27, 28). Klein and coworkers (29) used ion exchange and gel filtration to isolate dextranucrase from *S. sanguis* OMZ9. They observed a single protein in pure form with a 13% recovery. Affinity chromatography on Biogel-insoluble dextran and gel filtration on a hydrophobic support was used by McCabe and Smith to isolate the *S. mutans* enzyme (30). The yield was 65%, however the specific activity reported was relatively low.

The dextranucrase used in these studies was isolated from *Streptococcus sanguis* ATCC 10558 by Huang's procedure (24). This procedure involved concentration and dialysis of the culture fluids, which were then absorbed onto hydroxyl apatite. Batch-wise elution with increasing concentrations of phosphate buffer at pH 6.0 was carried out, and the fraction containing the enzyme was applied to a DEAE cellulose column. Two major peaks of activity, peaks A and B, were eluted with increasing ionic strength. The peak B enzyme was
further purified to homogeneity on a hydroxyl apatite column
developed by Parnaik (31). The final yield of enzyme by this scheme
is about 20% with a 100 fold purification, and the specific activity
is high, 170 units per milligram of protein.

One property of dextranucrase which makes it difficult to study
is its propensity to form large aggregates (32-35), which prevents it
from penetrating gel-filtration columns, or polyacrylamide gels. In
addition, investigators have observed multiple forms which differ with
regard to molecular weights (36), charge (37), or charge-to-mass ratios
(38, 39).

D. Mechanisms of Catalysis

1. Group transfer

A generalized model for enzymes involved in carbohydrate
synthesis and degradation was presented by Hehre and co-workers (40).
Polysaccharide synthetic enzymes such as dextranucrase form one
glycosidic bond at the expense of another with the simultaneous
transfer of a proton, hence these enzymes fall into the general
category of group transfer enzymes. This transfer at the anomeric
carbon can be illustrated as follows:

\[ \text{Glycosyl-X} + \text{H-R} \rightleftharpoons \text{Glycosyl-R + H-X} \]

where X groups can be,

\[
\begin{align*}
\text{-O-} & \quad \text{nucleoside,} \\
\text{-O-P-O-} & \quad \text{OH, and saccharides} \\
\text{-O-} & \quad \text{OH}
\end{align*}
\]

and where R groups can be

\[
\begin{align*}
\text{H}_2\text{O, monosaccharide, R-\text{NH}_2, polypreonol-O-P-OH, etc.} \\
\text{OH}
\end{align*}
\]
The glycosyl-X group is labeled the donor substrate and is represented by sucrose in the dextran sucrase reaction. The H-R is the acceptor substrate which may be a mono, oligo, or polysaccharide in the dextran sucrase reaction. It is well known that group transfer reactions involve two principal reaction mechanisms and both of these may be involved with polysaccharide (or complex carbohydrate) synthesizing enzymes. In one mechanism the glycosyl moiety becomes covalently linked to the enzyme prior to transfer to the acceptor. In the other mechanism, the intermediate is not covalently linked and may form a carbonium ion. The following is a discussion of these two types of mechanisms and how they apply to the dextran sucrase reaction:

2. **Carbonium ion mechanism**

The first step of this mechanism (41) involves the acid catalyzed protonation of the oxygen in the glycosidic bond. The protonated X group, as shown above, can leave the active site, thus producing a glycosyl C-1 carbonium ion, which is stabilized by the lone pair on the ring oxygen, and the major form contributing to the resonance hybrid is probably the oxocarbonium ion.
If water is the accepting group, as is the case in many carbohydrates, then attack by water will produce the hemiacetal form of the product. The final configuration at the anomeric carbon can be retention or inversion depending on the direction of attack of the acceptor molecule.

Since a carbonium ion is formed at the C1 carbon during catalysis then this atom is sp2 hybridized making the intermediate trigonal planar at the C1 carbon. This planarity will distort the species from a chair form to a half-chair form. Wolfenden (42) has proposed that molecules which mimic transition state species at the active sites of enzymes can be potent inhibitors of those same enzymes. Such is the case with lysozyme where a carbonium ion intermediate has been implicated. The δ-lactone of tetra-N-acetylchitotetraose which is sp2 hybridized at C1 binds to lysozyme 32 times stronger than the substrate tetra-N-acetylchitotetraose and is an effective inhibitor of lysozyme (43).

\[ \text{δ-lactone of tetra-N-acetylchitotetraose} \]
Other glycosyltransferases that are known to involve carbonium ion intermediates, such as glycogen phosphorylase, are inhibited by analogs that are trigonal planar at the anomeric carbon. In the case of dextranucrase, Parnaik (31) showed that δ-lactone of glucose did not inhibit the enzyme, even at concentrations ten times greater than the $K_m$ for sucrose which is 5mM. This evidence suggests that a carbonium ion may not be involved in the catalytic pathway of dextranucrase.

3. Covalent mechanism

The first step of this mechanism (44) involves a nucleophile which attacks at the C1 carbon in the glycosidic bond in a bimolecular nucleophilic substitution reaction (SN2) to form a covalent intermediate or glycosyl-enzyme intermediate. The $X$ group of the donor molecule leaves, allowing a second SN2 attack by an acceptor substrate resulting in a second inversion of configuration at the anomeric carbon. Two inversions of configuration at the C1 carbon necessitates that the product retain the configuration of the donor substrate. Such is the case with dextranucrase where the dextran has the same $\alpha$-configuration that is present in sucrose. This evidence for the dextranucrase reaction is consistent with the formation of a covalent intermediate or an SN1 carbonium ion mechanism with exclusive $\alpha$-side attack, which must be directed by the group transfer enzyme.

Some group transfer enzymes that form covalent intermediates may also catalyze an isotope exchange reaction. Although isotope exchange does not rule out a carbonium ion mechanism, a demonstration of such an exchange is consistent with a covalent mechanism. An
overall isotope exchange reaction can be illustrated as follows:

\[ R-X + X^* \xrightarrow{\text{Enzyme}} R-X^* + X \]

In this reaction \( X^* \) is radioactively labeled. In the case of a covalent intermediate, \( \text{Enz-R} \), it can be seen that the isotope exchange reaction is really the reversal of the first half reaction, i.e.,

\[ R-X + \text{Enz} \xrightarrow{} \text{Enz-R} + X \]
\[ \text{Enz-R} + X^* \xrightarrow{} \text{Enz} + R-X^* \]

An example of this last case is sucrose phosphorylase which was shown by Doudoroff (45) to catalyze an isotope exchange of \( [^{14}\text{C}] \)-fructose into sucrose. Later, Voet and Abeles (46) demonstrated that this enzyme also forms a covalent intermediate during catalysis. However, cellobiose phosphorylase and maltose phosphorylase, which both have carbonium ion intermediates, do not catalyze isotope exchanges (47).

The two half reactions for sucrose phosphorylase both show isotope exchange reactions, which can be represented by:

1. \( \alpha-D-\text{glucose-1-P} + ^{32}\text{P}_i \xrightarrow{} \alpha-D-\text{glucose-1-}^{32}\text{P}_i + \text{P}_i \)
2. \( \text{sucrose} + D-[^{14}\text{C}]\text{fructose} \xrightarrow{} [^{14}\text{C}]\text{sucrose} + D-\text{fructose} \)

Sucrose phosphorylase also catalyzes a number of glucosyl transfer reactions. For example:

\( \text{sucrose} + \text{P}_i \xrightarrow{} D-\text{glucose-1-P} + D-\text{fructose} \)

Of importance the reaction with the acceptor, \( \text{P}_i \), follows ping-pong kinetics and \( D-\text{glucose} \) acts as a competitive inhibitor (48). This enzyme has a broad acceptor specificity and in addition to \( \text{P}_i \) it catalyzes transfer to \( D-\text{fructose}, L-\text{sorbose}, D-\text{xylose}, L-\text{arabinose}, L-\text{arabinulose}, \) and \( \text{AsO}_4^- \).
The enzyme also catalyzes the hydrolysis of sucrose which is irreversible (48, 49). From the kinetic data it was calculated that the hydrolysis of the glucose-enzyme complex was less than 0.2 sec. Therefore, Voet and Abeles (46) noted that for the complex to be isolated, it had to be denatured rapidly or chemically modified to reduce the rate of hydrolysis. To inactivate the enzyme and thus prevent hydrolysis, sodium periodate was used to stop the reaction. Exposure to 6M urea in acid did not release the glucose from the enzyme. A similar glucosylated enzyme was isolated after stopping the reaction with acid. Pepsin digestion of the glucosylated enzyme from both the periodate treated and acid treated enzyme yielded glucosylated peptides. The glucosyl-enzyme bond was found to be unstable in mild base at pH8, which argues for an ester linkage with aspartic or glutamic acid or an O-glycosidic bond to serine or threonine. The specific amino acid involved in the covalent linkage could not be identified. Glucosyl enzyme intermediates have also been implicated with other glucosyl transferases such as methyl-D-glucosidase (50).

It has not been clearly established if a glucosyl enzyme intermediate is involved in the dextranucrase reaction. As with sucrose phosphorylase, dextranucrase from _S. sanguis_ also catalyzes an isotope exchange reaction (9) which will be discussed later in the introduction. Two other important features are the retention of the α configuration of sucrose in the dextran product, and the lack of inhibition by carbonium ion transition state analogs (31). In addition, Robyt and Walseth argued (51) that acceptor substrates such as glucose, fructose and maltose could release dextranyl and single glucosyl
residues from "charged" enzyme. In a typical experiment, enzyme was incubated with unlabeled sucrose for 30 min. The reaction mixture was passed over a Bio-Gel P6 column to separate the reacted or "charged" enzyme from low molecular weight compounds such as unreacted sucrose. The charged enzyme was reacted with $^{14}C$ maltose for 24 hours and analyzed by paper chromatography. A radioactively labeled trisaccharide was observed on the chromatogram. However, in a control reaction with uncharged enzyme plus $^{14}C$ maltose, a $^{14}C$-trisaccharide was also produced. This material was not present in the unreacted $^{14}C$ maltose.

In addition, Parnaik (31) noted that the acceptor-products observed were in 60-fold excess of that expected. During a reaction performed in the presence of sucrose, the polysaccharide release to acceptor was $2.5 \times 10^{-6}$ moles dextran/μ/min. This represented 0.4% of the total reaction where the rate of fructose release is 1 μmole fructose/μ/min.

On this basis, Parnaik argued that this could not be a major pathway for release of dextran chains from the enzyme.

In a separate experiment Robyt and co-workers (35) reacted immobilized dextranucrase with 1 μCi of $^{14}C$ sucrose for 10 hrs to allow the reaction to go to completion. After extensive washing radioactivity was observed to be bound to the immobilized enzyme. This form was called "charged" enzyme. The charged enzyme was heated at 95°C for 10 min at pH2 and the released material was analyzed by paper chromatography. Dextran and glucose were observed on the chromatogram. The radioactivity in $^{14}C$ glucose was 837 cpm which represented about 0.1% of the total radioactivity used in the reaction. No $^{14}C$ glucose was observed when the charged enzyme was chased with sucrose. The authors argued that a covalent intermediate was involved in the reaction.
E. Reactions Catalyzed by Dextranucrase

It is known that dextranucrase catalyzes the transfer of glucosyl residues to acceptors (52-55) and has the ability to produce dextran from sucrose in the absence of acceptors (52, 54, 55). Dextranucrase has been implicated in the catalysis of two other reactions, the branching of dextran (16) and the hydrolysis of sucrose (56, 57). In addition, dextranucrase has been shown to catalyze an isotope exchange reaction with sucrose and $^{14}C$fructose to produce $^{14}C$sucrose (9). All of these reactions, if they occur, would involve the participation of the donor substrate, sucrose. The following will include a discussion of the donor substrate specificity and the evidence concerning each one of the aforementioned reactions.

1. Donor Substrate Specificity

Of interest is the narrow specificity of dextran sucrose for the donor substrate, since sucrose appears to be the only naturally occurring donor molecule (58). Hehre and Suzuki have shown that lactulosucrose may be an effective donor for the Leuconostoc enzyme (2). Lactulosucrose reacts to form dextran at about one third the rate measured with sucrose. Lactulosucrose can be formed in the transferase reaction when a glucosyl residue is transferred from sucrose to lactulose ($\alpha$-D-6-β-D-galactosylfructose) by Leuconostoc dextranucrase. Genghoff and Hehre demonstrated that $\alpha$-D-1 fluorogluucose could also function as a donor (59). The fluorine atom is slightly smaller than the C-1 oxygen in sucrose and the fluorine bond contains about the same free energy of hydrolysis as the glycosidic bond in sucrose. Jung (58) demonstrated that $\alpha$-D-1 fluorogluucose is an effective donor substrate.
for the *S. sanguis* dextran sucrase in the de novo synthesis reaction where polymer is formed in the absence of added acceptor. In the presence of maltose, the glucosyl moiety of α-fluoroglucose was transferred to the disaccharide to form a series of oligosaccharides analogous to the reaction involving sucrose and maltose. Of significance, the $K_m$ and $V_{max}$ values for sucrose and α-fluoroglucose are very similar indicating that α-fluoroglucose is as effective a donor substrate as sucrose. In an attempt to find additional donor substrates Grier (60) synthesized a series of fluoromonosaccharides which were epimers or deoxy analogs of α-fluoroglucose. In every instance these related compounds did not function as donor substrates but were competitive inhibitors. This emphasizes the absolute specificity of dextran sucrase toward the glucose configuration of the donor substrate. This is consistent with the inability to discover other natural donor substrates for dextran sucrase.

2. **Transfer to Acceptor**

   (primer dependent polymerization)

   One of the most extensively studied reactions of dextran sucrase, is its ability to transfer single glucose units to a variety of acceptor substrates. In direct contrast to the narrow specificity of the donor substrate, the specificity toward acceptors is very broad (52–54). A variety of mono and oligosaccharides as well as dextran function as acceptors. Disaccharides such as maltose and isomaltose compete effectively with dextran to produce a series of oligosaccharides. A monosaccharide, α-methyl glucoside, has also been shown to be an effective acceptor. The most effective acceptors appear to be molecules that contain one or more
α-D-glucopyranosyl residues at the nonreducing end (13, 55). This preference for the α configuration may account for the observation that D-glucose is a less effective acceptor than α-methyl glucoside, since an equilibrium exists between the α and β anomers of D-glucose in solution.

The transfer of glucose residues to an acceptor such as isomaltose could conceivably occur at either the reducing or nonreducing end of the disaccharide. Walker (53) determined the direction of addition by reacting dextransucrase with \(^{14}C\) sucrose and isomaltose. The \(^{14}C\)-labeled isomaltotriose product was isolated and reduced with borohydride to form sorbitol at the reducing end of the molecule. After acid hydrolysis, the monosaccharides were separated by paper chromatography. Only 0.15% of the radioactivity migrated with sorbitol, with most of the radioactivity migrating as glucose, which shows that the transfer of \(^{14}C\) glucose residues to the isomaltose occurred at the nonreducing end. By a similar procedure, Jung (58) demonstrated that S. sanguis dextransucrase transfers \(^{14}C\) glucosyl residues from 1-α-fluoro \(^{14}C\) glucose to the nonreducing end of maltose. In support of a mechanism involving chain growth at the non-reducing end, Neely (13) suggested the simultaneous binding of sucrose at a donor site and acceptor at a receptor site. The glucosyl residue from the sucrose is then transferred to the acceptor molecule. Subsequently, glucosyl units are transferred to the non-reducing end of the terminal glucose residue of the growing chain.

Of interest is the fact that the product always has the proper configuration by containing an α-D-glucopyranosyl residue at the nonreducing end. Considering acceptor specificity, dextransucrase
will readily bind the newly formed product and thus will transfer another glucose molecule, eventually forming a polymer. This is one of two possible modes by which dextranucrase forms polymer. This mode of acceptor or primer dependent polymerization is a multi-chain mechanism which will be discussed in the next section.

Mayer and co-workers (55) examined the relationship between the acceptor and its product in the dextranucrase reaction. They established that the kinetics of the acceptor substrate reaction were consistent with the formation of a series of precursors and products by the transfer of glucose to the original acceptor. Each newly formed product differed from its precursor by only a single glucose residue. The data was consistent with a precursor product relationship where dextranucrase catalyzed the transfer of single glucose residues to the acceptor.

3. De Novo Polymerization

The alternative to primer dependent polymer formation is a primer independent polymerization or de novo synthesis. Mayer and co-workers (55) demonstrated that dextranucrase catalyzes the conversion of sucrose to dextran by de novo synthesis. The major difficulty in experiments of this type is the possibility that the enzyme preparation contains endogenous acceptors. In this regard they utilized highly purified enzyme preparations, in which no carbohydrate was detectable. In addition, the enzyme preparation was treated with dextranase and α-glucosidase and no effect was observed on the ability of dextranucrase to utilize sucrose. To further reduce the possible introduction of acceptors, sucrose was treated to remove any possible oligosaccharide or polysaccharide
contaminants. This did not alter the catalytic rate. Thus it appears reasonable that dextranucrase can catalyze two polymerizations, one in the absence of acceptors by de novo synthesis and the other by transferring glucosyl residues to acceptors.

As with any polymerization, these two modes of polymerization can be divided into three stages, chain initiation, propagation and termination. In the acceptor dependent polymerization, the introduction of an acceptor circumvents the requirement for initiation since the acceptor represents the start of the chain itself. A similar process could occur in de novo synthesis. If sucrose functions as an acceptor then the initial transfer of a glucosyl residue would be to sucrose. However, another possibility is that the initial transfer could occur to another glucosyl enzyme intermediate at an adjacent active site as proposed by Ebert and Schenk (34) and also by Robyt (35).

Chain propagation involves the transfer of new glucosyl residues to the nascent chain. The growth of the polysaccharide chain may continue until a molecular weight of several million has been attained (15). Chain propagation at an active site can occur by two general mechanisms:

1. A single-chain mechanism, wherein the enzyme remains associated with the growing polymer chain through a large number of propagative steps.

2. A multi-chain mechanism where glucosyl units are transferred to multiple chains which undergo continuous association and dissociation at the active site.
The acceptor dependent polymerization proceeds via a multi-chain mechanism where products may release and then reassociate to function as acceptors. In this manner there may exist a large number of growing chains per active site. Each newly formed product can function as an acceptor and thus repetitive transfers of glucosyl units results in chain elongation. On the other hand de novo synthesis is thought to occur by a single chain mechanism (16) where the growing polymer remains associated with the active site. Multiple transfers between linked sites is a proposed model for chain propagation (34, 35).

Chain termination for de novo synthesis could occur by dissociation of the polymer from the enzyme. The released polymer could then function as an acceptor in the primer dependent polymerization reaction and thus polymerization may continue as long as sucrose is present. The following discussion will concentrate on the limited amount of evidence concerning de novo synthesis and its possible mechanisms.

If sucrose itself is acting as an acceptor, then de novo synthesis by this mechanism would be analogous to the primer dependent mechanism in chain initiation. Considering the broad specificity of dextran-sucrase toward acceptors and that sucrose contains an $\alpha$ linked glucosyl moiety in a nonreducing end, then it seems likely that sucrose could function as an acceptor. In support of sucrose functioning as an acceptor, Behre (61) isolated a low molecular weight dextran (55,000-60,000 daltons) that contained fructose at its reducing end in a sucrose-like linkage which was susceptible to hydrolysis by yeast invertase. In addition, Stacey and co-workers (62) demonstrated that the trisaccharide glucose $\alpha 1-6$ glucose $\alpha 1-2$ fructose, served as
an effective acceptor for dextranucrase.

However, if sucrose were functioning as an acceptor one would expect a behavior similar to that observed with other acceptors, and clearly this is not the case. Using a preparation of dextranucrase which was isolated from _S. sanguis_ and which was free of endogenous acceptors, Mayer and co-workers (55) chromatographed the products of a reaction between $^{14}C$ sucrose and dextranucrase. The only products observed were nonmobile on paper chromatograms and appeared to be much higher molecular weight materials than seen with normal acceptors which form a series of oligosaccharides. Of importance, trehalose which can be considered an analog of sucrose did not exhibit any acceptor activity. In addition, Walker (53) was unable to detect any oligosaccharides in the early stages of dextran synthesis from sucrose by the _Leuconostoc_ enzyme. In an attempt to see if sucrose was incorporated into polymer, Parnaik (31) reacted $^{14}C$ sucrose, labeled only in the fructose portion, with _S. sanguis_ dextranucrase, but did not observe radioactivity being incorporated into product in a manner which is observed with other acceptors. The data indicated the absence of a terminal "sucrose" linkage which would be expected if sucrose served as the initiator. Further, if sucrose is functioning as an acceptor, then addition to the growing chain should be at the non-reducing end which is observed when acceptors are present. In another experiment, Parnaik reacted immobilized dextranucrase with $^{14}C$ sucrose and then washed away the unreacted sucrose. Unlabeled sucrose was then added to equal amounts of this "charged" enzyme for a period of 0.5, 1, 2, and 5 min. A reducing end analysis was performed on each reaction mixture and the radioactivity in sorbitol was shown to decrease with
increasing time indicating that addition was occurring at the reducing end in direct contrast to normal acceptor reactions. Prior to this, Robyt (35) had performed a similar experiment with the Leuconostoc enzyme and also observed growth occurring at the reducing end. These observations indicate that the enzyme itself is capable of initiating polymerization without the use of sucrose as an acceptor, and for this to occur requires the presence of at least two adjacent active sites which would act in tandem to form polymer.

Robyt (35) has proposed such a model for the biosynthesis of dextran. This model, as shown in Figure 3, requires the participation of two equivalent nucleophilic groups at the active site: $X_1$ and $X_2$. After binding 2 molecules of sucrose, both nucleophilic groups become glucosylated releasing 2 molecules of fructose. The C-6 hydroxyl group of one of the glucosyl units can then make a nucleophilic attack at the C-1 of the other glucosyl unit. This releases one of the nucleophilic units and forms an isomaltosyl moiety covalently bound to the enzyme. The free nucleophile is again glucosylated which attacks the isomaltosyl-enzyme linkage forming an isomaltotriosyl unit releasing the other nucleophile. Repetition of this process results in formation of polymer by chain propagation. The chain might be transferred to water or an acceptor resulting in chain termination. A similar mechanism for chain growth was proposed earlier by Ebert and Schenk (34), however their model does not require a covalent intermediate. This mechanism results in growth occurring at the reducing end consistent with Parnaik's and Robyt's findings. In addition, oligosaccharides may not be released in measurable quantities and the dextran propagation would be by single-chain mechanism resulting in
Figure 2. Model for Chain Growth at the Reducing End for Dextran-sucrase as Proposed by Robyt.

where:

$X_1, X_2$ = Two separate nucleophilic groups at the active site of dextran-sucrase

$O$ = glucose  
$\Delta$ = fructose  
$O\leftarrow O$ = sucrose  
$O\rightarrow O$ = two glucose units linked by an $\alpha-1,6$ glucosidic bond
Figure 2
dextrans with very high molecular weights. Bovey (15) has shown by light scattering measurements that molecular weights of de novo synthesized dextrans are very high, $50 \times 10^6$ daltons. More recent results by end group analysis indicate molecular weights $3-5 \times 10^5$ daltons (34).

4. Hydrolysis reaction

Another reaction which dextransucrase might catalyze is the hydrolysis of sucrose to form fructose and glucose. To date, there exist only a few publications which discuss this reaction in some detail. If hydrolysis occurs, glucose will be present in the reaction media, and trace amounts of glucose were first detected by Forsyth (63). Later, using dextransucrase isolated from *Leuconostoc mesenteroides*, Goodman and co-workers (56) in 1955 demonstrated sucrose hydrolysis activity was associated with this enzyme preparation. They inhibited invertase activity with $10^{-4}$ M silver nitrate, but did not demonstrate that the hydrolysis activity was directly associated with dextransucrase. In 1974, Fukui et al. (57) isolated a dextransucrase and an invertase from *S. mutans*. The isolated dextransucrase was shown to possess hydrolytic activity. The activity was diminished by the addition of an acceptor substrate, dextran. Based on this competition, the authors claimed that the *S. mutans* dextransucrase possesses the ability to hydrolyze sucrose. Invertase activity has also been observed in other dextransucrase preparations (33, 64), and it is unclear if some of this hydrolytic activity may be attributed to the presence of dextransucrase.
5. **The Formation of Branches**

Several mechanisms for branch formation have been proposed (16). A two enzyme reaction which would involve dextran sucrose and a separate branching enzyme or two other possible mechanisms which include the transferase capability of dextran sucrose.

As mentioned previously, Bovey (23) has argued for a two enzyme reaction which required a branching enzyme in addition to dextran sucrose. Bovey proposed that branches could be formed by scission and rearrangement of linear skeletal chains by the branching enzyme. This proposal stemmed from light-scattering and periodate-oxidation measurements during the early stages of dextran synthesis where he observed that the molecular weights of dextrans continue to increase after all the sucrose has been utilized. This indicated that branching and complexity of the dextrans were increasing. At higher temperatures this was more pronounced. The branching enzyme was believed to be more thermally stable than dextran sucrose and to be active only in the presence of magnesium ions. Subsequent workers (65, 66) failed to demonstrate a particular metal-ion requirement for branched-dextran synthesis or to isolate a separate branching-enzyme by means of refined separatory techniques. On this basis, Sidebotham (16) argued that dextran sucrose appeared to be responsible for the synthesis of both the skeletal chains and the branch linkages in dextrans. In addition, Ebert and co-workers (67) observed that the high molecular weights reported by Bovey of 2 to $5 \times 10^8$ are particle weights of associations of dextrans having molecular weights of approximately $3$ to $5 \times 10^5$. Therefore, increases in molecular weight attributed to a branching enzyme may be due to increased association of dextrans of constant molecular weight.
Observing that dextransucrase possessed transferase prompted Ebert and Brosche (68) to propose a mechanism which involves only dextransucrase. In this mechanism, a free dextran molecule functions as an acceptor for a dextranyl chain being synthesized by a single-chain mechanism on the enzyme. They tested this hypothesis by adding low molecular weight $^3$H dextran to a reaction mixture containing sucrose and dextransucrase, and observed that $^3$H label was being incorporated into high molecular weight dextran. However, this would also occur if glucosyl units were being added to the $^3$H dextran in the primer dependent reaction.

To further test this possibility, Robyt and Taniguchi (69) added $^{14}$C sucrose to immobilized dextransucrase, and then washed away unreacted sucrose. A low molecular weight dextran (7,000 daltons) was then added to the pulsed enzyme and $^{14}$C-dextran was released and subjected to acetalolysis. The products contained 92.7% $^{14}$C glucose and 7.3% $^{14}$C nigerose, which was labeled only at the reducing end. The nigerose indicates the presence of $\alpha$-1→3 linkages and the evidence supports the transfer of a labeled dextranyl or glucosyl moiety to the nonlabeled acceptor to form an $\alpha$-1→3 linkage.

A third mechanism of branch formation has been proposed by Hehre (70). In this mechanism, the branches are produced exclusively by transfers of D-glucopyranosyl groups from sucrose to the nonreducing ends of growing branch-chains. The author argued that the rapid synthesis of high molecular weight dextrans could occur by the simultaneous propagation of several branch-chains. Sidebotham (16) has argued that neither mechanism alone can account for all of the branching features observed in the dextran.
6. **Isotope Exchange Reaction**

The observation by Bourne, Peters and Weigel (71) that dextranulose could catalyze an isotope exchange reaction was the first demonstration that the enzyme's reaction with sucrose was reversible. Using *L. mesenteroides* dextranulose they observed the following reaction:

\[
\text{sucrose} + D\left(^{14}\text{C}\right)\text{fructose} \xrightarrow{\text{enzyme}} D\text{-fructose} + \left(^{14}\text{C}\right)\text{sucrose}
\]

Radiochromatograms showed four $^{14}$C-labeled products after incubating sucrose and D-$^{14}$C-fructose with dextranulose. One product was $\left(^{14}\text{C}\right)$sucrose. $\left(^{14}\text{C}\right)$Leucrose (5-O-a-D-glucopyranosyl-D-fructose) was also found and is formed by the transfer of glucose to the C-5 oxygen of D-fructose. The other two products were thought to be formed by successive transfer of glucose to sucrose which may be functioning as an acceptor. Both of these products yielded isomaltose, glucose, and $\left(^{14}\text{C}\right)$fructose on acid hydrolysis.

In our laboratory, Huang (9) demonstrated that the *S. sanguis* dextranulose could also catalyze an isotope exchange reaction between $\left(^{14}\text{C}\right)$fructose and unlabeled sucrose. The dextranulose was incubated with sucrose and a trace amount of $\left(^{14}\text{C}\right)$fructose. As a function of time, $\left(^{14}\text{C}\right)$sucrose was formed in increasing amounts as observed on paper chromatograms. An important control reaction was performed by incubating the enzyme with $\left(^{14}\text{C}\right)$fructose and unlabeled dextran in the absence of sucrose. Since only small amounts of $\left(^{14}\text{C}\right)$sucrose were observed, this indicated that the isotope exchange observed was due to reversal of the first-half reaction, the reaction with sucrose, rather than reversal of the overall transferase reaction. Reaction of the enzyme with $\left(^{14}\text{C}\right)$fructose, unlabeled sucrose, and unlabeled dextran as an
acceptor showed a diminished rate of $^{14}_C$ sucrose production relative to the reaction in the absence of acceptor. This suggests that the dextran and the $^{14}_C$ fructose are competing for the same form of the enzyme. In addition, Jung (58) in our laboratory observed that dextran-sucrase could catalyze an isotope exchange between $\alpha$-fluoroglucose and $^{14}_C$ fructose.

F. Purpose of Investigation

As mentioned earlier, it has not been clearly established that a glucosyl intermediate is involved in the dextran-sucrase reaction. Robyt et al. (51) has presented evidence to support such an intermediate. However, their interpretation is based on data that only represents a small fraction (0.4%) of the total reaction, and may not represent the major pathway (31). In addition, other inconsistencies in the data were noted. To provide direct evidence requires the isolation of an enzyme intermediate which is a competent catalyst. A competent transfer is one which approximates the normal catalytic rate of the enzyme. In addition, the products produced in such a transfer should represent a major part of the total reaction. Thus no direct evidence for a glucosyl intermediate exists. Nevertheless, several observations have been made which are consistent with a covalent intermediate such as the retention of the $\alpha$ configuration of sucrose in the dextran product and the lack of inhibition by carbonium ion transition state analogs (31). In addition, dextran-sucrase also catalyzes an isotope exchange reaction. Therefore, it seems reasonable that a glucosyl intermediate is involved in the reaction, and would be formed in a glucosylation reaction involving sucrose.
Assuming that such a glucosylation reaction exists, one can examine its potential involvement in the reactions catalyzed by dextran-sucrase. A representation of such an assumption is shown in Figure 3. The formation of the glucosyl intermediate would occur in the reaction with sucrose as shown in reaction 1. The reaction would be reversible as indicated previously by the isotope exchange experiments. Reaction 3 illustrates the transfer of glucosyl residues to acceptors. The reaction, as illustrated, would transfer a single glucosyl residue to each acceptor in the first transfer. Evidence for such a precursor product relationship was discussed earlier. It has been demonstrated that dextran-sucrase can catalyze the conversion of sucrose to dextran by de novo synthesis. Assuming the involvement of a glucosyl enzyme intermediate, the de novo synthesis reaction is illustrated by reaction 4. Based on the Robyt model (35) the product would be a dextranyl enzyme complex. Such a dextranyl-enzyme complex may undergo hydrolysis (reaction 5) or transfer to acceptor (reaction 6) and thus both would function as chain termination reactions. Preparations of dextran-sucrase have been shown to possess hydrolytic activity. Reaction 2 illustrates the potential involvement of a glucosyl intermediate in the hydrolysis of sucrose. No direct evidence exists for any of these last three reactions as denoted by the question marks appearing on the figure.

Thus, a key toward providing the initial evidence for such a pathway involves the isolation of glucosyl or dextranyl enzyme complexes. This investigation was carried out to isolate these possible species and to examine them to see if they are competent in transferring glucosyl or dextranyl residues. Another goal was to examine the possible involvement of covalent intermediates in each of the reactions described.
Figure 3. Possible Catalytic Pathways for Reactions Catalyzed by Dextranucrase

1. Reaction with sucrose
2. Hydrolysis reaction ?
3. Transfer to acceptor
4. De novo synthesis
5. Hydrolysis of dextranyl intermediate ?
6. Transfer of dextranyl intermediate ?

? Indicates reactions which have not been established for dextranucrase
Figure 3
Further evidence for common enzyme intermediates could be provided by examining possible competitive relationships between two or more reactions.
II. MATERIALS AND METHODS

A. Materials

1. Bacteria

Streptococcus sanguis ATCC 10558 was obtained from the American Type Culture Collection, Washington, D.C., and stored for laboratory use as a lyophilized powder at -20°C.

2. Enzymes and Proteins

The following enzyme preparations were purchased from Sigma Chemical Company (St. Louis, MO.): glucose-6-phosphate dehydrogenase (Type XV from Baker's yeast), hexokinase (Type F-300), phosphoglucose isomerase, invertase (from Baker's yeast), and bovine serum albumin. Pyruvate dehydrogenase was kindly provided by Dr. P.A. Frey. Dextranucrase was obtained using the procedure described in Methods.

3. Saccharides and Derivatives

Saccharides were obtained commercially from the following sources: sucrose from Mallinckrodt, Inc., Paris, KY.; D-fructose from J.T. Baker Chemical Co., Phillipsburg, N.J.; D-glucose, maltose, isomaltose, raffinose, and isomaltotriose from Sigma; sorbitol from Pfanstiehl Laboratories, Waukegan, IL.

Dextran T 10 and blue dextran 2000 were from Pharmacia Fine Chemicals, Piscataway, N.J. Dextran T 10 was dialyzed extensively, to remove low molecular weight oligosaccharides, and then lyophilized.

Radioactive saccharides were obtained as follows: \( \text{[U-}^{14}\text{C]} \) sucrose, (602 mCi/mmmole) from New England Nuclear, Boston, MA.; \( \text{[U-}^{14}\text{C]} \) maltose.
(5.9 mCi/mmole) and \( [6,6'\text{(n)}-\text{H}] \) sucrose (9.7 ci/mmole) from Amersham Corp. (Arlington Heights, IL.).

4. Chromatography Materials

Chromatography materials were obtained as follows: Sepharose 6B, Sepharose 6B-CL and Sephadex G-75 from Pharmacia; BioGel HTP from Bio-Rad Laboratories (Richmond, CA.); Whatman 1 MM and 3MM chromatography paper from Whatman, Inc. (Clifton, N.J.); diethylaminoethyl-cellulose (DEAE-cellulose) from Sigma.

5. Chemicals

Reagent grade chemicals and materials were obtained from the following sources: benzene, urea, sodium borohydride, and anthrone from Matheson, Coleman and Bell (Cincinnati, OH.); sodium phosphate from Mallinckrodt, Inc.; sodium thiocyanate, and boric acid from J.T. Baker Chemical Co.; Insta-Gel from Packard Instrument Co. (Downers Grove, IL.); Triton X-100, Tween 80, phosphatidyl ethanolamine, sodium dodecylsulfate (SDS), polyethylene glycol-300, sodium deoxycholate, 1,4-bis-[2-(5-phenyloxazolyl)]-benzene (POPOP), 2,5-diphenyloxazole (PPO), hydroxylapatite (suspension in 0.001 M phosphate buffer, pH 6.8, type 1), nicotinamide adenine dinucleotide phosphate (monosodium salt), adenosine-5'-triphosphate (disodium salt) and tris-(hydroxymethyl) aminomethane (trizma base) from Sigma; Brain Heart Infusion (dehydrated) from Difco Laboratories (Detroit, MI.). All other chemicals used in this study were of reagent or spectroscopic grade and were purchased from commercial sources.

B. Methods

1. Assay for Dextranucrase

Dextranucrase activity was determined by a measurement of the fructose produced during the following reaction:
sucrose + (glucose)\textsubscript{n} \xrightarrow{\text{dextran sucrase}} (glucose)\textsubscript{n+1} + fructose

The quantity of fructose was measured in the following coupled assay by a modification (24) of the procedure described by Carlson, Newbrun, and Krasse (72):

\[
\text{fructose} + \text{ATP} \xrightarrow{\text{hexokinase}} \text{fructose-6-phosphate} + \text{ADP}
\]

\[
\text{fructose-6-phosphate} \xrightarrow{\text{phosphoglucomutase}} \text{glucose-6-phosphate}
\]

\[
\text{glucose-6-phosphate} + \text{NADP}^+ \xrightarrow{\text{6-phosphogluconate dehydrogenase}} \text{6-phosphogluconate} + \text{NADPH} + \text{H}^+
\]

The formation of NADPH was measured spectrophotometrically at 340 nm. The assay system was a two-step procedure. In step one, sucrose (20 µmoles), Dextran T 10 (1 µmole), phosphate buffer, pH 6.0 (10 µmoles) were reacted with dextran sucrase in a total volume of 0.2 ml at 37°C for a specific period of time. The reaction mixtures were heated at 95°C for 2 min. A control was run in a similar manner except that the enzyme was heated at 95°C for 2 min. prior to the reaction. In step two, an aliquot of each reaction mixture was transferred to 0.1 ml solution containing 0.5 µmoles of ATP, 1.0 µmole MgCl\textsubscript{2}, 0.5 µmoles NADP\textsuperscript{+}, 10 µmoles Tris-HCl, pH 7.0, 0.01% NaN\textsubscript{3}, and 0.25 units each of hexokinase, phosphoglucomutase, and glucose-6-phosphate dehydrogenase. These reaction mixtures were maintained at 37°C for 15 min. at which time absorbances were read at 340 nm after dilution to 1.0 ml with H\textsubscript{2}O.

The enzyme activity was calculated by using the following equation where 1 unit is defined as that amount of enzyme catalyzing the formation of 1 µmole of fructose per min.
Activity (units) = \( \frac{\Delta A}{6.2} \times \frac{0.2 \text{ ml}}{X \text{ ml}} \times \frac{0.1 \text{ ml}}{Y \text{ ml}} \times \frac{1}{\text{time} \text{ (min)}} \)

where \( \Delta A = \) absorbance at 340 nm of experimental - absorbance at 340 nm of control

\[ X = \text{volume of enzyme solution in step 1} \]

\[ Y = \text{aliquot of step 1 transferred to step 2} \]

\[ \text{time} = \text{time of incubation of step 1}. \]

2. **Assay for Sucrose Hydrolysis**

Sucrose hydrolysis was measured by the appearance of glucose. The assay utilized the same coupled system used with the dextransucrase assay except phosphoglucone isomerase was omitted. Under these conditions NADPH is formed specifically by the oxidation of glucose. The level of phosphoglucone isomerase contaminating the assay enzymes was measured, and conditions were established such that less than 0.001 unit of isomerase was present during the glucose analysis. The absence of interfering amounts of isomerase was confirmed by incubating the coupled enzyme mixture (0.1 ml) with 0.1 \text{umole} of fructose at 37°C for 15 min. The absorbance at 340 nm was read together with a control which contained no fructose. The difference in absorbance between the experimental and the control was always less than 0.005 absorbance units.

3. **Purification of Dextransucrase**

The dextransucrase used in these studies was isolated according to the procedure developed by Huang (9) and modified by Parmaik (31). A brief description of this procedure will be given here.

The *S. sanguis* cells were grown on Brain-Heart infusion broth supplemented with 1% glucose at 37°C with light stirring. Prior to sterilization, the growth medium was ultrafiltered using an Amicon hollow fiber concentrator to remove high molecular weight contaminants.
During the cell growth the pH was maintained at 6.5 by adding 40% potassium hydroxide. Cells were harvested by batchwise centrifugation at 10,000 x g.

The cell-free culture fluid was concentrated to 1 liter at 4°C using the Amicon hollow fiber concentrator. The volume was maintained at 1 liter by adding water and the concentration continued until the conductance was less than 1 x 10^3 ohm^-1.

Hydroxyl apatite (100 g) was added to the concentrate and the pH adjusted to 5.7. The mixture was stirred slowly for 1 hour and the enzyme, which became bound to the hydroxyl apatite, was collected by centrifugation at 2,500 x g for 5 min. The precipitate was washed with five, 300 ml batches of 0.19 M potassium phosphate buffer at pH 6.0 at 4°C. The enzyme was eluted by washing with two, 300 ml batches of 0.5 M phosphate buffer and dialyzed overnight.

The dialyzed enzyme was applied to a DEAE-cellulose column (40 x 2.5 cm) that had been equilibrated with 0.01 M potassium phosphate buffer, pH 6.0. The column was washed with 350 ml of the same buffer, and then batchwise with 350 ml of 0.05M and 0.1 M, sodium chloride in buffer. The major portion of activity was present in the 0.05 M salt wash and this fraction was dialyzed overnight.

The dialyzed enzyme was applied to a Bio-Gel HTP column (2.5 x 5.0 cm) and washed with 0.2 M potassium phosphate buffer, pH 6.0. The enzyme was eluted with 0.5 M phosphate buffer and the peak fractions were pooled and dialyzed. The enzyme was quick frozen in liquid nitrogen and stored at -70°C. The specific activity ranged from 80 to 170 units/mg.
4. **Paper Chromatography**

Chromatographic separations were carried out on Whatman 1 MM or 3 MM filter paper by the descending method in solvent system I, butanone: acetic acid: H₂O saturated with boric acid (9/1/1 v/v); or in solvent system II, propanol: ethyl acetate: H₂O (6/1/3 v/v).

Standard sugars were visualized by the silver nitrate dip procedure (73).

5. **Radiological Procedures**

All counting was done in a Packard 460 CD liquid scintillation spectrometer. Radioactive chromatograms were cut into 1 cm strips and counted in 10 ml of scintillation fluid (15.2 g PPO, and 380 mg POPOP per gallon of spectral grade toluene). A dpm standard curve was prepared by counting a standard on Whatman 1 MM in the presence of increasing amounts of acetone as a quencher.

Aqueous samples were diluted to 3 ml and were mixed immediately after adding 5 ml of Insta-Gel.

Dual label counting of radioactive chromatograms was done by cutting the chromatogram into 1 cm strips. Each strip was allowed to stand 1 hour in 3 ml of H₂O. The strips were removed and the aqueous layer was mixed immediately after adding 5 ml of Insta-Gel. A dpm standard curve for dual label was prepared by counting standards in 3 ml of H₂O and 5 ml of Insta-Gel in the presence of increasing amounts of acetone as a quencher.

6. **Electrophoresis**

Polyacrylamide disc gel electrophoresis was carried out in the presence of SDS/Triton X-100 using conditions as described (28). The localization of activity on the gels was carried out in situ as
described (28) or by measuring dextranase activity and sucrose hydrolysis in eluates of 1 mm gel slices.

7. Preparation of Immobilized Enzyme

Dextranase was immobilized on hydroxyl apatite. For an individual reaction 2 units of dialyzed enzyme was stirred with approximately 100 mg of hydroxyl apatite, and allowed to stand at 4°C with occasional stirring. In this manner a minimum of 95% of the enzyme was absorbed on the hydroxyl apatite. The samples were centrifuged, and after removal of the supernatant fluid, the immobilized enzyme was immediately used by resuspending it in a 100 µl solution containing substrates.

8. Protein Analysis

Protein was determined by a modification of the Lowry procedure (74).

9. Reducing End Analysis

Samples were evaporated to dryness by vacuum and were dissolved in 50 µl of 0.01 M sodium borohydride, pH 12. The solutions were heated overnight at 50°C and neutralized with dilute acid. One ml of 1M HCl was added to each solution and the mixture was heated for 5 hours at 95°C to hydrolyze polysaccharides. The solutions were analyzed by paper chromatography in solvent system I.
III. RESULTS

A. Properties of Dextransucrase in the Presence of Denaturants

One major goal of this research was to determine if a covalent intermediate is involved in the dextransucrase reaction. As mentioned in the introduction, there is indirect evidence that a glucosyl intermediate is formed, but more concrete evidence was needed in order to establish this point conclusively. The most direct approach is to isolate such a species and demonstrate its properties. Many isolations of this type involve the use of chromatographic supports such as gel-filtration and polyacrylamide gel electrophoresis. Such techniques require the use of an unaggregated form of the enzyme to permit entry of the protein into the support and to provide a singular species of enzyme whose position of elution is well-defined. During the course of previous investigations on dextransucrase we have frequently observed behavior which suggested that the enzyme was aggregating. This apparent aggregation could occur in the absence of its substrates or product. For example, it was observed that the enzyme did not penetrate into electrophoretic polyacrylamide gels (28) or gel-filtration columns (see Figure 5A). This lack of penetration, prevented the use of these techniques not only for mechanistic studies, but for other studies as well. Therefore, the use of these chromatographic techniques to provide evidence for a covalent intermediate was contingent on finding conditions for the disaggregation of dextransucrase. In addition, the establishment of these conditions would also be important.
for enzyme purification and for performing structural studies on the
enzyme. For these reasons efforts were made to see if conditions for
disaggregation of the enzyme could be found, prior to the start of
mechanistic studies.

1. Activity of Dextranucrase in Detergents

Numerous attempts were made to find conditions which would
disaggregate the enzyme. Reagents, such as guanidine hydrochloride,
high ionic strength, heat denaturation, urea, potassium thiocyanate,
etc. (data not shown) were explored. None of these conditions yielded
an unaggregated form of the enzyme, and usually inactivated the enzyme.
Since mechanistic studies would require an active form of the enzyme,
it was important that any procedure developed would not result in
inactivation. SDS gel electrophoresis has been used to study the
molecular weight of dextranucrase which has been shown to vary from
40,000 (36) to 800,000 (32). The fact that the enzyme was mobile under
these conditions indicates that SDS breaks enzyme aggregates. However,
the enzyme was not active under these conditions. Earlier, Huang et al.
(24) demonstrated that dextranucrase retained 5.6% activity in a 6.9 mM
SDS solution and 80.5% in a 0.69 mM SDS solution. It seemed possible
that SDS solutions, at concentrations less than those used in SDS gel
electrophoresis, might be sufficient to cause disaggregation without
inactivating the enzyme. In addition, the ability to cause disaggregation
of dextranucrase may also be a property of other detergents. Therefore,
a study was performed to determine the effect of a variety of detergents
on the enzyme activity. To determine the effect of detergents on the
enzyme activity, dextranucrase was incubated at sub-CMC levels of
Tween 80, Triton X-100, SDS, sodium deoxycholate, polyethylene
glycol-300, and phosphatidyl ethanolamine, or combinations of these, and activity was determined as a function of time. The activity results are plotted on Figure 4 relative to an initial time control containing no detergent that was assigned an activity value of 100. Several detergents, SDS, Triton X-100, sodium deoxycholate, phosphatidyl ethanolamine, and a mixture of SDS plus Triton X-100, showed an initial stimulation of activity. However, with the exception of one detergent, all of the detergents and the control showed a net loss of activity as a function of time. Triton X-100 was unique, since not only did it stimulate activity, but it also stabilized the activity even in the presence of SDS, which by itself, inactivated the enzyme. The ability of these detergents to stimulate activity may also be of some significance. Thus the combination of SDS, which is known to disrupt enzyme aggregates, and Triton X-100, which stabilized the enzyme activity appeared to be a good candidate to cause disaggregation of dextranucrase with retention of activity. This possibility was examined in the following experiment.

2. Demonstration of Disaggregation by Detergents

As mentioned, aggregation prevents dextranucrase from penetrating gel-filtration columns. However, a small percentage does elute at the void volumes of these columns. For example, some activity does elute near the void volume of a Sepharose 6B column with an exclusion limit of $6 \times 10^6$ (see Figure 5A). In order to determine if the detergents caused dissaggregation, studies were carried out in which the enzyme was chromatographed on Sepharose 6B. Any retention of the enzyme on the column could then be viewed as dissociation.
Figure 4. The Effect of Detergents on Dextranucrase Activity

Dextranucrase (0.1 to 0.125 units) was allowed to stand at room temperature with the indicated concentrations of detergents in the presence of 0.01 M phosphate buffer, pH 6.0, X ... X no detergents.

X—X 0.005% SDS; ○—○ 0.01% Triton X-100; ●—● 0.005% SDS, 0.1% Triton X-100; □—□ 0.005% Polyethylene glycol 300 (PEG 300);
■—■ 0.005% SDS, 0.005% PEG 300; △—△ 0.01% Tween 80; ▲—▲ 0.005% SDS, 0.01% Tween-80; ●—● 0.01% sodium deoxycholate; ■—■ 0.01% phosphatidyl ethanolamine. At indicated times, aliquots were removed and assayed.
Figure 4
Purified dextran sulfate was mixed in 0.01 M sodium phosphate buffer, pH 6.0 with either: no detergent as a control, 0.01% Triton X-100, 0.005% SDS, or a mixture of 0.005% Triton X-100 and 0.005% SDS. The samples were maintained at room temperature for 1 hour and applied to column of Sepharose 6B equilibrated and eluted with the same buffered detergent solution. Fractions were assayed for activity. The peak fractions were pooled and total activity and protein were measured.

As shown in Fig. 5, the control had a peak of enzyme which eluted at or near the void volume. The behavior of this enzyme form was such that it appeared to be larger than pyruvate dehydrogenase complex, $M_v=4.8 \times 10^6$ (75). The recoveries of activity (3%) and protein (5%) were low. A measurement of dextran sulfate activity in the material taken from the top of the column showed that the majority of the enzyme had not penetrated into the bed. When the enzyme was chromatographed in the presence of Triton X-100 it eluted at the same position as the control and activity was detected at the top of the column which indicated that the enzyme was still aggregated. The recovery of activity was slightly improved at 10%. In the presence of SDS, the position of the activity peak shifted to a region of lower molecular weight and the recovery of activity was 53%. Since the protein recovery was 94%, it appeared that although disaggregation had occurred, enzyme activity was lost. This loss is consistent with the data seen in Figure 4. When a mixture of Triton X-100 and SDS was used, the elution pattern was the same as seen with SDS, but both the recovery of activity (79%) and protein (86%) were good. Similar data were obtained with a Tween 80/SDS mixture (data not shown). These
Figure 5. Gel Permeation of Dextran Sucrase in the Presence of Detergents

Purified dextran sucrase (1.5 to 2.0 units) was mixed with either:
A. 0.01 M sodium phosphate buffer, pH 6.0;  B. 0.01% Triton X-100 in sodium phosphate buffer, pH 6.0;  C. 0.005% SDS in sodium phosphate buffer, pH 6;  D. 0.005% Triton X-100 and 0.005% SDS in sodium phosphate buffer, pH 6 in a final volume of 1.0 ml. The samples were maintained at room temperature for 1 hr and then applied to a Sepharose 6B column (1.1 x 25.4 cm) which had been equilibrated with the indicated solutions. The enzyme was then eluted with the indicated solutions and the fractions were assayed for activity. The peak fractions were pooled, and total activity and protein were measured. The arrow in the figures show the position at which pyruvate dehydrogenase complex (M.W. = 4.8 x 10^6) eluted.
Figure 5

- Control: Recovery activity 3%, protein < 5%
- 0.01% Triton X-100: Recovery activity 10%
- 0.005% SDS: Recovery activity 53%, protein 94%
- 0.005% SDS + 0.01% Triton X-100: Recovery activity 79%, protein 86%
data demonstrate that SDS causes the dissociation of aggregate forms which had apparent molecular weights exceeding $4.8 \times 10^6$ and that non-ionic detergents such as Triton X-100 served to stabilize activity.

At the same time, Grahame (28) had performed gel electrophoresis experiments in detergents. These experiments, which were consistent with the data reported here, showed that SDS caused disaggregation and showed that Triton X-100 stabilized the enzyme activity. Seven major dextransucrase bands were observed on the gels and had molecular weights ranging from 230,000 daltons to 780,000. The detergent concentrations used in the gel electrophoresis experiments were used to establish conditions in the gel permeation experiment (Figure 5). Using conditions which provide an unaggregated and active form of dextransucrase, it was now possible to carry out mechanistic studies.

3. **Behavior of Disaggregated Dextransucrase After Reaction with Sucrose**

Since conditions have been found for disaggregation of dextransucrase, it was now possible to react the unaggregated enzyme with $[^{14}C]$sucrose to produce a "charged" form of the enzyme. This charged form could be chromatographed on a gel-filtration column in the presence of detergents to determine if radioactive label eluted with the enzyme from the column. The co-elution of the label with the enzyme would be consistent with the concept that it is covalently bound to the protein.

Dextransucrase was disaggregated with 0.005% SDS, 0.005% Triton X-100 and 0.1M sodium phosphate buffer at pH 6.0. The enzyme was reacted with $[^{14}C]$sucrose and applied to a Sepharose 6B gel-filtration column. The column was eluted with the same buffered detergent
solution used to disaggregate the enzyme. Fractions were collected and assayed for radioactivity and enzyme activity. The peak fractions containing the enzyme activity were pooled to measure for total enzyme and protein recovery.

As shown in Figure 6, only one peak of enzyme activity eluted from the gel-filtration column. This peak eluted at the same position (marked with an arrow on Figure 6) as observed with untreated and aggregated enzyme (see Figure 5A). The recoveries of enzyme activity and protein in this peak were 107% and 122% respectively which indicated that no material was filtered out on the column but that all of the enzyme was in a high molecular weight form. This experiment was performed under conditions where the enzyme produces dextran by de novo synthesis. Assuming all of the radioactivity that eluted with the enzyme is a polysaccharide, then a maximum molecular weight for this polymer can be computed. By assuming one active site per enzyme molecule, this polysaccharide would have a molecular weight of $3.6 \times 10^5$. This amount of polymer cannot account for the apparent increase in molecular weight, since the peak material appears to be larger than pyruvate dehydrogenase complex with a molecular weight of $4.8 \times 10^6$ (20). This indicates an association of the molecules. Under the conditions of this experiment, enzyme-enzyme association is disrupted. Therefore, it appears that an association is occurring between the protein and the sugars, which comigrate on the column, thus additional denaturing conditions were needed to disrupt possible non-covalent associations between the sugar and the protein.

4. Denaturation of Sucrose-Treated Enzyme

The previous experiment indicated that upon reacting dextran sucrase with [$^{14}C$]sucrose an association occurred between the
Figure 6. Gel Permeation in the Presence of Detergents of Dextran-
sucrase Reacted with sucrose

Purified dextransucrase (1.1 units) was disaggregated by
standing in 0.005% SDS and 0.1 M sodium phosphate at pH 6.0 for 15 min.
at room temperature. Triton X-100 was added to a final concentration
of 0.005% to stabilize enzyme activity and the mixture was allowed to
stand 1.8 hours. The enzyme was reacted with 1.1 μmoles of $[^{14}C]$sucrose
(5.0 μCi) and an aliquot chromatographed on a Sepharose 6B column
(1.1 x 24.0 cm) equilibrated with 0.1 M sodium phosphate pH 6.0, 0.005% 
SDS and 0.005% Triton X-100. Fractions were collected and assayed for
radioactivity (●) and enzyme activity (O). The enzyme peak fractions
were pooled and measured for enzyme and protein. The arrow in the figure
marks the position where aggregated enzyme elutes.
radioactivity and the enzyme. This association persisted in SDS. Since SDS may act to disrupt protein-protein interactions, it was felt that other denaturants such as urea or thiocyanate might be effective in disrupting other non-covalent interactions. The separation of the isotope and the protein on a gel-filtration column under these conditions would indicate that the two were not covalently bonded.

Dextranucrase was dissociated in 0.005% SDS and phosphate buffer. The enzyme was reacted with \(^{14}C\) sucrose for 1.5 min and sufficient solid urea was added to make the solution 8M. An aliquot of the denatured and inactivated enzyme was applied to a Sepharose 6B-CL column and eluted with SDS and 8M urea. Radioactivity was determined in each fraction.

Figure 7 shows that a major peak of radioactivity was observed in the same position as seen with aggregated enzyme in the preceding experiment. The peak was pooled. In addition, a pool was made of the fractions where unaggregated enzyme would normally elute, but which was devoid of isotope. Both pools were dialyzed to remove urea and assayed for protein. Each pool contained approximately 50% of the protein. Thus, it appeared that the aggregated form was glycosylated and denaturation in 8M urea did not result in disaggregation nor release of the isotope from the enzyme. Similar results were obtained in experiments (data not shown) using heat denaturation or denaturation in 3M sodium thiocyanate, a strong chaotrophic agent.

We can conclude from the experiments described that the native enzyme can be dissociated with SDS/Triton X-100, and that reaction of the enzyme with sucrose leads to reaggregation. Denaturants such as
Figure 7. Gel Permeation in the Presence of SDS and 8M Urea of
Dextranucrase Reacted with sucrose

Purified dextranucrase (1.1 units) was allowed to stand in
0.005% SDS and 0.01M sodium phosphate at pH 6.0 for 1 hour at room
temperature. The disaggregated enzyme was reacted with 1 μmole of
\(^{14}C\) sucrose (5.0 μCi) for 1.5 min and then 0.73 g of solid urea was
added. The volume increased to 1.5 ml which made the final urea
concentration 8M. An aliquot was chromatographed on a Sepharose 6B-CL
column (1.1 x 25.0 cm) equilibrated with 0.01 M sodium phosphate
pH 6.0, 0.005% SDS and 8M urea. Radioactivity was determined in each
fraction. The peak was pooled, as were fractions where unaggregated
enzyme would normally elute. Both pools were dialyzed and assayed for
protein.
Figure 7
SDS/Triton, urea, heat and thiocyanate did not restore the unaggregated form. Therefore, it is difficult to conclude that the persistence of the isotope with the protein indicates the presence of a covalent attachment of the sugars to the protein. However, during follow-up experiments (data not shown) where the sugar-protein aggregate was being examined two materials were observed: high molecular weight material, \( M_v > 4.8 \times 10^6 \) and a low molecular weight material, \( M_v < 5 \times 10^3 \). Paper chromatographic analysis of the low molecular weight material indicated that it was glucose and suggested that the glucose was released from the aggregate. The presence of glucose, during reactions of dextranucrase with sucrose, had previously been observed (56, 57, 63), and these preparations were shown to hydrolyze sucrose. However, it had not been clearly established that this activity was due to dextranucrase. The following series of experiments was designed to test this question.

B. Partial Reactions of Dextranucrase

If a glucosyl-enzyme intermediate is involved during catalysis, it might be possible to isolate the intermediate following a reaction with sucrose by some technique other than gel-filtration. It could then be possible to demonstrate that this intermediate is capable of participating in the reactions which dextranucrase is known to catalyze. These reactions are: transfer to fructose to form sucrose as indicated by the isotope exchange reaction, transfer to acceptor such as maltose to form a trisaccharide, or transfer to the dextran in a de novo synthesis of the polymer. If such an intermediate exists, it is possible that it may also transfer to water in a hydrolysis reaction. Significant hydrolysis of this type would dictate that the isolation of the intermediate must be sufficiently rapid to prevent
its complete hydrolysis. Several investigators had provided evidence that hydrolysis of sucrose occurred with various enzyme preparations (56, 57, 63, 64), but did not demonstrate that this activity was caused by dextransucrase. In the preceding experiment glucose was observed following a reaction of the purified enzyme with sucrose. Prior to developing a technique for isolation of an intermediate, it was important to establish whether or not dextransucrase from *S. sanguis* could hydrolyze sucrose.

1. **Hydrolysis of Sucrose by Dextransucrase**

Studies were designed to determine if sucrose hydrolysis occurred with the enzyme by measuring the formation of glucose. The coupled enzyme assay system for measuring release of fructose by dextransucrase uses hexokinase, phosphoglucoisomerase, and glucose-6-phosphate dehydrogenase (37). The system could also measure glucose, but by omission of phosphoglucoisomerase, the system becomes specific for the measurement of glucose and thus can be used as an assay for sucrose hydrolysis activity. In both assays the system is coupled to NADPH formation which is measured spectrophotometrically.

In the experiment, dextransucrase was allowed to react with 0.1M sucrose and aliquots were removed as a function of time and assayed for dextransucrase activity (fructose plus glucose) and sucrose hydrolysis (glucose) as just described. The results of these measurements are shown on Figure 8. The data clearly show increasing amounts of glucose being formed as a function of time. To determine if the hydrolytic activity might be associated with the dextransucrase, dextran a known acceptor (55) was included in a separate reaction. The dextran stimulated the dextransucrase activity, but diminished the rate of
Figure 8. **Formation of Glucose by Dextranucrase**

Dextranucrase (0.067 units) was reacted in the presence of 0.1 M sucrose (---) alone with 0.01 M Dextran T-10 (---) in a volume of 200 µl. At 15, 30, 45 and 60 min aliquots were withdrawn and assayed for fructose plus glucose (---), which is the standard analysis for dextranucrase, or for glucose (---) using the analysis described in Materials and Methods.
Figure 8

(--.--) Glu + Fru $\Delta A_{340}$

(--.--) Glu $\Delta A_{340}$

Time (min)

0 15 30 45 60

0.0 0.2 0.4 0.6 0.8
glucose formation. This indicated that dextran was competing with water for a form of the enzyme that was common to both reactions. The competition experiment suggests that sucrose hydrolysis is occurring at the dextransucrase active site, but additional evidence was sought to clearly establish this.

Another explanation for the apparent hydrolytic activity is the presence of an invertase as a contaminant in the enzyme preparation. This is a distinct possibility since many oral streptococci produce invertase (33, 57, 64). To determine if invertase was present, 1 unit of dextransucrase was subjected to polyacrylamide disc gel electrophoresis in the presence of SDS and Triton X-100 using conditions developed by Grahame (28). After electrophoresis the gel was sliced into segments about 1 mm in thickness. Enzyme activity was eluted from each slice by placing the segments in 100 µl of 0.1M phosphate buffer, pH 6 overnight at 4°C. The eluates were assayed for dextransucrase activity and sucrose hydrolysis. The results in Figure 9 show that both the dextransucrase activity and hydrolysis activity co-migrate which demonstrates that both of these activities are associated with the same protein. Furthermore, hydrolysis was not observed elsewhere on the gel. This indicated that the protein preparations were not contaminated with invertase. The same results were obtained by comparing the hydrolytic activity and the dextransucrase activity as determined in situ on a duplicate gel. These data are shown in Figure 10.

Another method for detecting invertase activity is based on the utilization of raffinose (0-α-D-galactopyranosyl-(1→6)-α-D-glucopyranosyl β-D-fructofuranoside) which is known to be a substrate for invertases that are produced by oral bacteria (72). Invertase action
Figure 9. Examination of Dextranucrase Activities Through the Use of Electrophoresis

Dextranucrase (1.0 unit) was subjected to electrophoresis in the presence of 0.005% SDS and 0.01% Triton X-100 as described (28). The gel was sliced into 1 mm segments and individually eluted in 0.1 M sodium phosphate buffer, pH 6.0 overnight at 4°C. Aliquots were removed from the eluates and assayed for dextranucrase activity (---) and hydrolytic activity by glucose formation (••).
Figure 9

Dextran sucrase \( \Delta A_{340}/100\mu l \)

<table>
<thead>
<tr>
<th>0.5</th>
<th>1.0</th>
<th>1.5</th>
<th>2.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.1</td>
<td>0.2</td>
<td>0.3</td>
</tr>
</tbody>
</table>

Glucose \( \Delta A_{340}/100\mu l \)

<table>
<thead>
<tr>
<th>0</th>
<th>0.1</th>
<th>0.2</th>
<th>0.3</th>
<th>0.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

Slice Number

10  20  30  40  50
Figure 10. Examination of Dextranucrase Activities Through the Use of Electrophoresis

Dextranucrase (1.0 unit) was applied to two separate polyacrylamide gels and subjected to electrophoresis in the presence of 0.005% SDS and 0.01% Triton X-100 as described (28). One gel was sliced into 1 mm segments and individually eluted in 0.1 M sodium phosphate buffer, pH 6.0 overnight at 4°C. Aliquots were removed from the eluates and assayed for hydrolysis activity (---). Dextranucrase was determined in situ (—) by exposing the duplicate gel to a sucrose solution overnight as described (28).
yields fructose as one of the products and can be followed by use of the same procedure for dextransucrase. However, raffinose is not a substrate for dextransucrase (34). Dextransucrase was incubated with raffinose which had been purified by paper chromatography. Aliquots were removed at 10, 30, 45, and 60 minutes, and assayed for the production of fructose using the coupled enzyme assay system. No hydrolysis of raffinose was observed under conditions which would have detected $1 \times 10^{-3}$ units of invertase. These results demonstrate that dextransucrase does hydrolyze sucrose and that the enzyme preparation used contains no detectable amount of invertase activity.

These initial studies of the hydrolysis of sucrose by dextransucrase used a soluble form of the enzyme. In the following two experiments an immobilized form was used to facilitate removal of the enzyme from its reaction media. We have observed that dextransucrase binds readily to hydroxyl apatite and remains fully active. This provides a rapid technique for removing the substrate from the enzyme. Using the immobilized enzyme, some of the characteristics of the hydrolytic reaction were studied.

Dextransucrase was immobilized on hydroxyl apatite (HA) and reacted with $\text{[}^{14}\text{C}]$ sucrose buffered at pH 6.0. The reaction was stopped after 1 min at room temperature by adding 5 ml of H$_2$O at 4°C. The mixture was immediately centrifuged and an aliquot of the supernatant fluid was examined using paper chromatography. The results shown in Figure 11 confirmed that glucose is formed and indicate that the immobilized dextransucrase as well as the native enzyme catalyzes the hydrolysis of sucrose. As expected, $\text{[}^{14}\text{C}]$ fructose and $\text{[}^{14}\text{C}]$ sucrose were also observed. This analysis could be used to determine the extent of
Figure 11. Hydrolysis of Sucrose by Immobilized Enzyme

Dextran sucrase (0.445 units), immobilized on hydroxyl apatite, was reacted with 0.2 μmoles of [U-¹⁴C] sucrose (6 x 10⁶ dpm) in the presence of 2 μmoles of Na₂HPO₄ buffer, pH 6.0, and 10 μg of SDS in a total volume of 0.5 ml. After 1 min at room temperature, 5 ml of H₂O at 0°C was added and the mixture immediately centrifuged. The supernatant fluid was removed and an aliquot was spotted on Whatman 1 MM, chromatographed in solvent system I and counted.
Figure 11
sucrose hydrolysis by the immobilized enzyme by measuring the quantity of glucose formed. The hydrolysis of sucrose also leads to the formation of fructose. Dextran formation can be determined indirectly by the difference between total fructose and glucose formed.

This method of data analysis was used in the following experiment to determine the amount of sucrose hydrolysis and dextran formation as a function of reaction time. Immobilized dextran sucrose was reacted with \( ^{14}\text{C} \) sucrose. Aliquots of the suspension were transferred to 5 ml of dilute buffer at 4°C at 0.25, 0.5, 1, 2, and 5 min. Each mixture was immediately centrifuged and an aliquot of the supernatant fluid analyzed by paper chromatography. Based on the percentage of radioactivity that migrated with glucose and fructose, the amounts of hydrolysis and dextran formation were computed as described. The ratio of these two reactions were plotted as a function of time as shown on Figure 12A. The plot shows that initially hydrolysis is the major activity at this sucrose concentration. With increasing reaction time the ratio rapidly decreases and by 5 min dextran formation has become the dominant activity. Since no acceptors were added to the reactions, dextran formation is occurring by de novo synthesis. Thus, it appears that at the longer times dextran formation competes with hydrolysis. This is similar to the observations made with the soluble system where hydrolysis activity declined in the presence of acceptors.

Another experiment was performed to examine hydrolysis activity and dextran formation as a function of sucrose concentration. Four reaction mixtures were prepared: 0.05 mM, 0.5 mM, 5.0 mM and 50 mM \( ^{14}\text{C} \) sucrose each in 10 mM sodium phosphate at pH 6.0. Each reaction mixture was added to immobilized enzyme and reacted for 1 min at room
Figure 12

A. The Hydrolysis of Sucrose as a Function of Time

Immobilized dextransucrase (1.8 units) was incubated with 0.55 μmoles of $[U^{-14}C]$ sucrose ($60 \times 10^6$ dpm), 50 μmoles of Na$_2$HPO$_4$ buffer, pH 6.0 in a total volume of 1 ml, at room temperature. Aliquots (200 μl) were transferred to 5 ml of 1 mM sodium phosphate buffer at pH 6.0 at 4°C at 0.25, 0.5, 1, 2, and 5 min. The mixtures were immediately centrifuged, and an aliquot of the supernatant fluid was chromatographed in solvent system I. The percent of the total radioactivity which co-migrated with fructose and glucose was measured. The ratio of hydrolysis versus dextran formation was computed and plotted as a function of time.

B. The Hydrolysis of Sucrose as a Function of Concentration

Reaction mixtures containing immobilized enzyme (1.8 units) 0.05 mM, 0.5 mM, 5.0 mM and 50 mM $[U^{-14}C]$ sucrose ($12 \times 10^6$ dpm) and 10 mM sodium phosphate, pH 6.0 were reacted for 1 min at room temperature. 5 ml of 1 mM sodium phosphate buffer at pH 6.0 at 4°C were added to each reaction mixture and the samples analyzed as described in 12A.
temperature. The reactions were stopped by adding 5 ml of dilute buffer at 4°C and an aliquot was analyzed by paper chromatography. The results in Figure 12b show that the rate of hydrolysis, relative to dextran formation, is greatest at low sucrose concentrations. With increasing sucrose concentrations, dextran formation becomes the dominant activity. Thus the two activities are competitive as a function of sucrose concentration. As pointed out, since there are no added acceptors, dextran is being formed by de novo synthesis. Thus hydrolysis is competitive with both the de novo synthesis reaction and with the transferase reaction as indicated by the competition experiment where dextran was added to the reaction media (Figure 8). These data suggest that a common enzyme form is involved in all three reactions. This could be a glucosylated form of the enzyme. The data also shows that the rate of hydrolysis under some of the conditions examined is sufficiently high that a rapid technique must be employed if a glucosylated intermediate is to be demonstrated.

In addition to demonstrating that the immobilized enzyme catalyzes the hydrolysis of sucrose, it was also observed that the isotope became bound when it was exposed to radioactive sucrose. In both of the previous experiments, each reaction mixture was centrifuged and the supernatant fluids were analyzed by paper chromatography. In addition, each pellet of immobilized enzyme was immediately resuspended in 5 ml of dilute buffer at 4°C and the supernatant fluid was removed after centrifugation. The wash was repeated and the pellet was resuspended in 0.5 ml of H₂O. An aliquot of the suspension was analyzed for radioactivity.
As shown in Figure 13, the amount of radioactivity bound to immobilized enzyme increased with time. A similar effect can be seen in Fig. 14 where the binding of $\text{[}^{14}\text{C]}$ glucose was shown to increase as a function of sucrose concentration. The effect leveled off which indicated a saturation of sites on the enzyme. Since the specific activity of the $\text{[}^{14}\text{C]}$ sucrose varied, the data in Figure 14 was plotted as $\mu$moles of $\text{[}^{14}\text{C]}$ glucose rather than radioactivity. These data indicate that the tightly bound material is a product of a reaction catalyzed by dextrantrasucrase. The following experiment was designed to analyze the composition of the tightly bound material.

It was observed that when the immobilized enzyme was heated at 93° C for 10 min the majority (up to 80%) of the tightly bound sugars were released into the supernatant fluid. In this manner, the supernatants of each reaction mixture were analyzed by paper chromatography. A typical chromatogram is shown in Figure 15. This chromatogram represents the products released by heat after the reaction of immobilized dextrantrasucrase with 0.05 mM$\text{[}^{14}\text{C]}$ sucrose for 1 min. Sucrose and fructose were below detectable limits, which indicates that the washes were effective in removing non-covalently bound material. Little non-mobile material was present in the washes as can be seen in Fig. 11, however, non-mobile material is present in the heat releasable material. This indicates that the non-mobile material was tightly bound. The material at the origin appears to represent some polymeric form. The presence of $\text{[}^{14}\text{C]}$ glucose demonstrates that this material was retained after extensive washing and was released when the immobilized enzyme was heated. The $\text{[}^{14}\text{C]}$ glucose observed represented 34% of the material released after heating and the non-mobile material was 60%.
The HA pellets from the experiment described in Figure 12A were washed twice with 5 ml portions of 1 mM sodium phosphate buffer, pH 6.0 at 4°C. Each pellet was resuspended in 0.5 ml of H2O and an aliquot of the suspension was removed and analyzed for radioactivity.
Figure 14. Binding of Radioactivity to Immobilized Enzyme as a Function of Sucrose Concentration

The HA pellets from the experiment described in Figure 12B were washed twice with 5 ml portions of 1 mM sodium phosphate buffer, pH 6.0 at 4°C. Each pellet was resuspended in 0.5 ml of H2O and an aliquot of the suspension was removed and analyzed for radioactivity.
Figure 14

Glucose (μmoles) x 10^5

Sucrose Concentration (mM)
The pellet of immobilized enzyme that had been charged in 0.05 mM $^{14}$C-sucrose (Figure 12B) was washed twice with 5 ml portions of 1 mM sodium phosphate buffer, pH 6.0 at 4°C. The pellet was resuspended in 0.5 ml of H$_2$O and heated at 93°C for 10 min. An aliquot was chromatographed on Whatman 1 MM in solvent system I and counted.
Figure 15
retention of glucose is consistent with the concept of a glucosyl intermediate.

In conjunction with the study of sucrose hydrolysis as a function of reaction time (Figure 12A), the binding and release of $^{14}C$ glucose was also determined. The results (Figure 16) show that initially the amount of glucose released increased with time, and then leveled off. This may be interpreted as saturation of sites on the enzyme. A similar effect was observed (Figure 17) when the heat releasable glucose was examined as a function of sucrose concentration. At concentrations above 5 mM the amount of glucose became maximal. This saturation behavior together with the retention of glucose by the immobilized enzyme is strongly suggestive of the presence of a glucosyl intermediate. If this is the case, it should be possible to demonstrate its involvement in the half-reactions catalyzed by dextransucrase. This question was investigated in the subsequent experiments.

2. Charged Dextransucrase Chased with Fructose

The previous experiments suggested that a glucosyl intermediate is involved in the dextransucrase reaction. This intermediate or charged form of the enzyme contained monomeric glucosyl residues, and some polymeric forms. While the data obtained are consistent with these forms being covalently bonded to the enzyme, additional studies were required to investigate this point. If the sugars are in an "activated" form, they ought to participate in the reactions catalyzed by dextransucrase. One such reaction is the isotope exchange reaction described by several investigators (2, 55, 58). Studies were carried out with the charged enzyme immobilized on HA to determine if sucrose could be formed if it was chased with fructose.
Figure 16. The Release of Glucose from Immobilized Enzyme as a Function of Reaction Time with Sucrose

The HA pellets from the experiment described in Figure 12A were washed twice with 5 ml portions of 1 mM sodium phosphate buffer at pH 6.0 at 4°C. Each pellet was resuspended in 0.5 ml of H2O and heated at 93°C for 10 min. An aliquot from each was chromatographed on Whatman 1 MM in solvent system I and counted. The % dpm appearing in glucose was plotted as a function of reaction time.
Figure 16

Time (min)
Figure 17. **The Release of Glucose from Immobilized Enzyme as a Function of Sucrose Concentration**

The HA pellets, from the experiment described in Figure 12B, were treated in the same manner described in Figure 16. The μmoles of glucose were computed and plotted as a function of sucrose concentration.
Figure 17

Sucrose Concentration (mM)

Glucose (μmoles) x 10^5

5 25 50

0 1.0 2.0 3.0
In the experiment, immobilized dextranulose was reacted with $^{14}C_sucrose$. After 1 min., 5 ml of cold buffer, pH 6.0 was added. The mixture was immediately centrifuged and the supernatant removed. This was followed by two more washes of the pellet with buffer. On the last wash, the suspension was divided into two equal portions prior to centrifugation. One pellet was resuspended in a solution containing fructose. After 1 min at room temperature the suspension was placed in a 93°C heating block for 2 min. An aliquot of the supernatant was examined by paper chromatography. A control reaction was run in an identical manner except H$_2$O was added in place of the fructose solution. After heating, 73% of the material bound to the HA in the fructose chase was released with heat and 44% was released in the control experiment.

As shown on Figure 18, the control reaction shows the appearance of $^{14}C_glucose (61\%$ of the total radioactivity) and non-mobile counts. There were no detectable amounts of $^{14}C_sucrose$ which demonstrates the removal of unreacted sucrose by the washing procedure. In the presence of fructose, the percentage of $^{14}C_glucose decreases relative to the control with the appearance of a new product that migrated with a sucrose standard. The new product was 68% of the total radioactivity on the chromatogram. The appearance of some $^{14}C_glucose in the fructose chase probably results from competition by the hydrolytic reaction. The counts at the origin of the chromatogram may be due to $^{14}C_dextran$ formed in the de novo synthesis reaction. These results provide direct evidence for a glucosyl-enzyme intermediate by demonstrating that the charged form of the enzyme is competent in transferring glucose residues to form a major product which appears
Dextranucrase, 3.4 units immobilized on HA, was reacted at room temperature with 0.01 μmoles of \[^{14}\text{C}]\text{sucrose (15 x 10^6 dpm)}, 2 μmoles of sodium phosphate buffer, pH 6.0 in a total volume of 200 μl. After 1 min the reaction was diluted with 5 ml of 1 mM sodium phosphate buffer, pH 6.0 at 4°C. The mixture was immediately centrifuged and the supernatant removed. This was followed by two more washes of the pellet with 5 ml of ice-cold buffer. On the last wash, the suspension was divided into 2 equal fractions prior to centrifugation. One pellet was resuspended in 100 μl of 100 mM fructose (○—○) and after 1 min at room temperature placed in a 93°C heating block for 2 min. An aliquot of the supernatant was chromatographed in solvent system I. A control reaction (○—○) was run simultaneously in an identical manner except 100 μl of H₂O was added in place of the fructose solution.
Figure 18

- Sucrose
- Glucose

Distance of Migration (cm)

dpm (%)
to be sucrose. In addition, the results demonstrate that the method is rapid enough to isolate this glucosylated form of the enzyme.

While the chromatographic identification of sucrose strongly suggested that it was being formed, it was important to verify this by more specific techniques. The approach employed was to determine the susceptibility of the product to invertase. Only $^{14}C$ glucose would be observed on the chromatogram, since unlabeled fructose was used in the preceding experiment.

To test this, the products from the fructose chase experiment were reacted with invertase. After 15 min., the reaction was spotted on Whatman 1 MM paper and chromatographed in solvent System I. A control reaction was performed in the same manner except a heat denatured invertase solution was used. The data (Figure 19) demonstrates that the product is hydrolyzed by invertase but is unaffected by the heat denatured invertase by comparison to Fig. 18. Thus, it can be concluded that when the charged enzyme was reacted with fructose, sucrose is formed and shows that the partial reaction with sucrose is reversible. More importantly, it demonstrates that the glucose on the charged enzyme is active in transfer reactions, and supports the idea that there is a glucosyl-enzyme intermediate. The possible involvement of such an intermediate in other reactions catalyzed by dextranucrase was examined in the following experiments.

3. **Charged Dextranucrase Chased with Sucrose**

Dextranucrase can utilize sucrose to form dextran by de novo synthesis. An insertion mechanism for this synthesis has been proposed (34, 35) which involves a glucosyl-enzyme intermediate. Evaluation of this issue was made to determine if the glucosyl
Figure 19. **Exposure of the Product of the Fructose Chase Experiment to Invertase**

An aliquot from the fructose chase experiment (Fig. 18) was reacted with 0.066 units of invertase in a total volume of 10 μl for 15 min at 50°C. The reaction (●) was spotted on Whatman 1 MM paper and was chromatographed in solvent system I. A control reaction (○) was performed in the same manner except a heat denatured invertase solution was used.
Figure 19
residues of the charged enzyme are active in de novo synthesis. The procedure used was similar to that of the previous chase of the charged enzyme (Fig. 18) except that unlabeled sucrose was employed instead of fructose.

In this experiment the immobilized dextranucrase was reacted with $[^{14}C]$ sucrose in buffer for 25 seconds. The cessation of the reaction and the subsequent washes were carried out as described (Fig. 20). The suspension was split into two equal fractions on the last wash. One pellet was resuspended in $H_2O$ for 5 sec. and heated at $95^\circ C$ for 10 min. as a control. Simultaneously, the other pellet was resuspended in a sucrose solution, allowed to react for 5 sec., and then placed in the heating block for 10 min. Aliquots of the reaction mixtures were chromatographed.

The results (Fig. 20) show a 5 fold decline in $[^{14}C]$ glucose in the chase with unlabeled sucrose relative to the control reaction. The glucose in the control reaction represents 14% of the total radioactivity, and in the sucrose chase it is 3%. The difference of 11% is important, since the corresponding increase is seen in the material at the origin, where the control is 80% and the sucrose chase is 92%. These data indicate that the majority of the glucose that had appeared in the control experiment was converted to non-mobile material in the sucrose chase. The origin material may represent a dextran-like polymer produced by the de novo synthesis reaction. Therefore, the same enzyme from that released glucose after heating may also be involved in de novo synthesis. The involvement of the charged enzyme in transferring glucosyl residues to an acceptor was examined in the next series of experiments.
Figure 20. Charged Dextransucrase Chased with Sucrose

Immobilized dextransucrase was reacted with 0.05 μmoles of \(^{14}C\)sucrose (67 x 10^6 dpm) in 100 μl of 0.1 M sodium phosphate buffer at pH 6.0 for 25 seconds at room temperature. The reaction was diluted with 5 ml of 1 mM sodium phosphate buffer, pH 6.0 at 4°C, immediately centrifuged and the supernatant removed. This was followed by two washes of the pellet with the same cold buffer. On the last wash the suspension was divided into 2 equal fractions. One pellet was resuspended in 0.5 ml of H₂O for 5 sec. and heated at 93°C for 10 min as a control (●). At the same time, the other pellet was resuspended in 0.5 ml of 50 mM sucrose (○), allowed to react for 5 sec., and then was placed in a 93°C heat block for 10 min. Aliquots were chromatographed in solvent system I.
Figure 20 Distance of Migration (cm)
4. Charged Dextranucrese Chased with an Acceptor

The ability of dextranucrese to catalyze the transfer of single glucosyl residues has been well established (53, 55). It was important to evaluate the ability of the charged enzyme to participate in such transfers. It was possible that the transfer pathway was distinct from that of de novo polymerization, even though indirect evidence indicated that they competed for the same form of the enzyme. This possibility was examined by reacting maltose, a good acceptor (55), with charged dextranucrese.

Immobilized dextranucrese was reacted with C14-sucrose as before, washed, and divided into two equal fractions. One fraction was reacted with maltose for 1 min at room temperature, and the reaction was stopped by heating. The other fraction functioned as a control by substituting buffer for maltose. Following a heat step, paper chromatography of the products was carried out in solvent system II, which will separate oligosaccharides.

The results (Fig. 21) show the appearance of glucose in the control with the complete absence of low molecular weight oligosaccharides. In the maltose chase experiment, the percentage of radioactivity glucose (3%) decreased significantly relative to the control (22%) with the appearance of a new 14C-labeled product. The new product migrated slightly farther than an isomaltotriose standard. These results are consistent with the idea that the charged enzyme had catalyzed the transfer of a glucosyl residue to maltose forming what appears to be a trisaccharide. No other oligosaccharides appeared on the chromatogram, indicating that the transfer of a glucosyl residue to maltose occurred only once.
Figure 21. Charged Dextranucrase Chased with Maltose

Immobilized dextranucrase (6.8 units) was reacted with 0.01 μmole of [14C]sucrose (15 x 10^6 dpm) in 200 μl of 1 mM sodium phosphate, pH 6.0 for 1 min at room temperature. The reaction was diluted with 5 ml of 1 mM sodium phosphate buffer, pH 6.0 at 4°C, immediately centrifuged and the supernatant removed. This was followed by two washes of the pellet with the same cold buffer. On the last wash, the suspension was divided into 2 equal fractions. One pellet was resuspended in 100 μl of 0.1 mM maltose (●—●) in 1 mM sodium phosphate buffer at pH 6.0 and allowed to stand 1 min and placed in 95°C heating block for 2 min. The control reaction (O—O) was carried out in an identical manner except for the substitution of 100 μl of 1 mM phosphate buffer for maltose. Aliquots were chromatographed in solvent system II.
Figure 21

Distance of Migration (cm)

isomaltotriose

glucose

dpm (%)

100

95

40

80

30

20

10

0
Since a small amount of glucose was observed in the maltose chase, it is possible that hydrolysis of the charged enzyme was competing with the transfer of glucosyl residues to maltose. A similar concept was indicated by the hydrolysis experiment (see Fig. 8) where transfer to a dextran acceptor and sucrose hydrolysis was thought to involve a common enzyme intermediate. The following experiment was designed to test this possibility.

The preceding experiment was repeated in the same way except after reacting with maltose for 1 min., 5 ml of ice-cold buffer was added. The cold supernatant fluid, which should contain non-covalent material, was removed after centrifugation and the pellet was heated in 0.5 ml of buffer for 2 min at 93°C. Aliquots of both the cold supernatant fluid and the hot supernatant fluid, which will contain material that was tightly bound, were analyzed by paper chromatography. A control reaction was performed in the same manner except buffer was used instead of maltose.

The results of the control reaction (Table 1) show that 92% of the material in the cold supernatant fluid was $^{14}C$ glucose (25,000 dpm). The $^{14}C$ glucose was released after extensive washing. This data is consistent with the concept that glucose is being released from the charged enzyme by hydrolysis. Most of the non-mobile material appears in the hot supernatant fluid which indicates that this material is tightly bound. In addition, 35% of the radioactivity on the chromatogram was $^{14}C$ glucose (30,000 dpm) which indicates that this material remained with the pellet and was tightly bound.

In the maltose chase, 95% of the material in the cold supernatant fluid was low molecular weight sugars. Trisaccharide was 82% and glucose was 13%. In the control reaction, 92% of the material in the
Table 1. **Charged Dextranucrase Chased with Maltose**

<table>
<thead>
<tr>
<th>Percent dpm at Origin</th>
<th>Trisaccharide</th>
<th>Glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sup. fluids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cold</td>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td>Hot</td>
<td>65</td>
<td>1</td>
</tr>
<tr>
<td><strong>Maltose Chase</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sup. fluids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cold</td>
<td>2</td>
<td>82</td>
</tr>
<tr>
<td>Hot</td>
<td>65</td>
<td>17</td>
</tr>
</tbody>
</table>

The procedure was the same as that in the preceding maltose chase (Figure 21) except after reaction with maltose for 1 min., 5 ml of 1 mM sodium phosphate buffer (4°C, pH 6) was added. The suspension was centrifuged and the cold supernatant fluid was removed. The pellet was heated at 93°C for 2 min. in 0.5 ml of 1 mM sodium phosphate buffer, pH 6. After centrifugation, the hot supernatant fluid was removed from the pellet. Aliquots of both the cold and hot supernatant fluids were analyzed by paper chromatography in solvent system II. A control reaction was performed in the same manner except buffer was used instead of maltose.
cold supernatant fluid was glucose with less than 1% trisaccharide. This data indicates that the transfer of glucosyl residues to maltose is competing effectively with the release of glucose from the charged enzyme. Since the transfer to acceptors probably involves a glucosyl-enzyme intermediate, this data is also consistent with the idea that this covalent intermediate can undergo hydrolysis. In addition, in the maltose chase, the majority of the non-mobile material was released only after heating which indicates that this material remained tightly bound after exposure to the acceptor.

To this point, it had been assumed that a trisaccharide was being formed during the maltose chase. Analogous to the fructose chase, it was important to show that a single glucosyl unit was being transferred to maltose. This could be done by determining the composition of the product in the maltose chase. The following experiment was designed to demonstrate that a trisaccharide was being formed.

Immobilized dextransucrase was charged in same manner as before, except $[^3H]$sucrose was employed instead of $[^{14}C]$sucrose. After washing, the $[^3H]$-charged enzyme was chased with $[^{14}C]$maltose, which had been purified by paper chromatography to radiochemical purity. An aliquot of the chase mixture was chromatographed in solvent system II, which will separate the unreacted $[^{14}C]$maltose from the apparent trisaccharide. The results (Fig. 22) show a peak of $^{14}C$ and $[^3H]$ which co-migrated in the same region as the isomalto-triose standard. The alignment of the peaks indicates that the two isotopes are associated with the same compound. Using the specific activity of the $[^{14}C]$maltose together with the amount of $^{14}C$
Immobilized dextranase (1.8 units) was reacted with 0.05 μmole of \(^3\)H-sucrose (11 x 10^6 dpm) in 100 μl of 1 mM sodium phosphate buffer at pH 6.0 for 30 sec. at room temperature. The reaction was diluted with 5 ml of 1 mM sodium phosphate buffer, pH 6.0 at 4°C, and immediately centrifuged and the supernatant fluid was removed. This was followed by two washes of the pellet with the same buffer. The pellet was reacted with 0.5 μmoles of \(^{14}\)C-maltose (6.6 x 10^6 dpm) in 50 μl of 1 mM sodium phosphate pH 6.0. After 10 min the reaction mixture was centrifuged and the supernatant removed. An aliquot was chromatographed in solvent system II on Whatman 1 MM. The chromatogram was cut into 1 cm strips and each strip was placed in a scintillation vial with 3 ml of water. After 1 hour the strips were removed and each vial was mixed immediately after the addition of 5 ml of Insta-Gel. The samples were counted using a dual label program. In the plot, \(^3\)H is represented by (O—O) and \(^{14}\)C by (O—O).
Figure 22

Distance of migration (cm) vs. dpm (%)

- Solid line: $^{14}$C
- Dashed line: $^3$H

isomaltotriose
appearing in the peak, the number of μmoles of $^{14}$C-maltosyl residues in the peak was determined to be $4.77 \times 10^{-3}$. Using the same procedure, the peak contains $4.92 \times 10^{-3}$ μmoles of $^{3}$H-glucosyl residues. Thus, there are 1.03 μmoles of glucosyl residues for every μmole of maltosyl residue showing that a trisaccharide was formed. We have shown that radioactivity is rapidly released from the charged enzyme by heating. In this experiment and the preceding one, the product of the transfer reaction is found in the supernatant fluid without heating. Thus, the transfer step results in the release of the glucosyl residue. This data demonstrates that charged dextranucrase can catalyze the transfer of a single glucosyl residue to an acceptor molecule such as maltose. Furthermore, it must be concluded that the glucosyl residues are activated for transfer, and hence must be in a covalent linkage.

Non-mobile material was not shown in Figure 22, since this material normally does not completely elute from the chromatogram and therefore, was not analyzed. Nevertheless, in the previous experiment (Table 1) the appearance of non-mobile material depended on the conditions used to terminate the reactions involving the charged enzyme. It appeared that this material was tightly bound. Thus, it was important to establish the composition of the non-mobile material. This question was examined in the subsequent experiments.

C. Analysis of Non-mobile Material

With reference to the behavior of the non-mobile radioactivity (Table 1), the majority of this material remained tightly bound and was released only after heating. This same behavior was observed after exposure to the acceptor, maltose. In addition, non-mobile material was observed in all previous charging reactions in which heat had been
used to stop the reaction. It had been assumed that origin material represented polymer produced by de novo synthesis and hence, would not migrate on the chromatogram. However, it was equally possible that the heat step released the enzyme from the hydroxyl apatite with sugars covalently bound. Under these conditions the sugars would be non-mobile.

In order to examine this question, immobilized dextranucrase was charged and then heated to stop the reaction. The supernatant fluid containing radioactive sugars was assayed for protein and was found to have less than 1% of the total protein that was bound to the HA. This data is consistent with the premise that non-mobile sugars represent a polymer of some type and that heat is sufficient to remove it from the immobilized enzyme. To support this concept, it was necessary to demonstrate that origin material was polymeric. This could be done by performing a reducing end analysis of the product released by heating. Reduction followed by acid hydrolysis will convert complex glucans to glucose and sorbitol which is derived from the reducing end. A reducing end analysis was performed on the product released in the control reaction (Figure 21). The control product was reduced with NaBH₄ at pH 12 overnight at 50°C and an aliquot was removed for examination. The remainder was hydrolyzed with 1M HCl for 5 hours at 95°C. Both reaction mixtures were examined by paper chromatography in solvent system I. The radioactivity located at the origin, and in glucose, and sorbitol were determined and are presented in Table 2.

As expected, the glucose in the untreated sample was converted to sorbitol after reduction. The fact that the amount of sorbitol
<table>
<thead>
<tr>
<th>Sample Treatment</th>
<th>% Radioactivity At</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Origin</td>
<td>Glucose</td>
<td>Sorbitol</td>
</tr>
<tr>
<td>Untreated</td>
<td>76.5</td>
<td>22.8</td>
<td></td>
</tr>
<tr>
<td>Reduced</td>
<td>74.3</td>
<td>0.8</td>
<td>22.7</td>
</tr>
<tr>
<td>Reduced and Hydrolyzed</td>
<td>0.8</td>
<td>72.3</td>
<td>24.8</td>
</tr>
</tbody>
</table>

An aliquot of the control reaction (Figure 21) was evaporated to dryness in vacuo. The residue was dissolved in 100 μl of 0.01 M NaBH₄, pH 12. The mixture was reacted at 50°C overnight and neutralized with dilute acid. An aliquot was removed for examination and the remainder was hydrolyzed in 1 M HCl for 5 hours at 95°C. Both reaction mixtures were analyzed by paper chromatography in solvent system I. The radioactivity located at the origin, and in glucose and sorbitol were determined. The data for the unreacted sample was taken from the control reaction in Figure 21.
was not greater than the original amount of glucose indicates that no glucose was released from the non-mobile material by treatment with base and heat. This further demonstrates that this material is polymeric and is not simply glucosyl residues covalently bonded to protein. Most covalent bonds between sugars and protein are cleaved under these conditions (77, 78, 79). After acid hydrolysis, the majority of non-mobile material was converted to glucose demonstrating that this material is polymer. At the same time there was a 2% increase in radioactivity in sorbitol. An average chain length can be determined from the following relationship:

\[
\frac{\text{glu} + \text{sorb}}{\text{sorb}} = \frac{72 + 2}{2} = 37
\]

This would correspond to a polymer with a molecular weight of about 6,000. It was recognized that the molecular weight calculation was based on a relatively small increase in percentage radioactivity in sorbitol, and may not have been accurate. Therefore, a more accurate method for determining the molecular weight was sought. If the glucose can be removed from the polymer prior to reduction and acid hydrolysis then any sorbitol observed would have originated from the reducing end of the polymer. This would provide a way of avoiding the large amount of sorbitol derived from glucose.

To remove the glucose from the supernatant fluid, an aliquot from the control reaction (Figure 21) was chromatographed on a G-75 gel filtration column. Fractions were collected and an aliquot was removed to determine the radioactivity. The results (Figure 23) show that two peaks of radioactivity were observed. The higher molecular weight material was 73.1% of the radioactivity which compared well to 76.5% of the isotope at the origin of the paper chromatogram.
An aliquot, from the control reaction (Figure 21), was applied to a G-75 gel permeation column (1.1 x 46.3 cm) and eluted with H$_2$O at 4°C at a flow rate of 0.18 ml/min. Fractions were collected and an aliquot counted in Insta-Gel.
Figure 23
(Fig. 21). The lower molecular weight component represented 25.8% of the isotope, which correlated with the 22.8% in glucose as determined by paper chromatogram. This correlation indicated that the polymer was separated from the glucose. No radioactivity was observed at the void volume of the column. This is consistent with the observation that heat removed the product from dextranucrase, which has a molecular weight of 210,000 and would be excluded from a G-75 column.

The polymeric fraction was pooled and reduced with NaBH₄ at pH 12 overnight at 50°C. After acid hydrolysis with 1M HCl for 5 hours at 100°C, the reaction mixture was chromatographed in solvent system I. The chromatogram showed 92.2% of the isotope in glucose and 5.7% in sorbitol. This indicates that the higher molecular weight peak from the G-75 column was a dextran polymer with an average chain length of 18. The molecular weight of this dextran is about 3,000 and is lower than the weight obtained in the previous experiment. Nevertheless, the results again indicate that the non-mobile material represents a dextran produced by de novo synthesis and that heat treatment is sufficient to remove this polymer from the enzyme.

D. Competition between Transfer to Acceptor and De Novo Synthesis

Another interesting observation made in the experiment described in Figure 21 was the fact that less polymer was present in the maltose chase experiment (38%) than in the control (77%). This data implies that the transfer of the glucosyl intermediate to an acceptor may be competitive with its transfer to polymer in the de novo synthesis measured by the presence of origin material. This competition was also
implied in the hydrolysis experiments where the transfer to acceptors and *de novo* synthesis of dextran were both found to be competitive with the hydrolysis reaction. It was suggested that a common intermediate was involved in all three reactions. Evidence has been provided for a glucosyl intermediate in both the *de novo* synthesis pathway and the transfer reaction. These data provide indirect evidence that these two reactions are competitive. The following experiment was designed to provide direct evidence for this premise.

Since origin material on the paper chromatogram represented polymer from *de novo* synthesis, then a measurement of origin material could be utilized to determine the extent of this reaction in the presence and absence of acceptors. Dextransucrase was incubated with \(^{14}C\)sucrose in 0, 1, 10 and 100 mM maltose for 1, 5, and 10 min., and each reaction was stopped by boiling for 2 min. Each reaction mixture was chromatographed in solvent system II, in which oligosaccharides will migrate from the origin. Based on the appearance of the chromatograms (data not shown) it was apparent from the precursor product relationship that material produced in the transfer of glucosyl residues to the maltose was migrating away from the origin as oligosaccharides. Therefore, the isotope appearing at the origin was used as a measure of the *de novo* synthesis. These results (Figure 24) show that counts appearing at the origin decrease with increasing concentrations of maltose. This trend was observed at all three time points, which demonstrates that the transfer of glucosyl residues to the maltose acceptor was competitive with the formation of dextran by *de novo* synthesis. That this direct competition was observed again indicates a common intermediate. Since both reactions apparently
Figure 24. **Dextran Formation by De Novo Synthesis as a Function of Acceptor Concentration**

Dextransucrase (0.25 units) was incubated with 6.5 μmoles of [14C]sucrose (1.32 x 10^6 dpm) and with either 0, 0.065, 0.65, or 6.5 μmoles of maltose and sodium phosphate buffer, pH 6.0 in a total volume of 65 μl. The reactions were stopped at 1, 5, and 10 min by boiling for 2 min. An aliquot of each reaction was spotted on Whatman 1 MM and chromatographed in solvent system II. After the chromatograms were counted, the % radioactivity appearing at the origin of the chromatograms was determined and plotted as a function of the maltose concentration at each one of the time points of 1 (---), 5 (-x-) and 10 min. (••).
Figure 24
involve a glucosyl enzyme intermediate suggests that the point of competition is this glucosyl enzyme form. The direct competition of these two reactions with the hydrolysis of sucrose indicates that a glucosyl intermediate is also involved in the latter reaction.
IV. DISCUSSION

It is clear that dextransucrase is involved in the production of dental carries and an understanding of enzyme mechanism may play a key role in solving this problem. One proposed mechanism involves a covalent intermediate (35); however, this type of mechanism has not been clearly established. Thus, one major goal of this research was to determine if a covalent intermediate is involved in the reactions catalyzed by dextransucrase.

One approach toward studying potential enzyme intermediates involves the use of gel-filtration columns. However, these initial studies were hampered by the inability of the enzyme to penetrate the chromatographic support. Only 3% of the enzyme eluted with an apparent molecular weight greater than $5 \times 10^6$, which indicated the presence of a large aggregate. Since up to 97% of the enzyme did not penetrate the support, it can be argued that the molecular weights are many times larger than this value. Therefore, the use of this chromatographic technique for mechanistic studies was contingent on finding conditions that would cause the disaggregation of dextransucrase. The fact that dextransucrase was mobile in SDS disc gel electrophoresis (32, 36), and that the enzyme remained active at low levels of SDS (24), indicated that this detergent might be a suitable candidate to cause disaggregation.

On this basis, a study was initiated to study the effects of detergents on properties of dextransucrase. Activity studies showed that, in general, detergents caused an initial stimulation of activity followed
by inactivation. Triton X-100 was unique in that not only did it stimulate, but it also stabilized the enzyme. This was true even in the presence of SDS, which by itself was an inactivator. Similar observations had been made by Miller and Robyt (80) who demonstrated that the enzyme produced by *Leuconostoc mesenteroides* was stabilized by the presence of non-ionic detergents. Harlander and Schactele (81) also observed stabilization of dextran sucrose activity when it was treated with lysophosphatidylcholine. Therefore, SDS and Triton X-100 were used in gel-filtration studies to examine their effect on the enzyme.

The ability of detergents to disaggregate dextran sucrose was demonstrated by gel permeation chromatography on Sepharose 6B. As previously noted, little of the untreated enzyme (M₅ > 4.8 x 10⁶) passed through the column. When SDS alone was employed, the enzyme was retained on the column demonstrating the ability of this detergent to cause disaggregation. While good recovery of protein was observed, only half of the activity was recovered. In the presence of Triton X-100, the enzyme remained in an aggregated state, but the yield was slightly greater than the control. The combination of Triton X-100 or Tween 80 with SDS resulted in good recovery of activity and dissociated the enzyme aggregate.

At the same time, similar observations were made in gel-electrophoresis experiments (28) performed in our lab by D. Grahame. The enzyme was unable to penetrate into the polyacrylamide gel unless SDS was present. However, little or no activity could be observed on such gels. This was in contrast to observations reported by Figures and Edwards (82) who found that Tween 80 alone in electrophoresis permitted migration. The authors suggested that this was due to dissociation
of an aggregate form of the enzyme, although there was no direct support for the idea. In the electrophoresis experiments performed in our lab, the combination of SDS with either Triton X-100 or Tween 80 permitted migration of the enzyme on the gels with resolution into five major bands of dextranucrase activity. These forms had molecular weights ranging from 230,000 to 760,000. The relationship between these forms is not understood and is currently being investigated. The column chromatography data, together with the electrophoresis data, clearly demonstrate that SDS causes disaggregation and the addition of non-ionic detergents such as Triton permitted full recovery of activity. Using these conditions, it was possible to study the disaggregated form of dextranucrase after reaction with sucrose.

After reaction with sucrose, an apparent increase in molecular weight of the unaggregated enzyme was observed. The reacted enzyme co-eluted with radioactive sugars which indicated an association between the protein and the sugar. This material eluted at the same position as observed with aggregated enzyme which showed that the apparent molecular weight of the reacted enzyme exceeded $4.8 \times 10^6$. Since the amount of sugar present could account for an increase in molecular weight of only $3.2 \times 10^5$, this indicated that the enzyme had reaggregated after reaction with sucrose. Under the conditions of the experiment where SDS/Triton X-100 are present, protein-protein interactions are disrupted. Since glucosylation of the enzyme led to reaggregation of the protein, this suggests that sugar-protein interactions are important in this type of aggregate formation. Using conditions without detergent present, Parnaik (31) observed co-elution of enzyme and $^{14}$C-sugars from a Bio-Gel P-200 column after reaction with $^{14}$C-sucrose. Analysis of the sugars
demonstrated that it was a polyglucan and represented dextran produced by de novo synthesis. It is reasonable to assume that this dextran could be involved in enzyme-dextran associations which would result in aggregate formation after reactions with sucrose.

Polyacrylamide gel-electrophoresis of the reacted enzyme also illustrated the same behavior (83). Reaction of the enzyme with sucrose yielded an enzyme form which was non-mobile in SDS/Triton gel electrophoresis. The fact that the treatment of the sucrose-pulsed enzyme with dextranase resulted in a pattern similar to native enzyme supported the idea that aggregation of this type involved intermolecular binding of saccharides and protein. In addition, it was observed that exogenously added dextran did not result in the same degree of aggregation on the gels as did the sucrose-pulsed enzyme. This suggested that the saccharide units on the pulsed-enzyme have a unique property.

Evidence was presented concerning attempts to dissociate the enzyme-sugar aggregate. The reacted enzyme was treated with 8M urea and chromatographed in the presence of SDS/urea. Under these conditions, the denatured enzyme still eluted from this sepharose 6B column at the same position as would the aggregated enzyme. The radioactive sugars remained with the protein in this study. Similar results were obtained with other denaturants. These data were consistent with the concept that the sugars were covalently attached, however, additional experiments were needed to establish this point.

If a covalent intermediate exists, it should be capable of participating in the reactions that the enzyme catalyzes. The demonstration of this is central toward establishing the involvement of an intermediate in the catalytic pathway. In order to do this, it must
be isolated in a form which could be utilized in such studies. Preliminary data had indicated that dextransucrase might catalyze the hydrolysis of sucrose. If the hydrolysis involved the intermediate, it would dictate that techniques for its isolation would have to be rapid. Investigations into this issue was further prompted by the observations of several investigators that hydrolysis of sucrose occurred with various dextransucrase preparations (56, 57, 63, 64); however, they did not demonstrate that this activity was caused by dextransucrase.

Evidence was presented in this study that clearly demonstrated the ability of dextransucrase to catalyze the hydrolysis of sucrose. Data was provided that ruled out contamination by invertase. Raffinose, which is not a substrate for dextransucrase (34, 84), but is a substrate for invertases produced by oral bacteria (76), was not hydrolyzed by this enzyme. This implied that the hydrolytic activity was caused by dextransucrase. This point was more firmly established by the demonstration of co-migration of hydrolytic activity and dextransucrase on polyacrylamide disc gel electrophoresis. The absence of hydrolytic activity elsewhere on the gel also indicated the absence of contaminating invertases.

In addition, the observation that hydrolysis and polymerization were competitive indicated a single enzyme was catalyzing both reactions. The data showed that the rate of hydrolysis was diminished in the presence of an acceptor substrate such as dextran. It was concluded that glucosyl transfer to water and to acceptor were competitive events.

Results from a study using an immobilized form of dextransucrase indicated that hydrolysis was also competitive with de novo synthesis.
When the dextransucrase reaction is carried out in the absence of added acceptors the principal reaction is de novo synthesis. The data indicated that as the rate of de novo synthesis increased, hydrolysis decreased. This was observed as a function of time, which may be related to the production of sufficient dextran to maximize the polymerization rate. It is known that dextran is an activator of dextran sucrase (55, 85, 86, 87, 88). This same relationship was observed as a function of sucrose concentration. This is consistent with the observation that the rate of dextran formation increased with increasing sucrose concentrations. It can be concluded that the hydrolysis of sucrose is competitive with both de novo synthesis and with glucosyl transfer to acceptor. This competition pattern indicated that a single enzyme form is common to all three reactions. Data that was presented here and from other investigators (9, 31, 35, 71) were consistent with the existence of a covalent intermediate. Therefore, the focal point of the competition could involve a glucosylated form of the enzyme, and its isolation would have to be rapid to prevent its complete hydrolysis.

Such a rapid technique was available. A system, first used by Parmak (31), involves the binding dextransucrase to hydroxyl apatite, and in the bound state the enzyme remains fully active. This provides a convenient and rapid means of removing enzyme from its substrate using either centrifugation or filtration. This system had been employed to study the competition between hydrolysis and de novo synthesis. During these reactions, it was observed that radioactive sugars remained bound to the immobilized enzyme after extensive
washing. The amount of radioactivity bound increased with reaction time. The same binding effect was observed as a function of sucrose concentration. The data showed that the system became saturated at elevated sucrose levels. An approximate $K_m$ of 3 mM was observed which is similar to the $K_m$ observed for the formation of dextran (55).

In addition, it was seen that heat treatment could release up to 80% of the sugars bound to the immobilized enzyme. Chromatography of the released material showed that glucose and non-mobile sugars were present. Similar observations were made by Robyt et al. (35) who observed non-mobile saccharides and glucose after reactions involving immobilized dextransucrase. In the study reported here, the amount of glucose released increased with reaction time and then leveled off. This was interpreted as saturation of sites on the enzyme. The same effect was observed as a function of sucrose concentration where at concentrations above 5 mM the amount of glucose became maximal. This saturation behavior, together with the retention of glucose by the immobilized or "charged" enzyme, was strongly suggestive of a glucosyl intermediate. The fact that this material could be released with heat indicated the presence of a relatively labile bond.

The preceding data suggested that a glucosyl intermediate is involved in the dextransucrase reaction. This intermediate or charged form of the enzyme contained monomeric glucosyl residues and some polymeric forms. The possibility that this charged enzyme could transfer glucosyl residues was tested by examining its
participation in the reactions catalyzed by dextransucrase. Three reactions that are catalyzed by dextransucrase were tested: isotope exchange (9, 58, 71), transfer to acceptors and de novo synthesis (55). In addition, evidence was sought to show participation of a glucosyl intermediate in the hydrolytic reaction established by this report.

The possibility that the charged enzyme could transfer glucosyl residues to fructose was tested first. This would be analogous to the isotope exchange reaction, but would involve an isolated intermediate rather than one produced in situ. During a chase of the charged enzyme with unlabeled fructose, a new product was formed that migrated with sucrose. The new product was readily hydrolyzed by invertase, thus demonstrating that sucrose was being formed. This showed that the charged enzyme could transfer single glucosyl residues, and provided the first direct evidence for an active glucosyl intermediate. Thus, reaction with sucrose leads to a glucosylation of the enzyme. The results of this experiment demonstrate the reversibility of this glucosylation reaction and the mechanism by which the isotope exchange reaction occurs. In addition, the data demonstrate that the isolation technique was sufficiently rapid to minimize the hydrolysis of the glucosyl intermediate.

The charged enzyme could also transfer glucosyl residues to maltose to form trisaccharides. Dual label experiments clearly demonstrated that a trisaccharide was being formed from one residue each of maltose and glucose. This shows that only a single glucosyl residue was being transferred to the acceptor. A corresponding
behavior was observed by Mayer and coworkers (55) when they examined the relationship between the acceptor and its product in the transfer reaction. Their data were consistent with a precursor product relationship where the enzyme catalyzes the transfer of single glucosyl residues to the acceptor. The data here demonstrate that capability.

As mentioned in the Introduction, Robyt and Walseth (51) argued that fructose and maltose could release dextranyl and single glucosyl residues from enzyme that had been reacted with sucrose. However, their chases required 24 hour reaction times and their control reaction with uncharged enzyme showed trisaccharide formation following exposure to maltose. Parmaik (31) noted that the rate of reaction with maltose was 0.4% of the expected rate, and pointed out other inconsistencies in their data. Since the observed rate was much slower than the normal rate of transfer, it is questionable that the "intermediate" being examined represents the major pathway for transferring glucosyl residues to acceptors.

The charged enzyme examined in this report exhibits properties significantly different from those associated with the enzyme form reported by Robyt. This charged enzyme catalyzes the transfer of glucosyl residues at rates comparable to the normal rates of transfer of glucosyl residues to acceptors. These reactions are facile and have been observed to occur with chases of 5 seconds or less. In addition, a major fraction of the product produced in these reactions results from the transfer of glucosyl residues to an acceptor. This demonstrates that this charged enzyme is a competent catalyst and
does represent the major pathway for transferring glucosyl residues to acceptors.

It has been pointed out that the technique employed in these studies was sufficiently rapid to minimize the possible hydrolysis of the glucosylated enzyme. However, such a hydrolysis was indicated when the charged immobilized enzyme was allowed to stand in aqueous media. The composition of the radioactivity, that was released, was 92% glucose and 8% non-mobile oligosaccharides. In the presence of acceptors, such as maltose, the amount of glucose released as monosaccharides was reduced to 13%. Instead, the majority of the glucosyl residues, 82%, were transferred to the acceptor. This is consistent with the data that indicated that hydrolysis and transfer to an acceptor compete for a common form of the enzyme. It can now be concluded that the glucosylated enzyme is the common form and can undergo hydrolysis. A consideration of this point again emphasizes the importance of rapid preparation and utilization of the charged enzyme, prior to the dissipation of the "charge" due to hydrolysis.

One important observation made in this maltose chase experiment was that the majority of non-mobile material remained bound to the immobilized enzyme after the maltose chase and in the control reaction. This is somewhat analogous to the tight binding of the dextran to the enzyme during the gel-filtration experiments. The majority of this non-mobile material was released into the supernatant fluid only after heating; however, protein was not observed in the supernatant fluid. Therefore, any covalent bonds that may have existed between the protein and the non-mobile material were cleaved by the heat.
Reducing end analysis of the non-mobile material showed that it was a polyglucan with an average chain length of about 18 and a molecular weight of 3,000. This material probably is a dextranyl oligomer produced by de novo synthesis. The structure of this dextran was not determined and may be different than the dextran produced during long reactions with sucrose.

A reducing end analysis was also performed on the mixture of sugars released by heat. The data indicated that there were approximately 12 monomeric glucosyl residues for every dextranyl chain, and suggests that there are multiple glucosyl sites for each dextranyl site. If the model for dextranucrase action proposed by Robyt et al. (35) is correct, then one would expect an equal number of glucosyl residues and dextranyl chains. It is uncertain why excess glucosyl sites are observed; nevertheless, the data is in direct conflict with the model proposed by Robyt and coworkers and warrants further examination.

Since the non-mobile material represents dextran produced by de novo synthesis, then reactions of this pathway could be examined. The possibility that the glucosyl residues of the charged enzyme were also active in de novo synthesis was tested. A chase of the charged enzyme with unlabeled sucrose showed a decrease in labeled glucose residues with a corresponding increase in non-mobile material. This data indicated that the glucosyl residues of the charged enzyme are incorporated into the dextran produced by de novo synthesis, and is consistent with the insertion mechanism (34, 35) proposed for this type of synthesis. Therefore, it is likely that a glucosyl enzyme
intermediate is also involved in de novo synthesis.

In a separate experiment, it was established that de novo synthesis and transfer to maltose were competitive. This again supports the idea that a single enzyme form is common to both reactions. Since both reactions involve a glucosyl form of the enzyme, it follows that this may be the point of the competition between these two reactions. Similar competitive behavior also exists between the hydrolytic reaction and both the de novo synthesis and transfer to acceptors. Previously, Huang (9) had demonstrated that isotope exchange was competitive with transfer to the acceptor, dextran. Since isotope exchange represents transfer to fructose, which involves a glucosyl enzyme intermediate, it is possible that the point of competition also involves this same intermediate. These data are consistent with the reaction pathway illustrated in Figure 25. In this pathway, the focal point is the glucosyl enzyme intermediate which we propose is formed in reaction with sucrose (Reaction 1). The reverse of reaction 1 represents the isotope exchange reaction by which sucrose can be formed (Reaction -1). The glucosyl intermediate has been shown to be active in transfer to acceptors (reaction 3) and in de novo synthesis (reaction 4). In addition, this same intermediate has been implicated in the reaction by which sucrose is hydrolyzed (reaction 2). The competition between these reactions is substantial reinforcement of the idea that this intermediate plays a central role.
Figure 25. **Proposed Pathway for Reactions Catalyzed by Dextranucrase**

1. Reaction with sucrose
2. Hydrolysis reaction
3. Transfer to acceptor
4. De novo synthesis
5. Hydrolysis of dextranyl intermediate ?
6. Transfer of dextranyl intermediate ?

? Indicates reactions which have not been established for dextranucrase
Figure 25
Evidence was presented here which is consistent with the insertion mechanism (35) proposed for reaction 4, but which was inconsistent with the 2 site mechanism proposed for this reaction. The insertion mechanism involves the formation of a dextranyl-enzyme intermediate. While evidence was reported in this dissertation that supports this concept, further work will be required to establish this point. More direct proof for a dextranyl-enzyme intermediate could be provided by showing its transfer to acceptor (reaction 6). If the insertion mechanism is correct, then glucosyl sites become dextranyl sites after one cycle. This would suggest the sites are equivalent and raises the possibility that the dextranyl-enzyme bond could also undergo hydrolysis (reaction 5); thus, it may be important to utilize a rapid isolation technique to establish the transfer of dextranyl residues to acceptors. In addition, the question of the reversibility of reactions 3 and 4 should also be addressed.

The nature of the apparent covalent bond between the sugars and the enzyme is not well characterized. The tight association between the sugars and protein seen in the gel-filtration experiments, even in the presence of denaturants, supports the idea of a covalent linkage. However, the strongest support comes from the fact that the sugars are catalytically competent. This must mean they are in an activated form, and thus are probably covalently bonded by a high energy bond to the protein. The facile hydrolysis of this, seen at room temperature and at elevated temperatures, is again consistent with this concept. The direct evidence for this will
require the isolation and characterization of a glucosylated amino acid following degradation of the labeled protein.

In addition, the basis for aggregation of the glucosylated enzyme must also be explored. This behavior may play a significant role in the cariogenic process and a study of this may lead to a new understanding of this phenomenon.
V. BIBLIOGRAPHY


128
22. Hakamori, S., J. Biochem. (Tokyo) 55 (1964) 205
23. Bovey, F. A., J. Polymer Sci., 35 (1959) 183
29. Klein, J. P., Schoeller, M., Frank, R., Biochim. 58 (1976) 1047
54. Hehre, E. J., J. Polymer Sci., 23 (1968) 239-244


64. Maynard, M., Kuramitsu, H., Infection and Immun., 23 (1979) 873-883


68. Ebert, K. H. and Brosche, M., Biopolymers, 5 (1967) 423


78. Nuenka, R. H. and Cunningham, L. W., J. Biol. Chem. 236 (1961) 2452


83. Grahame, D. A., unpublished results


