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Purification, surfactant stabilization, molecular weight studies, and divalent metal ion kinetics of dextranucrase from Leuconostoc mesenteroides NRRL B-512F

Arthur West Miller
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PURIFICATION, SURFACTANT STABILIZATION, MOLECULAR WEIGHT STUDIES, AND DIVALENT METAL ION KINETICS OF DEXTRANSUCRASE FROM LEUCONOSTOC MESENTEROIDES NRRL B-512F

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Purification, surfactant stabilization, molecular weight studies, and divalent metal ion kinetics of dextran sucrase from *Leuconostoc mesenteroides* NRRL B-512F

by

Arthur West Miller

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

Department: Biochemistry and Biophysics
Major: Biochemistry

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<tr>
<td>Bicine</td>
<td>N,N-bis(2-hydroxyethyl)glycine</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton</td>
</tr>
<tr>
<td>GTF-I</td>
<td>glucansucrase that synthesizes water-insoluble glucan</td>
</tr>
<tr>
<td>GTF-S</td>
<td>glucansucrase that synthesizes water-soluble glucan</td>
</tr>
<tr>
<td></td>
<td>( synonymous with <em>Streptococcus mutans</em> dextran sucrase)</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>i.r.</td>
<td>infrared</td>
</tr>
<tr>
<td>$\bar{M}_w$</td>
<td>weight average molecular weight</td>
</tr>
<tr>
<td>$\bar{M}_n$</td>
<td>number average molecular weight</td>
</tr>
<tr>
<td>MES</td>
<td>4-morpholineethanesulfonic acid</td>
</tr>
<tr>
<td>mol. wt.</td>
<td>molecular weight</td>
</tr>
<tr>
<td>M.W.</td>
<td>molecular weight</td>
</tr>
<tr>
<td>n.m.r.</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>PIPES</td>
<td>1,4-piperazinediethanesulfonic acid</td>
</tr>
<tr>
<td>pptn.</td>
<td>precipitation</td>
</tr>
<tr>
<td>S.D.</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>S.E.</td>
<td>standard error</td>
</tr>
<tr>
<td>S.E.M.</td>
<td>standard error of the mean</td>
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<tr>
<td>u.v.</td>
<td>ultraviolet</td>
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DEDICATION

To Alfred Tennyson,
for Ulysses
GENERAL INTRODUCTION

Dextran sucrase is one of a family of glucosyltransferases and fructosyltransferases, also called glucansucrases and fructansucrases, secreted by some species of Leuconostoc and Streptococcus [312, 343]. Glucansucrases polymerize the glucosyl moiety of sucrose to form branched polysaccharides that can be classified according to their main-chain linkages: α-(1→6) for dextrans, α-(1→3) for mutans, and α-(1→6) alternating with α-(1→3) for alternans [65]. The same enzymes responsible for the main-chain linkages also make the branch linkages [67, 68, 280, 284], which can be α-(1→2), α-(1→3), α-(1→4), or α-(1→6) [343].

The dextran made by the dextran sucrase of L. mesenteroides NRRL B-512F is used commercially as a blood plasma extender and to make specialty products such as dextran sulfate and Sephadex. This enzyme will frequently be referred to in this dissertation in the abbreviated form "B-512F dextran sucrase."

The mechanism of glucansucrase action appears to involve covalent glucosyl and glucanosyl enzyme intermediates (Figs. 1-2) [280]. Polymer synthesis (Fig. 1) occurs by the insertion of glucose residues at the reducing end of a glucanosyl-enzyme intermediate. Another type of transfer (Fig. 2), called an acceptor reaction, takes place when the enzyme-bound glucosyl or glucanosyl groups are transferred to a hydroxyl group of an acceptor compound, which can be a low-molecular-weight sugar (Fig. 2A), an unbranched segment of a glucan molecule (Fig. 2B), or a water molecule (Fig. 2C). When water is the acceptor, the reaction is sucrose hydrolysis or glucan chain termination. When an unbranched segment of a glucan molecule is the acceptor, the result is a branch linkage [280]. Glucansucrases can also transfer glucose residues from the nonreducing ends of glucans and certain oligosaccharides to other acceptors [26].

The relative rates of the different transferase reactions catalyzed by glucansucrases depend on the temperature and on the concentrations of enzyme, sucrose, preexisting glucan, acceptors, and various cations.
Figure 1. Proposed mechanism for glucan synthesis by glucansucrases

$X_1$ and $X_2$ are nucleophiles at the active site: $\textcircled{H}$ is sucrose; $\textcircled{O}$ is a glucose residue; $\textcircled{F}$ is a fructose residue; $\textcircled{B}$ indicates glucose residues joined by a main chain glucosidic linkage, either $\alpha-(1\rightarrow6)$ or $\alpha-(1\rightarrow3)$. 
Figure 2. Mechanism for the acceptor reactions of glucansucrases

$X_1$ and $X_2$ are nucleophiles at the active site; $\emptyset$ and $\blacklozenge$ are reducing end glucose residues; $\circ$ and $\bullet$ are nonreducing end glucose residues; $\longrightarrow$ is a main chain glucosidic linkage, and $\downarrow$ is a branch linkage. Acceptors are indicated by solid symbols. The acceptors shown in (A) and (B) are fragments of the glucan made by the glucansucrase represented, but the enzyme will transfer glucose and glucan groups to many other compounds.
A. TRANSFER TO NONREDUCING END OF ACCEPTOR

B. GLUCAN BRANCHING BY ACCEPTOR REACTIONS

C. HYDROLYSIS BY ACCEPTOR REACTIONS WITH WATER
Many glucansucrase-producing bacteria secrete a mixture of glucansucrases, fructansucrases, dextranase, fructanase, and invertase [286, 343], and the ability of these enzymes to use sucrose and/or the products of the other secreted enzymes as their substrates has complicated the study of glucansucrases.

Kinetic studies have been complicated both by the changeable structure and distribution of the enzyme products and by the difficulty in purifying the enzymes to homogeneity. A major difficulty in purification is the removal of glucan, which can affect glucansucrase properties at minute concentrations [237]. Binding studies and chemical modification studies have been hindered by the low purity of most glucansucrase preparations, and also by the strong tendency of glucansucrases to aggregate.

The dextranase from L. mesenteroides NRRL B-512F is an unusually good candidate for purification and for kinetic studies. L. mesenteroides NRRL B-512F secretes only one glucansucrase, and relative to most glucansucrase-producing bacteria, it secretes only small amounts of other enzymes that act on sucrose or dextran. B-512F dextranase has relatively little sucrase activity, so that if the concentration of acceptors is low, almost all of the sucrose consumed is used to synthesize a highly soluble dextran with about 5% branching, placing it among the least branched dextrans known [164].

The parts of this dissertation on metal ion kinetics were undertaken to fill the gap in the literature on metal ion effects, which have been largely ignored. Metal ions change the rates of glucan synthesis and the linkage ratios in the glucans synthesized [242, 286, 339]. The mechanistic scheme shown in Figs. 1-2 does not explain this. It was soon found that purified B-512F dextranase, from which dextran had been removed, was irreversibly inactivated by inhibitory metal ions. This led to a search for stabilizing compounds (Part I), to permit studies of metal ion kinetics to continue. The discovery that purified dextranase preparations contained dextranase stabilizers in turn led to efforts to obtain more purified dextranase preparations (Parts II and III), so that the mechanisms of stabilizer action could be examined.
Part VI of this dissertation, on molecular weight determination, was undertaken to determine whether dextran sucrase was a dimer, as was suggested by the presence of two functionally identical nucleophiles ($X_1$ and $X_2$) in Figs. 1-2. In the course of this investigation, an artifact was found in the periodic acid-Schiff stain that made the stain unreliable for detecting glycansucrase activity on polyacrylamide gels. This artifact is the subject of Part VII.

Explanation of Dissertation Format

This dissertation is presented as a series of parts in journal format, since many of the parts have been submitted for publication in condensed form, or will be. In the hope that the dissertation may be valuable as source material for graduate students continuing the same or similar work, more data are presented than in the corresponding manuscripts, and the discussions are longer and give more coverage to details of experimental technique. References are listed only once, near the end of the dissertation.

The work was performed entirely by myself, under the guidance of Dr. John F. Robyt, with the exception of Part II, where Steve Eklund provided herculean technical assistance. In addition, Dr. Robyt prepared the purified enzyme used in Part I, and Joe Couto prepared some of the crude enzyme concentrate used.
LITERATURE REVIEW
Stability of Dextranucrase in the Absence of Detergents

Robyt and Walseth [286] found that purified B-512F dextranucrase, from which dextran had been removed by dextranase treatment, lost activity rapidly over a period of days unless dextran were added back. Stability increased with added dextran concentration to a concentration of 4 mg/ml. Miller and Robyt [237] found that temperature increase, dilution, and physical manipulation increased the rate of activity loss.

Dextranase treatment of other L. mesenteroides glucansucrases [158, 339, 343] and of Streptococcus glucansucrase [54] had previously been found to decrease stability. While in some cases loss of activity may have been caused by protease, this was not true for the procedure of Robyt and Walseth [286]. The enzyme solution produced by the method of Robyt and Walseth, if sufficiently concentrated and not disturbed by manipulation, lost no activity at 4° for over a year.

The general topics of enzyme stability and enzyme stabilization have been reviewed [13, 69, 182, 224, 354]. For glucansucrases specifically, stability has been increased by adding glucan [286], calcium [172, 212, 237], or detergents [237] as stabilizers. Calcium, which stabilizes many exocellular bacterial enzymes [354], has been implicated both directly [172, 212] and indirectly (by the irreversible inactivation that takes place in the presence of EDTA [158, 205]) in the stability of L. mesenteroides glucansucrases. Stabilization by detergents is discussed below.

Effects of Lipids and Detergents on Dextranucrase

Stabilization of glucansucrases by detergents has been problematic, and often overshadowed by the effects of detergents on other enzyme properties or on enzyme production. The literature will be discussed chronologically, and not by detergent effect (such as stabilization or
increase in enzyme production).
Umesaki et al. [341] found that for S. mutans OMZ 176, 1 mg/ml Tween 80 did not affect glucansucrase stability, glucansucrase activity, or bacterial growth. Tween 80 did not solubilize membrane-bound glucansucrase or increase the amount of intracellular glucansucrase. Tween 80, Tween 60, and Emanon 4115 (also a polyoxyethylene-based detergent) increased GTF-I production and shifted the fatty acid composition of the bacterial membrane toward longer chain fatty acids.

Wittenberger et al. [355] found that the low amounts of glucansucrase produced by S. salivarius ATCC 25975 in a chemically defined medium were enhanced by Tween 80, but not by oleic acid, methyl oleate, or sucrose. Activation of the glucansucrase by Tween 80 varied from 0 to 30%.

Russell [290] found that a variety of nonionic detergents fully reactivated a mixture of glycosyltransferases from S. mutans strain Ingbritt that had been inactivated with SDS. Harlander and Schachtele [136] found that phosphoglycerides activated both GTF-S and GTF-I from S. mutans 6715, and that the activation was independent of activation by dextran. They found a sigmoidal kinetic response to phospholipids. $K_m$ was unchanged, and $V_{max}$ was increased. Some of these findings were later extended to the phospholipids of human sera and oral fluids [300].

Burckhardt and Guggenheim [36] had earlier found activation of S. mutans OMZ 176 glucansucrases by oral fluid.

Kuramitsu et al. [201] found that both GTF-I and GTF-S from S. mutans GS5 were inhibited by lipoteichoic acid, which is known to inhibit other enzymes. The inhibition was competitive with sucrose and with dextran T10, and was less for liposome- or cell-bound activity than for soluble activity. Deacylated lipoteichoic acid, which was not inhibitory, caused the formation of enzyme aggregates.

Figures and Edwards found that at high ionic strength, Tween 80 improved purification yields [97], and also that Tween 80 reduced the aggregation state [98] of the glycosyltransferases of S. mutans 6715 and FA-1. Luzio et al. [215] found that S. sanguis dextransucrase, which
had been purified in aggregated form, was activated and then inactivated in the presence of nonionic detergents, but was not disaggregated. SDS disaggregated the enzyme, but also inactivated it. The combination of SDS and a nonionic detergent disaggregated the enzyme without inactivating it.

Shimamura et al. [311] found that *S. mutans* 6715, which in the absence of Tween 80 produced only two glucansucrases, which were both activated by dextran, produced a third glucansucrase in the presence of Tween 80, which was not activated by dextran. Miller and Robyt [237] found that any of a wide variety of nonionic detergents stabilized *L. mesenteroides* NRRL B-512F dextranucrase. Their results are discussed in detail in Part I of this dissertation.

Ono et al. [258] reported detergent effects much like those found by Figures and Edwards [97, 98] and by Luzio et al. [215]. Specifically, 90% of the glucansucrase activity in a preparation purified from *S. mutans* 6715 was lost during gel filtration unless both SDS and a nonionic detergent were present, in which case only 10% of the activity was lost. SDS in combination with a nonionic detergent reduced the size of the glucansucrase aggregates, but nonionic detergent alone did not. Ono et al. [258] concluded that the effect of detergents was on protein-protein interactions and not on protein-glucan interactions.

Jacques et al. [161] found that Tween 80 was more effective at promoting the production of *S. salivarius* dextranucrase than other members of the Tween series of detergents, and that Triton X-100 was ineffective. Detergents often increase the production of microbial enzymes [93]. This may be by increasing membrane fluidity [161] and permeability [93] or, found less often [93], by protecting the enzymes from inactivation.
PURIFICATION AND PROPERTIES

Reviews

The purification and properties of the glucansucrases from Streptococcus have been reviewed extensively [52, 127, 128, 133, 239, 255, 343], and for those from Leuconostoc less recently [86, 248, 312, 336, 343]. Dextranucrase from L. mesenteroides NRRL B-512F is quite similar to streptococcal glucansucrases in terms of mechanism and most properties; e.g., glucan synthesis from the reducing end [283, 287], branching [68, 284] and other transferase [26] reactions, size (see Part VI of this dissertation), and pH optimum [172, 239, 255, 310, 312]. Differences in properties are the calcium dependence [237], higher $K_m$ [52, 84, 127, 239, 244, 247, 255, 320] and nonactivation by dextran [255, 286, 343] of the B-512F enzyme. These differences should probably be thought of as quantitative differences rather than as qualitative differences [68, 255].

Dextranucrase from L. mesenteroides

Table I summarizes the recent literature on B-512F dextranucrase purification, and also older literature where notably high specific activities or low carbohydrate contents were obtained. Because glucansucrase purification has been reviewed in four doctoral dissertations [63, 64, 345, 360] and three master's theses [171, 181, 223] from Iowa State University during the period 1977-1983, with particular emphasis on B-512F dextranucrase by Walseth [345] and by Kaboli [171], only the recent literature will be described in detail here. Numerical values for specific activity and carbohydrate content are given in Table I rather than in the text. Kinetic properties and molecular weight determinations are described in other sections of this review.

Robyt and Walseth [286] purified B-512F dextranucrase by concentrating and dialyzing the culture supernatant. treating the concentrate
Table I. B-512F dextranucrase purifications by year of publication

<table>
<thead>
<tr>
<th>Year</th>
<th>Authors</th>
<th>Ref.</th>
<th>Specific Activity</th>
<th>Yield</th>
<th>Mg carbohydrate per mg protein</th>
<th>Method</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>1955</td>
<td>Tsuchiya et al.</td>
<td>337</td>
<td>17-50 U</td>
<td>75%</td>
<td>Not given</td>
<td>Ethanol pptn.; alternatively,</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>per mg solids</td>
<td></td>
<td></td>
<td>ammonium sulfate pptn.</td>
<td></td>
</tr>
<tr>
<td>1957</td>
<td>Bailey et al.</td>
<td>11</td>
<td>0.02 U</td>
<td>19%</td>
<td>Carbohydrate 7-8% of solids</td>
<td>Growth in presence of maltose,</td>
<td>Low carbohydrate content. Activity is &quot;primed&quot; by maltose.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>per mg solids</td>
<td></td>
<td></td>
<td>ethanol pptn.</td>
<td></td>
</tr>
<tr>
<td>1959</td>
<td>Bovey</td>
<td>30</td>
<td>10 U</td>
<td>Not</td>
<td>Dextran content &quot;appreciable&quot;</td>
<td>Ammonium sulfate pptn.</td>
<td>Ammonium sulfate coprecipitates less dextran than ethanol.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>per mg solids</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1959</td>
<td>Braswell and Stern</td>
<td>33</td>
<td>35 U</td>
<td>Not</td>
<td>Not given</td>
<td>Ethanol pptn., low-pH pptn., hydroxyapatite</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>per mg solids</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>per mg solids</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1962</td>
<td>Ebert and Schenk</td>
<td>84</td>
<td>95 U/mg protein</td>
<td>Low</td>
<td>0.031</td>
<td>Methanol and ammonium sulfate pptn., hydroxyapatite</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(lower limit)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Year</td>
<td>Authors</td>
<td>U/mg</td>
<td>Protein (%)</td>
<td>Protein Yield (%)</td>
<td>Purification Method</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-------</td>
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<td>------</td>
<td>-------------</td>
<td>-------------------</td>
<td>---------------------------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1979</td>
<td>Robyt and Walseth</td>
<td>286</td>
<td>53</td>
<td>33%</td>
<td>Dextranase, gel filtration</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1979</td>
<td>Lawford et al.</td>
<td>205</td>
<td>Not given</td>
<td>Not given</td>
<td>Ammonium sulfate pptn. (+egg albumin), DEAE-cellulose</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1980</td>
<td>Kaboli and Reilly</td>
<td>172</td>
<td>34</td>
<td>54%</td>
<td>Ultrafiltration, gel filtration</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1980</td>
<td>Kobayashi and Matsuda</td>
<td>188</td>
<td>72</td>
<td>2%</td>
<td>Hydroxyapatite, Sephadex G-100, low yield.</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Purified enzyme acts</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Sepharose 6B, Aminobutyl-Sepharose 4B on raffinose.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1980</td>
<td>Lopez and Monsan</td>
<td>212</td>
<td>120</td>
<td>82%</td>
<td>Ultrafiltration, gel filtration</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1981</td>
<td>Monsan and Lopez</td>
<td>238</td>
<td>122</td>
<td>96%</td>
<td>Gel filtration</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>One-step purification.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1984</td>
<td>Paul et al.</td>
<td>263</td>
<td>&gt;170</td>
<td>95%</td>
<td>Phase partition (dextran/PEG)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>One-step purification.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1985</td>
<td>Yokoyama et al.</td>
<td>357</td>
<td>Not given</td>
<td>25%</td>
<td>Ethanol/CaCl₂ pptn, gluconolactone-Sepharose</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Remove 99.0% of dextran originating in culture</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1985</td>
<td>Miller and Robyt</td>
<td>170</td>
<td>30%</td>
<td>0.0007</td>
<td>Dextranase, DEAE-cellulose</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Part II of this dissertation</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
with dextranase, removing the added dextranase and other impurities on a Bio-Gel A-5m column, and concentrating the dextransucrase peak from the column over an Amicon PM10 membrane. The resulting preparation was very pure enzymatically (free of dextranase, levansucrase, and sucrose phosphorylase), and had a fairly high specific activity, but had a high carbohydrate content, mostly in the form of a high-mannose polysaccharide.

Kobayashi and Matsuda [188] purified B-512F dextransucrase to a higher specific activity and to a much lower carbohydrate content than did Robyt and Walseth. The carbohydrate in the purified preparation consisted mostly of dextran that was tightly bound to the enzyme. The procedure had many steps and a low yield. Dextranase from Chaetomium gracile, which is not commercially available, was used.

The purified enzyme was homogeneous by three types of gel electrophoresis, and did not contain glucosidase or dextranase, as determined by failure to detect products from native B-512F dextran or isomaltotetraose. The preparation acted on raffinose, generally not considered to be a substrate for dextransucrase [286], to produce fructose, melibiose, and a trace of leucrose. Because the reaction on raffinose was stimulated by dextran, the authors did not believe that it was caused by levansucrase or invertase.

Besides action on raffinose, properties of the purified B-512F dextransucrase of Kobayashi and Matsuda [188] that were different than those found in this dissertation included a molecular weight of 64,000-65,000, a pH optimum of 6.0, a pH stability range of 7-9 at 50, a high degree of sucrase activity, no affinity for Sephadex G-100, and activation by 0.5 mM SDS.

Recently, Matsuda and coworkers have described another method for purifying the dextransucrases from six strains of L. mesenteroides, including B-512F [357]. The terminal step of the procedure is the removal of most of the dextran on a gluconolactone-Sepharose column. This column bound part, but not all, of the dextransucrase in the applied preparations, but almost none of the carbohydrate. For B-512F dextransuc-
erase, two-thirds to three-quarters of the activity bound. While the procedure was much simpler than the earlier method [188], a substantial amount of carbohydrate remained in the purified preparation.

No evidence was presented that the chromatography on gluconolactone-Sepharose was an affinity step. The dextranoses were eluted from this column with salt. The method of preparing the gluconolactone-Sepharose [328] results in negative charges produced by the hydrolysis of the lactone, and in positive charges from the coupling of ethanolamine groups to block reactive sites on the column not already blocked by the coupling of lactone. The ability of a 0.2 M NaCl to elute the bound enzyme makes a specific enzyme-column interaction unlikely (see pp. 99-102 in Scouten [306]).

Monsan and coworkers have developed two one-step methods for purifying B-512F dextranuose from culture supernatant [238, 263]. One method consists in its entirety of the gel filtration of highly active culture supernatant, giving a dextranuose preparation with very high specific activity and in very high yield [238]. This method, which is related to that of Kaboli and Reilly [172], depends for its usefulness on the six-fold increase in the production of dextranuose (from 1.5 to 9 U/ml) given by the continuous addition of sucrose to the bacterial culture, relative to adding sucrose only at the beginning [212].

The second method [263], for which few experimental details are yet available, gives a dextranuose preparation with even higher yield and specific activity by means of phase partition in a dextran-poly(ethylene glycol) two-phase solvent system. In the same paper [263], it was reported that three times as much dextranuose could be produced per unit time by continuous culture as could be produced by the fed-batch culture that had been used for the gel filtration method [238]. Enzyme concentration reached higher levels in batch-fed cultures, but each batch took many hours to process.

In both of these purification methods, levansucrase was removed. The only clear drawback of either method was the high dextran content of the purified dextranuose.
A method for purifying B-512F dextranucrase using DEAE-cellulose chromatography has been described by Lawford et al. [205]. Details are given in the discussion section of Part II.

Kobayashi et al. [189] have purified dextranucrase from *L. mesenteroides* NRRL B-1416 by chromatography on DEAE-cellulose, aminopropyl-Sepharose, and Sepharose 6B.

**Glucansucrases from Streptococcus**

The glucansucrases and fructansucrases of *S. mutans* IB, Bl4, and OMZ 175 have been purified and characterized by Sato et al. [298]. The glucansucrases make water-soluble glucans, and the fructansucrases are inulinsucrases. Furuta et al. [109] have cleanly separated GTF-I and GTF-S from *S. mutans* 6715 using ammonium sulfate precipitation, chromatofocusing, and phenyl-Sepharose CL-4B or hydroxyapatite chromatography. A monoclonal antibody to GTF-I was used to show the separation of the two enzymes.

Using ultrafiltration, DEAE-Sepharose chromatography, and preparative isoelectric focusing, Tsumori et al. [338] have purified a glucansucrase from *S. mutans* HS6 that makes a linear α-(1→3) glucan. The purified enzyme contained 6% carbohydrate, and had a pH optimum of 6.0, a $K_m$ of 1.2 mM sucrose, and a specific activity of 48.3 U/mg protein.

Ono et al. [258] have purified glucansucrases from *S. mutans* 6715 and from some other strains of *S. mutans* by ammonium sulfate precipitation and affinity chromatography on Sephacryl S-200, followed by one or more of a variety of other column steps. For alkylamine-Sepharose columns, it was found that yield decreased with increasing alkyl chain length, and that the enzyme bound irreversibly when alkyl groups were longer than C6. For hydroxyapatite and ion-exchange columns, including 1% dextran T10 in the eluent gave better resolution and higher yields. It was found that a very small contamination of the enzyme sample with dextran prevented the enzyme from binding to glucan-based affinity columns.
The purified glucansucrase migrated on a gel filtration column with $M_r 2 \times 10^6$ in the absence of detergents, or in the presence of only a nonionic detergent. This was reduced to $M_r 6 \times 10^5$ if SDS and a nonionic detergent were used together. The enzyme migrated in the void volume when high-molecular-weight dextran was added to it, but was returned to $M_r 6 \times 10^5$ if dextran T10 was then added.

The resolution of an endodextranase inhibitor from the glucansucrases of *S. mutans* 6715-49 is described by Hamelik and McCabe [129]. The relevance of this is that the presence of the dextranase secreted by *S. mutans* can be masked if the inhibitor is not removed, leading to spurious results in studies of glucansucrase reactions.

Some details on the affinity chromatography of glucansucrases that are not available elsewhere are given by Inoue and Smith [156].
Inferences made about dextransucrase mechanism from kinetic studies have been reviewed by Sidebotham [312] and by Robyt [280]; however, neither the kinetic mechanism of dextran polymerization nor of acceptor reactions is known. For this reason, this section will review a large number of dextransucrase reactions that should be considered, or which it might be possible to exploit, in kinetic studies of mechanism.

General Mechanism

Stringer and Tsuchiya [320], using mixtures of α-methyl glucoside and sucrose, concluded that the mechanism of acceptor reactions, and by extension the mechanism of dextran formation, was sequential rather than ping pong for B-512F dextransucrase. Neely and Thompson [251] also concluded from the effect of dextransucrase concentration on rate that a sequential mechanism was likely. Kobayashi and Matsuda [187], from a study using mixtures of B-512F dextransucrase and either glucoamylase or endodextranase, concluded that the mechanism of dextran polymerization was ping pong bi bi. Kinetic studies of levansucrase from Bacillus subtilis have been also interpreted as showing a ping pong bi bi mechanism for levan synthesis in the presence of pre-existing levan [48].

Bovey [31] derived a steady-state kinetic equation for B-512F dextransucrase that contained two terms, one for the formation of high-molecular-weight dextran by a single-chain mechanism, and one for the formation of low-molecular-weight dextran by acceptor reactions. Ebert and coworkers [83, 85, 86] also derived a rate equation to account for acceptor reactions and substrate inhibition in addition to dextran polymerization by a single-chain mechanism.

Bovey’s mechanism accounted for observations that acceptor reactions reduced the rate of high-molecular-weight dextran synthesis, and that both $K_m$ and $V_{max}$ increased with acceptor concentration. By his scheme, $K_m$ and $V_{max}$ would be predicted to increase linearly with acceptor concentra-
tration, and substrate inhibition would occur in the formation of high-molecular-weight dextran, but not in the formation of acceptor products. There were unexplained deviations from the model at low sucrose and high acceptor concentrations. Stringer and Tsuchiya [320] also found experimental deviations from their model.

Sucrose Hydrolysis

Initial rate studies where only polysaccharide formation is measured may seriously underestimate the reaction rate because of sucrose hydrolysis by dextranucrase. Sucrose hydrolysis by glucansucrases has been briefly discussed by Ciardi et al. [53]. For dextranucrase from S. sanguis ATCC 10558, Luzio and Mayer [214] found that the ratio of sucrose hydrolysis to dextran synthesis decreased with increasing sucrose concentration, and that the ratio also decreased during the course of reaction.

Highly purified B-512F dextranucrase preparations were found by Robyt and Walseth [286] and by Paul et al. [263] to give 0.5 and 3.9 mole percent of glucose from sucrose, respectively. Matsuda and coworkers [188, 357] also found sucrose hydrolysis by purified B-512F dextranucrase. The amount of glucose formed, which could be as high as 32 mole percent [357], was unaffected by the presence of clinical dextran [188], but was reduced by aggregation of the enzyme [357].

Effect of dextran Dextran can decrease the amount of sucrose hydrolysis. This is discussed below under "Activation by Dextran."

Effect of temperature Lawford et al. [205] found that the mole fraction of glucose produced by purified B-512F dextranucrase decreased with decreasing temperature, from 34% at 30° to 20% at 10°. (Tanaka et al. [329] have also reported the corresponding finding for levansucrase; i.e., that the ratio of reducing sugar release to polymer synthesis by levansucrase decreases toward unity as the temperature decreases from 30° to 4°.) This result may be difficult to reconcile with the finding by Tsuchiya et al. [337] that lowering the reaction temperature
increases the fraction of low-molecular-weight products produced by B-512F dextransucrase, a feature of acceptor reactions which has been exploited in the preparative biosynthesis of isomalto-oligosaccharides [169, 314].

Substrate Inhibition by Sucrose

Whether assayed by fructose release or by dextran formation, dextransucrase is inhibited by high sucrose concentrations. Neely [245] estimated a sucrose inhibition constant of 3.9 M for a B-512F preparation having a $K_m$ of 35 mM. For B-512F dextransucrase, maximum activity occurs at about 200 mM sucrose [138, 245]. For other glucansucrases, it is often lower (e.g., 50 mM [99]).

Stringer and Tsuchiya [320] found that $\alpha$-methyl glucoside prevented substrate inhibition for B-512F dextransucrase. Mayer et al. [226], for dextransucrase from S. sanguis ATCC 10558, found the more general result that dextran and all acceptors prevented substrate inhibition, and that they also caused a linear increase in $V_{\text{max}}$ and $K_m$ (by reducing sugar assay). For the same dextransucrase, Jung and Mayer [170] found that when glucosyl fluoride was used instead of sucrose, the onset of substrate inhibition occurred at nearly the same concentration as with sucrose. As with sucrose, maltose or dextran T10 eliminated the substrate inhibition. Figures and Edwards [99] isolated an S. mutans glucansucrase fraction that had no measurable substrate inhibition, but the isolation procedure may have caused this fraction to be contaminated with glucan that prevented inhibition.

Models to explain these results have been described above under "General Mechanism."

Enzyme Concentration

A handful of papers, all but one using B-512F dextransucrase, have reported effects of dextransucrase concentration. Neely and Thompson
(see also "General Mechanism" above) found that increasing the enzyme concentration first increased and then decreased the reaction rate. Tsuchiya et al. [337] found that increasing the enzyme concentration shifted the molecular weight of the dextran synthesized to lower values. The shift was less at lower temperatures. Braswell et al. [34] found that decreasing the enzyme or the sucrose concentration increased both the molecular weight of the dextran and the amount of branching. Increasing the temperature did the same. Tsumuraya et al. [339] found for L. mesenteroides IAM 1046 dextran sucrase that neither temperature nor concentration of enzyme, sucrose, or fructose affected the amount of branching, although branching did decrease below pH 5.0.

**Activation by Dextran**

Added dextran has frequently been found to activate the glucansucrases of Streptococcus but not of Leuconostoc [280], although Kobayashi and Matsuda reported that dextran activated purified B-512F dextran sucrase, decreasing the $K_m$. Activation by dextran is not fully understood, but does not result from a substrate ("primer") requirement for dextran [99, 109, 193, 226, 261, 283, 311]. In many cases, the activation is correlated with an increase in branching reactions, especially where an increase in the rate of insoluble glucan formation is observed.

In other cases, the activation appears to be caused by an increase in glucan synthesis at the expense of a reduction in sucrose hydrolysis. Tsumori et al. [338] found that sucrase activity for purified GTF-I was high both in the presence and absence of dextran T10, but that glucan synthesis was increased twenty-fold by dextran T10 from a minute rate in its absence. The authors speculated that consecutive α-(1→6) linkages were needed for an added glucan to be activating. Fukui et al. [107] found that GTF-I from S. mutans 6715 had high sucrase activity in the absence of dextran, and that insoluble glucan was formed at a very low rate. Adding a small amount of dextran T10 increased the rate of insoluble glucan synthesis to nearly the rate of reducing sugar release.
Figures and Edwards [99] found that the rate polymer synthesis by GTF-I from S. mutans 6715 was increased by a factor of three by dextran when either sucrose or glucosyl fluoride was used as the substrate. With glucosyl fluoride, fluoride release was the same in the presence or absence of dextran, at a rate corresponding to the maximum rate observed for polymer synthesis. Ono et al. [258] found that GTF-I from S. mutans 6715 was more inhibited by periodate-oxidized dextran than GTF-S, but that fructose release was not inhibited. Fructose release by one purified glucansucrase fraction was remarked to be high at low dextran concentration.

Nonlinear kinetics

For the dextransucrase from L. mesenteroides B-1416, whose activity is much more sensitive to dextran concentration than that of B-512F dextransucrase [189], dextran was found to give hyperbolic mixed-type activation [189]. At fixed sucrose concentration, plots of 1/v vs. 1/[dextran] curved downward with increasing dextran concentration. It was concluded that independent sucrose and dextran sites existed, with sucrose able to bind to both. Very similar results have been observed by Mayer et al. [226] for dextransucrase from S. sanguis ATCC 10558, by Chludzinski et al. [51] and Shimamura et al. [310] for dextransucrase from S. mutans 6715. Chludzinski et al. [51] showed a Hill plot suggesting that sucrose and dextran competed for a common site.

The existence of multiple dextran sites on single enzyme molecules cannot be concluded from these observations alone. Double-reciprocal plots necessarily show the curvature described, over some range of activator concentration, for nonessential mixed-type activators that bind to only one site on an enzyme (see pp. 227-242 in Segel [307]). Kinetic cooperativity can also occur for monomeric, single-site enzymes with nonequilibrium mechanisms [252]. Curved plots would also result if dextran were an essential activator present in minute, unmeasured amounts in enzyme or sucrose preparations (all commercially available sucrose contains dextran [164]) or if multiple enzyme forms with different dextran
dependence were present.

Inhibition by Dextran

A small number of reports exist of the inhibition of *S. sanguis* and *S. salivarius* dextranucrases by clinical dextran [176, 350, 355]. The noncompetitive inhibition of GTF-I from *S. mutans* by dextran [99, 239], on the other hand, is probably only a reduction in insoluble glucan formation concomitant with an increase in soluble glucan formation.

Acceptors inhibit the formation of polysaccharide, but this is not enzyme inhibition as it is conventionally viewed because it is the result of an increase in the formation of low-molecular-weight acceptor products [282]. For example, a large number of disaccharides "inhibit" insoluble glucan formation by *S. mutans* GTF-I [309], with maltose being a competitive inhibitor [239]. An inhibition that may be real in the conventional sense is the competitive inhibition by fructose of polysaccharide formation by *S. mutans* dextranucrase [50].
Relation to branching reactions

The branching of glucans synthesized by glucansucrases occurs by acceptor reactions [280, 284]. The activation of glucansucrases by dextran can be partly explained on the basis of an increase in branching reactions. This may also account for the synergistic activation that occurred when two glucansucrase fractions of low activity isolated from *S. mutans* OMZ 176 were combined [193].

Small oligosaccharides are not readily branched by most glucansucrases. Thus, a mixture of sucrose, glucose, and B-512F dextranucrase gave only linear acceptor products up through an oligosaccharide size of at least 11 glucose residues [314]. Linear isomalto-oligosaccharides smaller than isomaltotetraoside are not branched by GTF from *S. mutans* OMZ 176, and very little branching occurs with acceptors smaller than isomaltotetraoside [344]. Activation of glucansucrases by the addition of acceptor (branchable) dextran or of low-molecular-weight acceptors therefore may not result from a fast catalytic rate for acceptor reactions relative to main-chain polymerization, but rather from an increase in the rate of single-chain (reducing end) synthesis occurring by the release of large, covalently bound, sterically hindering glucanosyl groups from the enzyme [280, 281], or by the prevention of their formation.

"Reverse" and "disproportionating" acceptor reactions

Isotope exchange experiments [29, 114, 226] show that B-512F dextransucrase will exchange the fructose moiety of sucrose with free fructose. Similarly, sucrose and leucrose are both produced by dextransucrase from the non-natural substrates glucosyl fluoride [114] and lactulosucrose (4-F-β-galactosylsucrose) [139] in the presence of fructose.

Glucansucrases slowly transfer glucose residues from the nonreducing ends of the glucans and acceptor products that they synthesize to other acceptors [26, 228]. Some free glucose is produced during these reactions. McCabe and Hamelik [228] found that this "disproportionating" transferase activity did not consistently associate with either GTF-S or
GTF-I when these S. mutans enzymes were separated, and concluded that they were caused by a branching enzyme distinct from GTF-S and GTF-I. Binder et al. [26] found disproportionating transferase activity in all purified glucansucrase preparations from a variety of strains of L. mesenteroides and S. mutans, and concluded that the activity was integral to glucansucrase mechanism.

### Metal Ions

Literature on the effects of metal ions on glucansucrases is sparse, and is nearly all represented in Table II. This literature is discussed in Parts I, IV, and V of the dissertation. A good summary is also given on pp. 20-21 of Walseth [345].

In general, metal ions are inhibitory, or at least not activating. The exception is the reversal by Ca$^{2+}$ of the EDTA inhibition of dextran-sucrase from strains B-512F, B-1299, and IAM 1046 of L. mesenteroides [158, 186, 188, 205, 249, 286, 339]. Ca$^{2+}$ also stabilizes these dextran-sucrases [158, 172, 205, 212, 237, 286].

Nearly all monovalent and divalent metal ions appear to increase the proportion of α-(1→3) linkages relative to α-(1→6) linkages [242, 339]. By contrast, the effect of anions on glucansucrases is slight [242, 299].

### Alternative Substrates

Sucrose is the only naturally occurring substrate for dextran-sucrase. Because alternative substrates can be used to draw conclusions about kinetic mechanisms [276], a list of alternative substrates for dextran-sucrase is given here.

- Glucosyl fluoride [114], lactulosucrose (4F-β-galactosylsucrose) [139], α-D-glucopyranosyl α-L-sorbofuranoside [227], and p-nitrophenyl α-D-glucoside [24] are glucose donors for dextran formation. For S. sanguis dextran-sucrase, $K_m$ and $V_{max}$ are nearly the same for glucosyl
Table II. Effect of metal ions and metal ion chelators on glucansucrases

<table>
<thead>
<tr>
<th>Authors</th>
<th>Ref.</th>
<th>Enzyme Source</th>
<th>Strongly Inhibiting*</th>
<th>Moderately Inhibiting*</th>
<th>Weakly Inhibiting or Non-inhibiting*</th>
<th>Activating</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chludzinski et al.</td>
<td>51</td>
<td>S. mutans</td>
<td></td>
<td></td>
<td>Citrate, Phosphate</td>
<td></td>
</tr>
<tr>
<td>Carlsson et al.</td>
<td>44</td>
<td>S. sanguis</td>
<td></td>
<td></td>
<td>EDTA</td>
<td></td>
</tr>
<tr>
<td>Hehre</td>
<td>138</td>
<td>L. mesenteroides</td>
<td></td>
<td>Mn^{2+}, Zn^{2+}</td>
<td>Cu^{+}, Ag^{+}b</td>
<td></td>
</tr>
<tr>
<td>Huang et al.</td>
<td>150</td>
<td>S. sanguis</td>
<td>Hg^{2+}</td>
<td></td>
<td>Citrate, Phosphate</td>
<td>EDTA</td>
</tr>
<tr>
<td>Itaya and Yamamoto</td>
<td>158</td>
<td>L. mesenteroides</td>
<td></td>
<td></td>
<td>EDTA</td>
<td></td>
</tr>
<tr>
<td>Kobayashi and Matsuda</td>
<td>184</td>
<td>L. mesenteroides</td>
<td></td>
<td>Ca^{2+}, Cu^{2+}</td>
<td>Fe^{3+}, Cu^{2+}, Fe^{3+}e</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>NRRL B-1299</td>
<td></td>
<td></td>
<td>Mg^{2+}, Co^{2+}, Mn^{2+}, Zn^{2+}, EDTA,</td>
<td>o-phenanthroline</td>
</tr>
<tr>
<td>Kobayashi and Matsuda</td>
<td>186</td>
<td>L. mesenteroides</td>
<td></td>
<td>Mn^{2+}, Fe^{3+}, Ca^{2+}, Mg^{2+}</td>
<td>Mg^{2+}f'</td>
<td>Fe^{2+}, Co^{2+}, Ca^{2+}, Mg^{2+}</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NRRL B-1299</td>
<td></td>
<td></td>
<td>Mg^{2+}f'</td>
<td>Fe^{2+}, Co^{2+}, Ca^{2+}, Mg^{2+}</td>
</tr>
<tr>
<td>Kobayashi and Matsuda</td>
<td>188</td>
<td>L. mesenteroides</td>
<td>Cu^{2+}, Fe^{3+}, Ca^{2+}, Mg^{2+}</td>
<td></td>
<td>Mg^{2+}f'</td>
<td>Fe^{2+}, Co^{2+}, Ca^{2+}, Mg^{2+}</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NRRL B-512F</td>
<td></td>
<td></td>
<td>Mg^{2+}f'</td>
<td>Fe^{2+}, Co^{2+}, Ca^{2+}, Mg^{2+}</td>
</tr>
</tbody>
</table>
Characterization of the strength of inhibition is arbitrary, because experimental conditions varied. Generally, >85% inhibition was considered strong, 40–85% inhibition was considered moderate, and <40% inhibition was considered weak.

Less than 0.1 mM.

When equimolar metal ion was added, the inhibition was partly reversed by Ca$^{2+}$, Sr$^{2+}$, Ba$^{2+}$, and Mg$^{2+}$ in the relative amounts Ca$^{2+} >$ Sr$^{2+} >$ Ba$^{2+} >$ Mg$^{2+}$. The inhibition was not reversed at all by Co$^{2+}$, Mn$^{2+}$, Zn$^{2+}$, or Cu$^{2+}$.

"Enzyme II" only.

"Enzyme I" only.

"N" form (aggregated enzyme) only.

"I" form (monomer) only.

Both in presence and absence of EDTA.

Not inhibiting in the presence of added dextran.

Strongly inhibiting in the presence of added dextran.
Table II (Continued)

<table>
<thead>
<tr>
<th>Authors</th>
<th>Ref.</th>
<th>Enzyme Source</th>
<th>Strongly Inhibiting</th>
<th>Moderately Inhibiting</th>
<th>Weakly Inhibiting or Non-inhibiting</th>
<th>Activating</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lawford et al.</td>
<td>205</td>
<td><em>L. mesenteroides</em></td>
<td>EDTA&lt;sup&gt;k&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hallmark</td>
<td>249</td>
<td><em>L. mesenteroides</em></td>
<td>EDTA, Oxalate&lt;sup&gt;k&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Robyt and Waisath</td>
<td>286</td>
<td><em>L. mesenteroides</em></td>
<td>Hg&lt;sup&gt;2+&lt;/sup&gt;, Cu&lt;sup&gt;2+&lt;/sup&gt;, Zn&lt;sup&gt;2+&lt;/sup&gt;, Cd&lt;sup&gt;2+&lt;/sup&gt;, Ca&lt;sup&gt;2+&lt;/sup&gt;, Sr&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Pb&lt;sup&gt;2+&lt;/sup&gt;, Fe&lt;sup&gt;3+&lt;/sup&gt;, EDTA&lt;sup&gt;k&lt;/sup&gt;, Co&lt;sup&gt;2+&lt;/sup&gt;, Ni&lt;sup&gt;2+&lt;/sup&gt;, Mg&lt;sup&gt;2+&lt;/sup&gt;, Mn&lt;sup&gt;2+&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Scales et al.</td>
<td>299</td>
<td><em>S. mutans</em></td>
<td>Fa&lt;sup&gt;n&lt;/sup&gt;</td>
<td>Ag&lt;sup&gt;+&lt;/sup&gt;, Hg&lt;sup&gt;2+&lt;/sup&gt;, Cd&lt;sup&gt;2+&lt;/sup&gt;, Pb&lt;sup&gt;2+&lt;/sup&gt;, Ca&lt;sup&gt;2+&lt;/sup&gt;, Mg&lt;sup&gt;2+&lt;/sup&gt;, Ni&lt;sup&gt;2+&lt;/sup&gt;, Mn&lt;sup&gt;2+&lt;/sup&gt;, Co&lt;sup&gt;2+&lt;/sup&gt;, EDTA&lt;sup&gt;k&lt;/sup&gt;, Na&lt;sup&gt;+&lt;/sup&gt;, K&lt;sup&gt;+&lt;/sup&gt;, NH&lt;sub&gt;4&lt;/sub&gt;&lt;sup&gt;+&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tsunuraya et al.</td>
<td>339</td>
<td><em>L. mesenteroides</em></td>
<td>Cu&lt;sup&gt;2+&lt;/sup&gt;, Hg&lt;sup&gt;2+&lt;/sup&gt;, FeSO&lt;sub&gt;4&lt;/sub&gt;, FeCl&lt;sub&gt;2&lt;/sub&gt;, PCMB&lt;sup&gt;n&lt;/sup&gt;, ZnSO&lt;sub&gt;4&lt;/sub&gt;, EDTA&lt;sup&gt;k&lt;/sup&gt;, Ba&lt;sup&gt;2+&lt;/sup&gt;, Mn&lt;sup&gt;2+&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>k</sup> Inhibition completely reversed by Ca<sup>2+</sup>.

<sup>1</sup> Inhibition completely reversed by Ca<sup>2+</sup>, and partly by Sr<sup>2+</sup> and Ba<sup>2+</sup>.

<sup>m</sup> Caused greater inhibition in the presence of EDTA.

<sup>n</sup> A mixture of dextranucrase and levansucrase.
μ-Chloromercuribenzoate.

The EDTA-inactivated enzyme could be reactivated by Ca\(^{2+}\), but only partly or not at all by Mg\(^{2+}\), Cu\(^{2+}\), Ba\(^{2+}\), Zn\(^{2+}\), or Fe\(^{2+}\).
fluoride as for sucrose [170].

6-Thiosucrose is a substrate for the formation of an insoluble thioglucan by S. mutans GTF-I, and can also be used by GTF-S from S. mutans 6715 and by B-512F dextran sucrase to donate 6-thioglucosyl groups to acceptors [25].

Inhibitors

Enzyme inhibitors can be used kinetically to draw mechanistic conclusions [105]. Some description of many of the known inhibitors of dextran sucrase is given here.

Chemically synthesized sucrose analogs

The only glycosyl fluoride which has been found to be a substrate for dextran sucrase is glucosyl fluoride. Other α-hexosyl fluorides are competitive inhibitors, while their β anomers are noncompetitive inhibitors with higher $K_i$ values [125].

Derivatives of sucrose, methyl α-glucoside, methyl β-glucoside, and methyl α-mannoside in which the 6 and/or 6' hydroxyl groups have been replaced with amino groups are competitive, uncompetitive, or noncompetitive inhibitors, depending on the specific derivative, of polysaccharide formation by the glucansucrases of S. mutans 6715 [330, 331]. The 6,6'-dihalo- and dideoxysucroses are also glucansucrase inhibitors [25]. B-512F dextran sucrase is inhibited competitively by 6,6'-dideoxysucrose and in a mixed fashion by 6,6'-dibromosucrose (A.W. Miller and J. F. Robyt, unpublished results). Monomethyl sucrases, which are not substrates for B-512F dextran sucrase [87], are another class of potential inhibitors.

Oligosaccharides

The levansucrase acceptor product xylsucrose [10], and a number of other uncommon oligosaccharides such as maltosylsucrose, inhibit both soluble and insoluble glucan synthesis by S. mutans glucansucrases
[154], although only for xylsucrose was it determined that acceptor products were not formed [154]. The inhibition of B-512F dextranucrase by fructose, raffinose, and melibiose has been cited earlier in the review. Other unusual oligosaccharides which have been found not to be dextranucrase substrates, such as galsucrose, planteose, and several others [95], may also be inhibitors. Periodate-oxidized dextran inhibits S. mutans GTF, to a degree dependent on the size of the dextran and the degree of oxidation [259].

Miscellaneous

Pyridoxal phosphate inhibits S. mutans glucansucrase in a pure noncompetitive fashion [332]. The inhibition, which reaches 99+% at 30 mM pyridoxal phosphate, requires 2 h preincubation with the enzyme to develop fully and is reversed by dialysis. Other pyridine analogs than pyridoxal phosphate, such as 4-pyridoxic acid and 3-hydroxypyridine, are also inhibitory [332], although they do not contain aldehyde groups.

Ribocitrin is a glycoside consisting of three riboses linked α-(1→3), with the α-linked aglycon (S)-homocitric acid [326]. This glycoside inhibits insoluble glucan formation noncompetitively with respect to sucrose (50% inhibition at 18 μg/ml) [325] and competitively with respect to dextran T10 [323] in a mixture of GTF-S and GTF-I from S. mutans E49. The insoluble glucan is made by the action of GTF-I on the product of GTF-S [324]. Ribocitrin inhibits insoluble glucan synthesis by inhibiting GTF-S [324].

Mutastein, a preparation from Aspergillus terrus strain M3328 containing 85% protein and 6.5% carbohydrate, inhibited glucan formation by both GTF-S and GTF-I from S. mutans OMZ 176 [192]. GTF-I was inhibited much more than GTF-S. Mutastein, which was inactivated by pronase, also inhibited dextran synthesis by B-512F dextranucrase, by about 50% at 20 μg/ml [192].

Several flavonoids inhibit reducing sugar release by S. mutans dextranucrase by over 50% at 15–120 μg/ml flavonoid, but do not inhibit the enzyme more than 70% even at much higher concentrations [152]. The
fluorescein dye eosin yellowish, used because of its relation to the photoinactivating dye rose bengal, is competitive with sucrose for the dextransucrase from *S. sanguis* 804 [41]. (Dextran protected the enzyme from photoinactivation by rose bengal, leading to the conclusion that the dye site overlapped both the sucrose and dextran sites [41].) *S. mutans* GTF is inhibited by the α-glucosidase inhibitors 1-desoxynojirimycin, N-methyl-desoxynojirimycin, and acarbose [192]. The kinetic effects of lipids and detergents has been discussed in an earlier section of this review.
MOLECULAR WEIGHT

The discussion that follows is concerned primarily with aggregation and other causes of multiple glucansucrase molecular weights, and not with specific molecular weight values, which are given in Table III. Additional information on these topics can be found in the review articles cited in the section of this review dealing with dextran sucrase purification, and also in Refs. 116, 123, 136, 201, 215, and 301.

Aggregation

Under nondenaturing conditions, glucansucrases aggregate strongly, with particle sizes often ranging up into millions of daltons [98, 215, 286, 290]. For example, glucansucrase aggregates of 0.5–50 MDa have been found for S. mutans 6715 [255]. The aggregation is frequently too high to allow penetration into chromatographic and electrophoretic media [44, 98, 286, 299, 357]. The aggregation is mediated by glucan [116, 200, 286], but also occurs in its absence [116, 201, 286, 301]. Aggregation has also been correlated with phospholipids [136] and with deacylated lipoteichoic acids [201].

Carlsson et al. [44] found that precipitates formed from purified S. sanguis 804 dextran sucrase on dialysis could be dissolved with urea or glycine. Kobayashi and Matsuda [183, 186] found that a low-molecular-weight form ("I") of L. mesenteroides NRRL B-1299 dextran sucrase was converted to a high-molecular-weight aggregate form ("N") on aging, and that the conversion was accelerated by NaCl. Newman et al. [256] found that 1.55 M ammonium sulfate converted part of a 100–110 kDa glucansucrase from S. mutans 3209 into a high-molecular-weight aggregate that synthesized a glucan with a higher percentage of α-(1→6) linkages.

Compared to glucansucrases from S. mutans cultures grown in conventional glucose medium, glucansucrases of lower molecular weight are isolated from cultures grown in chemically defined medium [301], cultures grown on fructose rather than glucose [116], or cultures grown in
Table III. Molecular weights reported for glucansucrases by year of publication

<table>
<thead>
<tr>
<th>Year</th>
<th>Ref.</th>
<th>Authors</th>
<th>M.W. (in thousands)</th>
<th>Method</th>
<th>Species and Strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>1962</td>
<td>84</td>
<td>Ebert and Schenk</td>
<td>284</td>
<td>ultracentrifugation</td>
<td>L. mesenteroides NRRL B-512F</td>
</tr>
<tr>
<td>1974</td>
<td>106</td>
<td>Fukui et al.</td>
<td>170 (GTF-S)</td>
<td>SDS-PAGE</td>
<td>S. mutans HS-6</td>
</tr>
<tr>
<td>1974</td>
<td>183</td>
<td>Kobayashi and Matsuda</td>
<td>42 (extracellular &quot;I&quot; form),</td>
<td>Ferguson plot</td>
<td>L. mesenteroides NRRL B-1299</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>&gt;450 (extracellular &quot;N&quot; form),</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>74 (intracellular form)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1974</td>
<td>241</td>
<td>Mukasa and Slade</td>
<td>400-2000</td>
<td>gel filtration</td>
<td>S. mutans HS-6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(mixed enzyme complex)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1975</td>
<td>184</td>
<td>Kobayashi and Matsuda</td>
<td>79, 69 (both intracellular)</td>
<td>SDS-PAGE</td>
<td>L. mesenteroides NRRL B-1299</td>
</tr>
<tr>
<td>1975</td>
<td>200</td>
<td>Kuramitsu</td>
<td>45 (GTF-S), 190, 235 (make mostly 1G)</td>
<td>gel filtration and SDS-PAGE</td>
<td>S. mutans GS-5</td>
</tr>
<tr>
<td>1975</td>
<td>299</td>
<td>Scales et al.</td>
<td>&gt;800 (mixed enzymes)</td>
<td>gel filtration</td>
<td>S. mutans FA-1</td>
</tr>
<tr>
<td>1976</td>
<td>51</td>
<td>Chludzinski et al.</td>
<td>94</td>
<td>gel filtration</td>
<td>S. mutans 6715</td>
</tr>
<tr>
<td>1976</td>
<td>54</td>
<td>Ciardi et al.</td>
<td>160 (5 forms) (GTF-I) C, 220, 225 (both GTF-I) d, 185, 272 (both GTF-S) e</td>
<td>Ferguson plot</td>
<td>S. mutans 6715</td>
</tr>
<tr>
<td>1976</td>
<td>116</td>
<td>Germaine and Schachtele</td>
<td>40</td>
<td>gel filtration</td>
<td>S. mutans 6715 and S-19</td>
</tr>
<tr>
<td>Year</td>
<td>Reference</td>
<td>Charge Isomers</td>
<td>Gel Filtration</td>
<td>Species</td>
<td></td>
</tr>
<tr>
<td>------</td>
<td>-----------</td>
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<td>----------------</td>
<td>---------</td>
<td></td>
</tr>
<tr>
<td>1976</td>
<td>Kobayashi and Matsuda</td>
<td>48 (&quot;I&quot; form) (extracellular)</td>
<td>SDS-PAGE</td>
<td>L. mesenteroides NRRL B-1299</td>
<td></td>
</tr>
<tr>
<td>1977</td>
<td>Callaham and Heitz</td>
<td>1000-2000</td>
<td>gel filtration</td>
<td>S. sanguis 804</td>
<td></td>
</tr>
<tr>
<td>1977</td>
<td>Ciardi et al.</td>
<td>175 (GTF-S), 150 (GTF-I)</td>
<td>gel filtration</td>
<td>S. mutans 6715</td>
<td></td>
</tr>
<tr>
<td>1978</td>
<td>Russell</td>
<td>200 (GT-I)</td>
<td>SDS-PAGE</td>
<td>S. mutans strain Ingbritt</td>
<td></td>
</tr>
<tr>
<td>1979</td>
<td>Figures and Edwards</td>
<td>225-250 (GTF-I)</td>
<td>gel filtration</td>
<td>S. mutans 6715</td>
<td></td>
</tr>
<tr>
<td>1979</td>
<td>Huang et al.</td>
<td>102</td>
<td>gel filtration</td>
<td>S. sanguis ATCC 10558</td>
<td></td>
</tr>
<tr>
<td>1979</td>
<td>Russell</td>
<td>132, 140 (mostly GTF-I)</td>
<td>SDS-PAGE</td>
<td>S. mutans 3209</td>
<td></td>
</tr>
<tr>
<td>1979</td>
<td>Smith et al.</td>
<td>422 (smallest active complex)</td>
<td>gel filtration</td>
<td>S. mutans 6715</td>
<td></td>
</tr>
</tbody>
</table>

- Intracellular form has two charge isomers.
- Get a GTF-S complex of smaller size after some additional purification.
- The 5 forms of 160 kDa differ in charge.
- The 220 and 225 kDa forms differ in charge.
- The 185 and 272 kDa forms have similar charge.
- Cluster of different forms all with molecular weights close to 200 kDa.
- Also find 95, 80, and 70 kDa forms of fructansucrase.
<table>
<thead>
<tr>
<th>Year</th>
<th>Ref.</th>
<th>Authors</th>
<th>M.W. (in thousands)</th>
<th>Method</th>
<th>Species and Strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>1980</td>
<td>188</td>
<td>Kobayashi and Matsuda</td>
<td>64-65</td>
<td>SDS-PAGE and Ferguson plot</td>
<td>L. mesenteroides NRRL B-512F</td>
</tr>
<tr>
<td>1980</td>
<td>256</td>
<td>Newman et al.</td>
<td>100-110</td>
<td>ultracentrifugation</td>
<td>S. mutans 3209</td>
</tr>
<tr>
<td>1981</td>
<td>294</td>
<td>Russell</td>
<td>132, 140, and 150</td>
<td>SDS-PAGE</td>
<td>S. mutans 3209</td>
</tr>
<tr>
<td>1982</td>
<td>107</td>
<td>Fukui et al.</td>
<td>180 (GTF-I)</td>
<td>SDS-PAGE</td>
<td>S. mutans 6715-15</td>
</tr>
<tr>
<td>1982</td>
<td>215</td>
<td>Luzio et al.</td>
<td>231, 260, 280, 339, 485, 682, and 780</td>
<td>Ferguson plot</td>
<td>S. sanguis</td>
</tr>
<tr>
<td>1982</td>
<td>310</td>
<td>Shimamura et al.</td>
<td>149 +/- 5 (ultracentrifugation) or 160 (SDS-PAGE) (GTF-S)</td>
<td>ultracentrifugation and SDS-PAGE</td>
<td>S. mutans 6715</td>
</tr>
<tr>
<td>1983</td>
<td>180</td>
<td>Kenney and Cole</td>
<td>162, 138 (both GTF-S), 153 (GTF-I)</td>
<td>SDS-PAGE</td>
<td>S. mutans 3209</td>
</tr>
<tr>
<td>1983</td>
<td>193</td>
<td>Koga et al.</td>
<td>150 (GTF-S), 180 (GTF-I)</td>
<td>SDS-PAGE</td>
<td>S. mutans OMZ 16</td>
</tr>
<tr>
<td>1984</td>
<td>123</td>
<td>Grahame and Mayer</td>
<td>156, 174</td>
<td>SDS-PAGE</td>
<td>S. sanguis ATCC 10558</td>
</tr>
<tr>
<td>1984</td>
<td>298</td>
<td>Sato et al.</td>
<td>173, 156 (both GTF-S)</td>
<td>SDS-PAGE</td>
<td>S. mutans 1B, Bi4, and OMZ 175</td>
</tr>
<tr>
<td>1985</td>
<td>109</td>
<td>Furuta et al.</td>
<td>160 +/- 3 (GTF-S), 165 +/- 3 (GTF-I)</td>
<td>SDS-PAGE</td>
<td>S. mutans 6715</td>
</tr>
</tbody>
</table>
State that molecular weight and shape are close to those of IgG or IgA.

Also find a 79 kDa fructansucrase.

Also find 84 and 76 kDa fructansucrases for serotype c strain, and 106 and 84 kDa fructansucrases for serotype e and f strains.

Strains IB, BL4, and OMZ 175 are of serotypes c, e, and f, respectively.
medium depleted of traces of sucrose and glucan by invertase [200, 301] or glucanases [200], or by cultures where dextran T10, a low-molecular-weight dextran ($\tilde{M}_n^{\sim}5000$), has been included in the medium [200]. Kura-mitsu [200] found that all the enzyme produced during growth under these alternative, low-glucan conditions was in a 45 kDa form and made only soluble glucan, compared with the 190 and 235 kDa forms that predominated otherwise, and which produced mainly insoluble glucan. These 190 and 235 kDa forms were not reduced in molecular weight (as measured by SDS gel electrophoresis) by dextranase or mutanase alone, but part could be reduced by dextranase and mutanase acting together. By nondenaturing gel filtration, the 190 and 245 kDa forms were aggregates of >500 kDa.

Germaine and Schachtele found that glucansucrase from cultures of S. mutans 6715 grown on fructose [116] or in chemically defined medium [301] formed aggregates that were dissociated by 1 M KCl, while those grown on glucose formed dextran-induced aggregates that were not dissociated even by 3 M KCl. Salt-dissociable aggregates were more activated by dextran T10 [301] than salt-stable aggregates.

Tween 80 has been used to prevent [97] or break up [98] glucansucrase aggregates. In two cases where Tween 80 or other nonionic detergents did not disaggregate dextran-sucrase, the combination of a nonionic detergent with 0.005% SDS did [215, 258].

The effect of aggregation and salt on glucansucrases extends beyond changes in molecular weight. For example, aggregation and salt are reported to influence the relative amounts of $\alpha$-(1\(\rightarrow\)6) and $\alpha$-(1\(\rightarrow\)3) linkages in the glucans synthesized [242, 256]. These effects are beyond the scope of this review.

Other Causes of Multiple Molecular Weights

Proteolysis

Some of the multiplicity in glucansucrase molecular weight can be explained by proteolysis. Russell [294] found 132, 140, and 150 kDa glucansucrase forms in the presence of the protease inhibitor phenyl-
methylsulfonyl fluoride, but only 132 and 140 kDa forms in its absence. He also found [294] more of a 74 kDa glucan-binding protein that had no glucansucrase activity was formed in the absence of phenylmethylsulfonyl fluoride than in its presence. Kenny and Cole [180] found that a 138 kDa glucansucrase was derived from proteolysis of a 162 kDa form.

Grahame and Mayer [123] found that a dextranucrase from S. sanguis was initially secreted entirely in a 174 kDa form, which was converted after secretion entirely to a 156 kDa form. Heat treatment stopped the conversion completely, but several protease inhibitors did not. Of these inhibitors, EDTA slowed the conversion the most.

**Endogenous dextran**

Kobayashi and Matsuda [188] have found that isolated (non-aggregated) molecules of B-512F dextranucrase may differ in molecular weight due to having different amounts of very tightly bound dextran. Parnaik et al. [262] have also commented on the larger, less dissociable aggregates of S. sanguis dextranucrase formed by brief incubation with sucrose, relative to those formed on adding dextran T10 or preformed S. sanguis dextran.

**Molecular Weight Determination by Radiation Inactivation**

Enzymes are inactivated by ionizing radiation at a rate proportional to their volume ("target size"), a phenomenon that has been empirically related to the molecular weight required for enzyme function [178]. This molecular weight excludes bound lipids and carbohydrates [19].

The molecular weight determined by radiation inactivation can correspond to a monomer or to an oligomer (i.e., to functionally coupled subunits) [19, 178]. Changes in the functional molecular weight can be used to study changes in subunit interaction [19, 221].

Before irradiation, enzyme samples must be purged of oxygen and either frozen or lyophilized, to prevent inactivation by OH• and O2•− radicals and by hydrogen peroxide formed by primary ionizations that
occur outside the volume of the enzyme molecule. These reactive species can diffuse to the enzyme and inactivate it [82, 178]. The sensitivity of enzymes to ionizing radiation increases with increasing temperature [177]. Also, the most important assumption of the method has been challenged [151], which is that a single primary ionization occurring within the volume of an enzyme molecule completely destroys the molecule's biological function.

The low dose rates when the radiation source is 60Co increase the amount of inactivation taking place by secondary mechanisms (mechanisms other than inactivation caused by primary ionizations within the volume of the enzyme molecule), making it desirable to calibrate the irradiation apparatus with enzymes of known molecular weight [18]. The presence of nonionic detergents, especially polyoxyethylene detergents such as Triton X-100, causes faster inactivation [19]. Detergents can also change the apparent enzyme size by causing the functional coupling or uncoupling of subunits [19].
THE PERIODIC ACID–SCHIFF STAIN

Description, Usage, and Specificity of the PAS Stain

The periodic acid–Schiff (PAS) stain is widely used to detect glycoproteins on electrophoretic media, including polyacrylamide gels. The staining procedure consists of generating aldehydes by the periodate oxidation of carbohydrate, which are visualized by the Schiff reaction for aldehydes. The PAS stain is usually considered to be specific for carbohydrate, although preexisting aldehydes and certain lipids will also stain, as well as periodate-oxidizable amino alcohols, such as free or N-terminal serine and threonine [174, 210, 264].

Protein-Staining Artifacts of the PAS Stain

Proteins that are not glycoproteins have been reported [16] to stain on polyacrylamide gels by one commonly used PAS staining procedure [359] due to protein oxidation by periodate. Nearly all proteins stained. The intensity of the staining was proportional to moles of periodate consumed by protein in solution.

A better known artifact is caused by the binding of the positively-charged, amphiphilic Schiff dye compound to SDS [121, 349]. The dye binds both to free SDS and to SDS that is bound to protein. Because procedures to remove SDS from polyacrylamide gels can leave small amounts of protein-bound SDS, especially when the SDS preparation contains alkyl sulfates with chain lengths longer than C10, proteins can stain on SDS gels. Maintaining an acid pH during destaining is also recommended to help prevent staining artifacts [121, 349].

A case of protein staining that may or may not be related to these two artifacts has also been reported [268]. Bovine serum albumin, which is not a glycoprotein, stained initially, but its color faded on storage, unlike the stable color given by fibrinogen.
Procedures for the PAS Stain

The PAS stain has been used histochemically since the 1940s [231]. Reviews of its histochemical and histological use can be found in Lillie and Fullmer [210] and in Pearse [264].

For detecting glycoproteins on polyacrylamide gels, commonly referenced PAS staining procedures are those of Clarke [56], Zacharius and Zell [359], Fairbanks et al. [94], and Segrest and Jackson [308].

Segrest and Jackson [308] found that the color of oxidized glycoprotein bands intensified over 18 h in Schiff’s reagent. Gels were stored in Schiff’s reagent. Matthieu and Quarles [225] found that placing gels in a metabisulfite solution for 8 h after Segrest and Jackson’s 18 h incubation in Schiff’s reagent doubled the glycoprotein band intensities. Konat et al. [195] increased the sensitivity of the Matthieu and Quarles procedure twenty-fold by heating the gels in an acidic, alcoholic solution of metabisulfite after incubation in Schiff’s reagent.

Trivedi et al. [334] have described a PAS stain that increases sensitivity by reducing the background, which is done by using high acetic acid and methanol concentrations in the destaining washes. Kapitany and Zebrowski [173] have described a procedure that improves on earlier methods for glycoprotein detection on polyacrylamide gels by using stronger oxidizing conditions and more controlled washing to remove periodic acid.

Alternatives to the PAS Stains

Methods for detecting carbohydrate, either in the form of polysaccharide or in the form of glycoprotein, on a variety of electrophoretic media can be classified as either using periodate oxidation or not using periodate oxidation. Until recently, the PAS stain was the most important of the methods using periodate oxidation. However, the Schiff reaction for visualization of periodate-oxidized carbohydrate is being
supplanted by more sensitive and more selective methods [110, 130]. These are principally silver stains [77, 101, 335] and stains using fluorescent chromophores [88, 110]. One silver stain [77] has over 60 times the sensitivity of the PAS stain for glycoproteins, and another [335] has 500 times the sensitivity of the PAS stain for lipopolysaccharides. Silver stains are often nonspecific [113], however, and have stained proteins not thought to be glycoproteins [77, 101, 335]. Even without periodate oxidation, silver stains will visualize heme-containing proteins [101].

As a group, methods using periodate oxidation are losing ground to methods that avoid the nonspecific staining caused by nonspecific [16, 55] periodate oxidation. An example is the thymol-sulfuric acid procedure [110, 275] derived from a well-known method for the quantitation of carbohydrates in solution. Of increasing importance [130] are methods using lectins and antibodies coupled to fluorescent chromophores or to the chromogenic enzymes horseradish peroxidase and alkaline phosphatase [38]. These methods offer increased selectivity as well as increased sensitivity. A method combining sensitive enzymatic detection with periodate oxidation has recently been reported [119]. In this method, the chromogenic enzymes are coupled to periodate-oxidized carbohydrate with a bifunctional reagent.

The PAS stain still has some advantages. It can be used easily on rod gels, unlike silver stains. No special equipment is needed to see the stained bands, unlike methods using fluorescent chromophores. The PAS stain is much more stable than the thymol-sulfuric acid stain [275], which fades within a few hours. Finally, the PAS stain uses commercial reagents that can be obtained quickly and cheaply.

**Mechanism of the PAS Stain**

The mechanism of the PAS stain will be discussed under four overlapping categories: (1) characterization of the Schiff reaction product, (2) reaction kinetics and intermediates, (3) stoichiometry of the
Schiff reaction and the PAS stain, and (4) role of bisulfite washes.

Characterization of the Schiff reaction product

Schiff's reagent is compounded of basic fuchsin, hydrochloric acid, and potassium metabisulfite or some other source of sulfur (IV) oxide [174]. Other acids have been substituted for hydrochloric acid [174]. Basic fuchsin is a mixture of three closely related triarylmethane dyes. The general chemistry and photochemistry of these dyes is reviewed in Refs. 3, 4, and 91. A good, very brief review of the Schiff reaction is given in Ref. 71.

The chemistry of the Schiff reaction is complex and not well understood, but the colored product from the reaction of the Schiff dye with aldehyde is known not to be a Schiff base, but an aminoalkylsulfonate (Fig. 1). This has been shown by n.m.r. spectroscopy [279], i.r. spectroscopy and elemental analysis [254], u.v. and visible spectroscopy in comparison with model compounds [243], the stabilizing effect of a bisulfite rinse [134], pH dependence of the dye color [274], dye substituent effects [71], and chromatography of the staining components in the dye [20]. The product is different than that obtained by the treatment of aldehydes with basic fuchsin alone [27, 72, 148, 272] or with "aldehyde-fuchsin" [272]. It is likely, however, that linkages other than aminoalkylsulfonate linkages, including Schiff base linkages, are present to a degree under some conditions [80].

Reaction kinetics and intermediates

The Schiff reaction probably goes through a Schiff base intermediate [115, 134, 135, 235, 279], although there is disagreement on the structure of the actual reactants and on the order in which they react [20, 115, 134, 135, 235, 279]. It is also possible that no Schiff base intermediate is involved [71]. The kinetics of staining and destaining during the Schiff reaction have been studied as part of the Feulgen test for DNA [79, 80]. Hörmann et al. [147] have studied the kinetics of color formation in the Schiff reaction as a function of
Figure 1. The Schiff reaction with aldehydes

The leuco form of the Schiff dye (top right) reacts with either one or two equivalents of bisulfite and aldehyde (RCHO) to give a colorless intermediate (middle left), which dissociates to give a colored product (bottom right).
The diagram shows the chemical reaction and structure of a dye. The reaction is:

$$2RCHO + 2HSO_3^- + 2H^+ + H_3N^+ ightleftharpoons \text{LEUCO FORM OF DYE (COLORLESS)}$$

The leuco form of the dye is shown on the left, and the colored form is shown on the right. The reaction involves the addition of formaldehyde, sulfuric acid, and hydrogen ions to form the colored form of the dye. The leuco form is depicted as a colorless compound, while the colored form contains a positive charge on the nitrogen atom.
bisulfite ("SO₂") concentration.

The Schiff reaction product with acetaldehyde is more stable than the one with formaldehyde [135, 147]. With acetaldehyde, color formation is followed by decolorization. This may result from the loss of acetaldehyde from Schiff complexes, which are favored kinetically, to bisulfite–acetaldehyde adducts, which are favored thermodynamically.

In the PAS stain, oxidation is always incomplete because of short oxidation times [17, 70, 273]. In test reactions in solution, monosaccharides oxidized more quickly than polysaccharides [70]. Among polysaccharides, dextran oxidized especially slowly [70].

**Stoichiometry of the Schiff reaction and the PAS stain**

One molecule of dye in Schiff’s reagent can theoretically react with up to six equivalents of aldehyde [134]. Molecular modeling of the staining reaction with both simple and complex carbohydrates shows that steric hindrance prevents the quantitative reaction of oxidized polysaccharides with dye [273], and that Schiff base linkages, being less flexible than aminoalkylsulfonate linkages, considerably reduce the degree of reaction for steric reasons [135].

Even with simple aldehydes, there is evidence that the dye rarely reacts with more than two equivalents of aldehyde. Color intensity is proportional to the square of the weight concentration of aldehyde for deoxyribose [135] formaldehyde [135, 147, 304], acetaldehyde [304], glutaraldehyde [304], and glucose [304], but only to the first power for DNA [135] and for malto-oligosaccharides larger than maltose [304]. (See also Ref. 80.) Based on these findings, Scott and Harbinson [304] proposed that Schiff’s reagent reacts primarily with dialdehydes. Carbohydrates whose color intensity is linear with weight concentration contain pairs of neighboring aldehydes. Scott and Harbinson tested their proposal with molecular models [304].

Electronic factors contribute to the preference for disubstitution of the Schiff dye with aldehyde. Successive aldehyde additions to the leuco (colorless) form of the dye increasingly promote a dissociation
reaction that gives the less reactive colored form of the dye [279].

Precipitation of the Schiff reaction product [254, 304], which is especially likely with polyaldehyes [304], suggests that crosslinked polymers are formed. This is supported by studies of the polymerization of mixtures of formaldehyde and basic fuchsin [42].

Role of bisulfite washes

The word "bisulfite" is applied generically here to the various sulfite, bisulfite, metabisulfite, and sulfur dioxide treatments used after the incubation in Schiff's reagent in staining procedures that include the Schiff reaction. Bisulfite washes have been used in the Feulgen reaction and the PAS stain ever since they were described by Feulgen and Rossenbeck in 1924 [96]. The washes have been omitted from only a few PAS staining procedures [77, 173].

The function and usefulness of the bisulfite washes are not clear. They have been claimed to increase [94, 135] and stabilize [94] color formation, but they have also been found to cause no increase in color [77, 81] or to cause a loss in color [80]. It may be that the bisulfite drives the Schiff reaction to completion [134], which would both increase and stabilize the color. It may be that the bisulfite prevents recolorization of the unsubstituted (unreacted) or monosubstituted dye before it can be washed away (refs. 17, 72, 304, and pp. 612-616 in Lillie and Fullmer [210]). Bisulfite washes may prevent the Schiff's reagent (leucofuchsin) from being air-oxidized to give a "spurious color reaction" (ref. 302 and pp. 612-616 in Lillie and Fullmer [210]).
PART I

STABILIZATION OF DEXTRANSUCRASE FROM LEUCONOSTOC MESENTEROIDES NRRL B-512F BY NONIONIC DETERGENTS, POLY(ETHYLENE GLYCOL), AND HIGH-MOLECULAR-WEIGHT DEXTRAN
ABSTRACT

Dextranucrase (3 International Units/ml culture supernatant) was obtained by a modification of the method of Robyt and Walseth [J. F. Robyt and T. F. Walseth. Carbohydr. Res. 66, 95-111 (1979)] from a nitrosoguanidine mutant of Leuconostoc mesenteroides NRRL B-512F selected for high dextranucrase production. Dialyzed, concentrated culture supernatant (crude enzyme) was treated with immobilized dextranase and chromatographed on a column of Bio-Gel A-5m. The resulting, purified enzyme lost activity rapidly at 25° or on manipulation, as did the crude enzyme when diluted below 1 U/ml. Both enzyme preparations could be stabilized by low levels of high-molecular-weight dextran (e.g., 2 µg/ml), poly(ethylene glycol) (e.g., 10 µg/ml PEG 20,000), or nonionic detergents (e.g., 10 µg/ml Tween 80). The stabilizing capacity of poly(ethylene glycol) and of dextran increased with molecular weight. Calcium had no stabilizing action in the absence of other additions, but reduced the inactivation that occurred in the presence of 5 mg/ml bovine serum albumin or high concentrations (>50 µg/ml) of Triton X-100. In summary, dextranucrase could be stabilized against activity losses caused by dilution or by thermal inactivation through the addition of low concentrations of nonionic polymers (dextran, PEG 20,000, methyl cellulose) or of nonionic detergents at or slightly below their critical micelle concentrations.
INTRODUCTION

Dextran sucrase (E.C. 2.4.1.5) polymerizes the glucosyl moiety of sucrose to form dextran, an α-(1-6)-linked glucan with α-linked branches [165]. It is elaborated by Leuconostoc and Streptococcus species, and is primarily exocellular, although it is also found bound to the cell surface. The dextran sucrase from Leuconostoc mesenteroides NRRL B-512F, which synthesizes a dextran that has 95% α-(1-6) and 5% α-(1-3) branch linkages [211], is only produced when induced by sucrose [250]. Consequently, the crude enzyme contains a great deal of dextran. Following a purification procedure that removed dextran (as well as many other impurities), Robyt and Walseth [286] found that the enzyme lost half its activity after storage for two days at 4°C or -15°C, irrespective of the presence of 25% (v/v) glycerol. This loss of activity could be prevented by adding back dextran. Subsequently, Miller and Robyt [236] found that dilute enzyme, whether crude or purified, lost activity rapidly at 25°C. This loss could also be prevented by the addition of dextran. In order to perform kinetic studies that would be handicapped by the presence of dextran, a search was conducted for alternative stabilizers. The results of this search have been previously reported in abstract form [236], and will be described here in detail.
MATERIALS AND METHODS

Materials

Leuconostoc mesenteroides B-512F was obtained from the Northern Regional Research Center (formerly Northern Regional Research Laboratory, NRRL) (Peoria, IL). Tween 80 and PEG 20,000 were from Fisher Scientific Co. (Pittsburgh, PA). Zwittergent 3-12 was from Calbiochem-Behring Corp. (La Jolla, CA). PEGs 400, 600, 1,000, and 6,000 were from J. T. Baker Chemical Co. (Phillipsburg, NJ). Bio-Gels P-2 and A-5m were from Bio-Rad Laboratories (Richmond, CA). Nonidet P40 was from Bethesda Research Laboratories, Inc. (Gaithersburg, MD). Dextranase S was from Swiss Ferment Co. (Basel, Switzerland). Octylglucoside, methyl cellulose (viscosity of 2% aqueous solution = 4,000 centipoises), Triton X-100, Lubrol WX, Lubrol PX, sodium taurocholate (approximately 98%; synthesized from cholic acid), mannan from Saccharomyces cerevisiae, bovine serum albumin (crystallized and lyophilized), Penicillium funiculosum dextranase (Grade I, chromatographically purified), and dextrans of various molecular weights were purchased from Sigma Chemical Co. (St. Louis, MO). Molecular weights (for Fig. 17) are weight average molecular weights. Native dextran (industrial grade dextran from Sigma, molecular weight 5-40 x 10^6) was precipitated twice from aqueous solution with two volumes of ethanol. \([U-^{14}C]sucrose\) was from New England Nuclear (Boston, MA) and ICN (Irvine, CA). \([fructose-U-^{14}C]sucrose\) was from New England Nuclear. All other chemicals were of reagent grade.

Mutation and Culture

Leuconostoc mesenteroides NRRL B-512F was grown in sucrose medium [286], treated with N-methyl-N′-nitro-N-nitrosoguanidine [1], and plated on agar medium containing sucrose. Colonies showing exceptional polysaccharide formation were selected. The dextranucrase activity in the resulting culture supernatant was 3 U/ml, compared to 0.02 U/ml for the
parent organism. The dextrans produced by crude and purified dextranu-
scrase derived from this culture supernatant by the procedure described in
the next paragraph were identical to the dextran produced by the dextran-
sucrase from the B-512F parent, as judged by $^{13}$C-n.m.r. spectra and by
the distribution of *Penicillium funiculosum* dextranase hydrolysis prod-
ucts (glucose, isomaltose, and branched tetra-, penta-, and hexa-
saccharides), which showed that the dextran was 4-5% branched.

### Enzyme Purification

The culture supernatant was dialyzed and concentrated on a
Bio-Fiber 80 miniplant (Bio-Rad Laboratories, Richmond, CA). The concen-
trate was centrifuged at 12,000 x g for 1 h to remove a particulate
suspension. The centrifuged preparation (crude enzyme) had an activity
of 90 U/ml and contained less than 0.02 U/ml of levansucrase, as judged
by failure to incorporate $^{14}$C from [fructose-$^{14}$C]sucrose into methanol-
insoluble polymer [118]. The specific activity of the crude dextran-
sucrase was 98 U/mg protein (as bovine serum albumin), based on the
protein determination of Bradford [32]. Dextranase S (an endodex-tra-
nase) was immobilized to beads of ethylenediamine-derivatized Bio-Gel P-2
that had been treated with glutaraldehyde [287]. Crude dextranuoscrase
was incubated with immobilized dextranase and passed over a 2.5 x 60 cm
column of Bio-Gel A-5m. The pooled, active fractions constituted
purified enzyme.

### Preincubations and Assays

Preincubations were performed in 0.05 M sodium acetate buffer
(pH 5.0) at various temperatures. Dextranucrase assays were performed at
the preincubation temperature by following the incorporation of label
from [U-$^{14}$C]sucrose into methanol-insoluble polymer [118]. One unit of
enzyme is defined as the amount that will incorporate 1 umol of D-gluc-
cose into polysaccharide in one minute at 25° and pH 5.
RESULTS

Any manipulation of purified enzyme resulted in loss of activity. No loss of activity was observed from crude enzyme in the temperature range examined (4°C–30°C) unless it was diluted to less than 1 U/ml (Fig. 1). The progressive inactivation that occurred then, when plotted semilogarithmically against time, was often biphasic or otherwise complex, but became more linear as enzyme concentration was reduced, and was completely linear (showed simple exponential decay) at initial enzyme concentrations of 0.01 U/ml or less. Complex inactivation curves are well-known for enzymes in general (Laidler [204], pp. 336–403), and have been observed previously for dextranucrase [172]. The rate of inactivation increased sharply at temperatures above 25°C, although it occurred even at 4°C (Fig. 2). Inactivation occurred at equal rates in both glass (hydrophilic surface) and polystyrene (hydrophobic surface) vessels (Fig. 3), making it unlikely that inactivation was caused primarily by the binding of the enzyme to the vessel walls. Fig. 4 is suggestive of denaturation at the air-buffer interface, because it shows that a nearly constant amount of activity was lost from a tube of enzyme every time the surface layer was disturbed.

Bovine serum albumin, which is commonly added to enzyme preparations to stabilize them, unexpectedly increased the rate of inactivation of dextranucrase (Fig. 5). Calcium, while by itself having no effect on the rate of inactivation, prevented the increase caused by bovine serum albumin. Only the increase was prevented: the inactivation in the presence of both 5 mg/ml bovine serum albumin and 5 mM added calcium occurred at virtually the same rate as in the absence of either.

The inactivation could be stopped at any time by adding to the dilute enzyme solution a sufficiently high concentration of any of the stabilizers listed in Table I. This is shown for Tween 80 in Fig. 6. The concentration dependence for Tween 80 is shown in Fig. 7, which also illustrates the point that part of the activity, but only a part, was often retained at stabilizer concentrations between the non-stabilizing
<table>
<thead>
<tr>
<th>Stabilizer</th>
<th>Enzyme preparation</th>
<th>Temperature of preincubation and assay (°C)</th>
<th>Lowest tested concentration giving any stabilization (µg/ml)</th>
<th>Lowest tested concentration giving complete stabilization (µg/ml)</th>
<th>Critical micelle concentration in water (µg/ml)</th>
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<tbody>
<tr>
<td>Tween 80</td>
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<td>13</td>
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<tr>
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<td>50</td>
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<td>Lubrol PX</td>
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<td>100</td>
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<tr>
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<td>0.08</td>
<td>2</td>
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</tbody>
</table>

a From references [126, 140, 142, 288]. Recalculated from molarity where necessary.

b No stabilization at this concentration.

c Complete stabilization was not observed. Stabilization was nearly complete at 100 µg/ml, but above this concentration, inactivation began to occur.

d Lowest tested concentration (40 µg/ml) gave complete stabilization.
and fully stabilizing concentrations given in Table I.

Although the concentration range over which stabilization developed was investigated with only one enzyme preparation (either crude or purified) for each stabilizer, all of the stabilizers were effective with both preparations. For most of the stabilizers, only a few concentrations (usually in the form of serial, tenfold dilutions) were examined for their stabilizing effect. Therefore, the concentrations in Table I are given as "lowest tested concentration" rather than as "lowest concentration."

Anomalous results were obtained with Zwittergent 3-12 and Triton X-100. Increasing the concentration of these detergents only decreased the rate of enzyme inactivation up to a point. As the concentrations were increased above 100 μg/ml for Zwittergent 3-12 (Fig. 8) or 50 μg/ml for Triton X-100 (Fig. 9), at which concentrations almost complete stabilization was observed, the rate of inactivation began to increase again. For Triton X-100, the most likely cause of this effect was considered to be attack by the oxidizing impurity known to be present in commercial preparations of this detergent [8, 277].

Curiously, while calcium did not prevent the loss of activity caused by high concentrations of Zwittergent 3-12, it did prevent the loss caused by high concentrations of Triton X-100. For example, at 5 mg/ml Triton X-100, where inactivation occurred much more rapidly than in the absence of Triton X-100, the addition of calcium to 0.2 mM resulted in complete stabilization of the enzyme (Fig. 10). Results were the same for Nonidet P40, a detergent structurally almost the same as Triton X-100, but made by another manufacturer.

High dilution of crude or purified enzyme into buffers containing stabilizers gave an apparent activation relative to controls not containing stabilizers (shown for Tween 80 in Fig. 11). Some of this apparent activation resulted from inactivation of the control enzyme before and during its initial assay. Because of the complex and somewhat unpredictable shape of the inactivation curves (see Fig. 7), the control velocities for Fig. 11 were not corrected for this inactivation. Above
10 μg/ml Tween 80, however, where complete stabilization occurred, the additional activation must be real.

Calcium and zinc affected enzyme stability. Calcium had no observable effect in the absence of other additions (Fig. 12), but as noted above, the addition of calcium prevented increases in the inactivation rate caused by high concentrations of bovine serum albumin or Triton X-100. Progressive inactivation was also caused by EDTA, and the addition of Tween 80 did not prevent this inactivation (Fig. 13). Likewise, zinc caused inactivation of the purified enzyme (Fig. 14), but not of the crude enzyme (Fig. 15), even in the presence of Tween 80 and a small amount of calcium. These results were presumably due to the removal or displacement of stabilizing calcium from the enzyme.

Both poly(ethylene glycol) and dextran stabilized the enzyme to an extent dependent on their molecular weight (Fig. 16, respectively). A single polymer concentration (5 mg/ml) was used for the experiments shown in these two figures. Lower concentrations were tested only for the highest molecular weight dextran and poly(ethylene glycol) used for Fig. 16 (Table I). High-molecular-weight dextran stabilized the enzyme at extraordinarily low concentration (Table I and Fig. 17).
Figure 1. Effect of dilution on the stability of crude dextransucrase

Initial enzyme activity (U/ml): ● 9; ▲ 0.9; □ 0.09.
Figure 2. Effect of temperature on the stability of crude dextran-sucrase

Initial enzyme activity was 0.3 U/ml. Temperature (°C):
- O, 15;
- ●, 20;
- Δ, 25;
- ▲, 30.
Figure 3. Inactivation of purified dextranucrase on transfer and subsequent storage

Dextranucrase (0.4 U/ml) was placed in glass (○) or polystyrene (●) tubes and stored at 4°C. Assays were performed at 27.5°C.
Figure 4. Loss of activity from purified enzyme as functions of length of storage at 4°C (▲) and of number of manipulations (○) after initial transfer of enzyme solution to a glass storage vessel.
Figure 5. Effect of 5 mg/ml bovine serum albumin on the stability of crude dextran sucrase in the presence (●) and absence ( ○ ) of 5 mM added calcium

△, no added calcium or bovine serum albumin. Initial enzyme activity was 0.09 U/ml.
After 2.5 h incubation, enzyme solution (0.15 U/ml before incubation) was diluted with an equal volume of water (○) or 10 mg/ml Tween 80 (●). Activities plotted after this dilution were multiplied by 2 as a correction factor. △, enzyme activity before dilution.

Figure 6. Stabilization of crude dextranucrase by Tween 80
Figure 7. Effect of Tween 80 concentration on the stability of purified enzyme

Initial enzyme activity was 0.2 U/ml. Tween 80 concentration (μg/ml): □, 0; ▲, 1.2; △, 2.5; ●, 5; ○, 10.
Figure 8. Effect of Zwittergent 3-12 concentration on the stability of purified dextransucrase

Initial enzyme activity was 0.2 U/ml. Preincubation solutions contained 1 mM calcium. Zwittergent 3-12 concentration (μg/ml): ▲, 1; □, 10; ●, 100; ○, 1000.
Figure 9. Effect of Triton X-100 concentration on the stability of crude enzyme in the absence of added calcium

Initial enzyme activity was 0.09 U/ml. Triton X-100 concentration (μg/ml): ○, 0; ▲, 0.5; △, 5; ●, 50; □, 500; ■, 5000.
Figure 10. Effect of added calcium on the stability of crude dextran sucrase in the presence of 5 mg/ml Triton X-100

Initial enzyme concentration was 0.09 U/ml. Calcium added (mM): ■, 0; ▲, 0.12; ○, 0.25.
Figure 11. Activation of purified dextranucrase by Tween 80

Each type of symbol represents a different experiment.
Figure 12. Effect of calcium on the stability of crude dextranucrase

Initial enzyme activity was 0.3 U/ml. Calcium concentration (mM): ○, 0; ●, 10.
Figure 13. Effect of EDTA on the stability of crude dextran sucrose in the presence (closed symbols) and absence (open symbols) of 5 mg/ml Tween 80

EDTA concentration (mM): ○, 0; △, 10. Initial enzyme activity was 0.15 U/ml Tween 80 and 0.7 mM calcium.
Figure 14. Effect of Zn$^{2+}$ on the stability of purified dextransucrase in the presence of 3 mg/ml Tween 80 and 0.7 mM calcium

(A) Fraction of initial activity (0.14 U/ml in the absence of Zn$^{2+}$) remaining with time. Zn$^{2+}$ concentration (mM): ○, 0; ●, 2.5; △, 5; ▲, 10.

(B) Rate of inactivation as a function of Zn$^{2+}$ concentration. When the ordinate axis in panel (A) is recalibrated in units of ln(activity), the slopes of the inactivation curves drawn in the figure become equal to negative one times the first-order decay constants for activity loss. Smaller decay constants therefore represent less rapid loss of activity, and a zero value represents complete stability.
% Initial Activity

Hours of enzyme incubation at 27.5°C before assay

First-Order Decay Constant (h⁻¹)

$[\text{Zn}^{2+}]$ (mM)
Figure 15. Effect of Zn$^{2+}$ on the stability of crude dextranucrase in the presence (△, ▲) and absence (○, ●) of 2.5 mg/ml Tween 80

Initial activity in the absence of Zn$^{2+}$ was 0.09 U/ml.

Zn$^{2+}$ concentration (mM): △. ○. 0; ▲. ●. 7.
Figure 16. Effect of poly(ethylene glycol) and dextran molecular weight on the stability of crude dextransucrase

(A) Poly(ethylene glycol) Dextransucrase (initial activity, 0.3 U/ml) was incubated with 5 mg/ml poly(ethylene glycol), with calcium present at 1.5 mM. Plots of log (activity) vs. time were linear for 1.5 h. Derivation of first-order decay constants is described in the legend to Figure 14. The intercept on the ordinate axis is the decay constant in the absence of poly(ethylene glycol).

(B) Dextran Dextransucrase (initial activity, 0.14 U/ml) was incubated with 5 mg/ml dextran, with calcium present at 1.5 mM. Plots of log (activity) vs. time were linear for 3 h. Derivation of first-order decay constants is described in the legend to Figure 14. The intercept on the ordinate axis is the decay constant in the absence of added dextran.
First-Order Decay Constant ($h^{-1}$)

First-Order Decay Constant ($h^{-1}$)

Dextran Molecular Weight ($\times 10^3$)

PEG Molecular Weight ($\times 10^3$)
Figure 17. Effect of high-molecular-weight dextran concentration on the stability of crude dextranucrase

Initial activity was 0.15 U/ml. Dextran concentration (ug/ml): ▲. 0; △. 0.08; ●. 0.4; ○. 2.
DISCUSSION

Dextranucrase was inactivated in temperature-dependent fashion (Fig. 2) at the low enzyme concentrations (less than 1 U/ml) required for accurate kinetic assays (Fig. 1). The shapes of the inactivation curves (Figs. 2, 5-7, 9, 12, and 15) suggest a complex inactivation process ([172], pp. 336-403 in Laidler [204], pp. 339-373 and 471-484 in Ghélis and Yon [120]).

Enzymes often undergo a time-dependent inactivation following dilution to low concentration ([5], p. 43 in Fromm [104]), for reasons that include surface denaturation ([5], p. 11 in Dixon and Webb [74], p. 43 in Fromm [104]), binding of inactivators [182], and dissociation of active oligomers into inactive subunits [23]. One method commonly used to prevent this inactivation is to increase the total protein concentration by adding a large amount (1-2 mg/ml) of an inert protein such as bovine serum albumin [5]. This was not effective in the present case (Fig. 5). Because of the ability of bovine serum albumin to bind many ions, lipids, and other molecules, it is not inert in many systems, as it was not in this one. No other proteins were tested for their stabilizing ability, although this has been recommended for cases where serum albumin does not work [5].

Another method to prevent inactivation is to add ligands (substrates, products, or effectors) of the enzyme to the enzyme solution ([354], pp. 43-44 in Fromm [104]). While dextran was effective at low concentration (Table I and Fig. 17), it was our original purpose to find an alternative stabilizer.

Nonionic polymers other than dextran that stabilized the enzyme were methyl cellulose and poly(ethylene glycol) (Table I). At high concentration (0.5% w/v), both poly(ethylene glycol) and dextran showed a molecular weight dependence for the stabilization (Fig. 16), with the largest polymers being much better stabilizers than the smallest ones. At this relatively high concentration, one way the polymers might be stabilizing the enzyme would be by raising its effective concentration
through steric exclusion [278]. However, both PEG 20,000 and high-
molecular-weight dextran stabilized the enzyme at concentrations of only
a few μg/ml (see Table I), where steric exclusion could not be a signifi-
cant factor. It is more likely, therefore, that both poly(ethylene
glycol) and dextran stabilize the enzyme by binding to it.

The stabilization given by a low concentration (170 μg/ml) of the
largest polymers was greater than that given by a high concentration
(0.5% w/v, or 5000 μg/ml) of the smaller ones. In the case of dextran,
this could be caused by structural differences. Low-molecular-weight
dextrans are produced by acid hydrolysis of high-molecular-weight dex-
tran. Acid hydrolysis cleaves α-(1→3) linkages faster than α-(1→6)
linkages [312], with the result that the low-molecular-weight dextrans
are less branched. There is no corresponding possibility for poly(ethyl-
ene glycol), which is an unbranched polymer. Takeda and Hizukuri [327]
found that at the low concentration of 0.04%, poly(ethylene glycol)
became a better stabilizer of sweet potato β-amylase as the molecular
weight of the poly(ethylene glycol) increased.

The most interesting stabilizers, because most unexpected, were the
detergents. All of the nonionic and mild, ionic detergents tested stabi-
лизирован коричневый цемент. Концентрации, в которых они стабилизировали
enzyme (Table I) are so low as to suggest that stabilization may be mediated
through detergent monomers rather than micelles. This is not certain,
because critical micelle concentrations were not determined under the
conditions of enzyme precubation.

Detergent effects have been described previously for streptococcal
glucansuсrases, but reports have not been consistent. For example,
Figures and Edwards [97, 98] found that Tween 80 reduced the aggrega-
tion state of glucansuсrases from Streptococcus mutans 6715 and improved
purification yields. By contrast, Luzio et al. [215] found that dex-
transuсrase from S. sanguis ATCC 10558 was not disaggregated by nonionic
detergents. However, in the presence of nonionic detergents at or below
the critical micelle concentrations, their enzyme was disaggregated in
active form by 0.005% SDS, which in the absence of nonionic detergents
inactivated the enzyme. Similar results in reversing the effects of SDS with nonionic detergents were earlier observed by Russell [290] for the glucansucrases of *S. mutans* OMZ 176. Wittenberger et al. [355] found that Tween 80 activated the enzyme from *S. salivarius* up to 30%. Luzio et al. [215] found an initial activation by nonionic detergents and poly(ethylene glycol), but except for Triton X-100, this was followed by inactivation. The results of this study support some activation (Fig. 11), but not subsequent inactivation (Fig. 6).

The effects of detergents may be related to the membrane origin of glucansucrases, as discussed by Figures and Edwards [98]. In this context, it was found by Harlander and Schachtele [136] that phospho-glycerides stimulated the glucansucrases of *S. mutans* 6715. Interestingly, they also found that the activation by phospholipids was independent of (additive with) the activation by dextran. This argues that the mechanisms of stabilization by dextran and detergents may also be different.

Calcium has not been reported to affect the activity or stability of streptococcal glucansucrases. However, it has been shown to affect both of these properties for the dextranases from *L. mesenteroides* NRRL B-512F [172, 205, 286] and *L. mesenteroides* IAM 1046 [158, 339]. The studies cited demonstrated that EDTA inhibited the *Leuconostoc* enzyme, and that calcium best reversed the inhibition. Furthermore, the amount of activity that could be recovered after incubation with EDTA decreased with the length of time that the enzyme was kept calcium-deficient. This was observed here (Fig. 13) even in the presence of an otherwise stabilizing concentration of Tween 80. Therefore, calcium and detergents have separate stabilizing roles. Calcium was also found to prevent increases in the rate of inactivation that occurred in the presence of bovine serum albumin (Fig. 5) and Triton X-100 (Figs. 9 and 10), but not the increase that occurred in the presence of Zwittergent 3-12 (Fig. 8). Calcium was not stabilizing in the absence of such inactivating agents (Fig. 12).

Zinc is known to inhibit *Leuconostoc* dextranases [286, 339]. Fig. 14 shows that it also has a strong influence on dextranucrase
stability. Even in the presence of Tween 80 and calcium, zinc inactivates the enzyme, most likely by displacing the stabilizing calcium. Fig. 14b shows that the rate of inactivation does not increase linearly with zinc concentration. More than one zinc-binding site may therefore be involved. Interestingly, zinc did not affect the rate of inactivation of crude enzyme either in the presence or absence of Tween 80 (Fig. 15), which may be related to the presence of dextran.

In summary, we find three classes of stabilizer for dextranucrase: nonionic (and mild ionic) detergents, nonionic polymers, and calcium. The mechanisms of stabilization, which may be different for the different classes of stabilizer, remain unknown, as do the mechanisms of inactivation, although some of the data hints at the involvement of oxidation (Fig. 10) or of conventional surface denaturation [146, 216] at the air interface (Figs. 3 and 4).
PART II

MILLIGRAM TO GRAM SCALE PURIFICATION AND CHARACTERIZATION OF DEXTRANSUCRASE FROM LEUCONOSTOC MESENTEROIDES NRRL B-512F
ABSTRACT

DEAE-cellulose chromatography has been optimized to permit the facile purification of gram quantities of dextranucrase (sucrose: 1,6-α-D-glucan 6-α-glucosyltransferase, EC 2.4.1.5) from Leuconostoc mesenteroides NRRL B-512F to high specific activity (90-130 U/mg protein) and low carbohydrate content (0.2-0.7 mg/mg protein) compared with previous procedures. The purified enzyme can be stored indefinitely without loss of activity. By adding an affinity chromatography step using Sephadex G-200, hundred-milligram amounts can be made with even less carbohydrate (0.1 mg/mg protein), with higher specific activity (170 U/mg protein), and in over 50% overall yield. Adding a final ion-exchange step using DEAE-Trisacryl M gave ten-milligram amounts without detectable carbohydrate (<1 ug/mg protein). Levansucrase and dextranase were undetectable after the affinity chromatography step.
INTRODUCTION

Dextranulcerase (sucrose: 1,6-α-D-glucan 6-α-D-glucosyltransferase, EC 2.4.1.5) [239, 312, 343], elaborated by species of Leuconostoc and Streptococcus, polymerizes the glucosyl moiety of sucrose to form dextran, an α-(1→6)-linked glucan with α-linked branches [343]. We are interested in dextranulcerase as a general model for polysaccharide biosynthesis. As is usual in enzymology, studies are limited by the supply and quality of purified enzyme. In particular, experiments that are difficult without large amounts (many milligrams) of very pure dextranulcerase include chemical modification studies, isolation and characterization of the unusual covalent intermediates involved in the mechanism of this enzyme [283, 287], and crystallization. No glucansucrase has yet been crystallized or sequenced.

Current purification procedures for the purification of dextranulcerase from L. mesenteroides B-512F [188, 238, 286] either fail to remove important impurities, especially polysaccharide, or suffer from low yields. Here, a combination of ion-exchange and affinity chromatography has been optimized to give a dextranulcerase preparation with very low carbohydrate content and a high specific activity in good yield. The method can be adapted to produce milligram or gram quantities.
MATERIALS AND METHODS

Materials

Penicillium funiculosum dextranase (Grade I, chromatographically purified), DEAE-cellulose (medium mesh, 0.93 meq/g), DEAE-Sephacel, and dextran of various sizes, were from Sigma Chemical Co. (St. Louis, MO). DEAE-Trisacryl M and Ultrogel HA were from LKB Instruments, Inc. (Gaithersburg, MD). Sephadex (40-120 μm dry bead diameter for all types used), Sephacryl S-200, dextran T10, and dextran T2000 were from Pharmacia Fine Chemicals (Piscataway, NJ). Carbowax PEG 20,000 and Tween 80 were from Fisher Scientific Co. (Pittsburgh, PA). Polyethylene glycols 400 (av. mol. wt. 380-420) and 6,000 (av. mol. wt. 6,000-7,500) were from J. T. Baker Chemical Co. (Phillipsburg, NJ). Bio-Gels P-4 (50-150 mesh) and A-5m were from Bio-Rad Laboratories (Richmond, CA). Hydroxyapatite (high resolution dry powder, lot 110015) was from Calbiochem-Behring Corp. (La Jolla, CA). [U-14C]sucrose was purified by paper chromatography (two ascents at 85°C in 1-butanol/pyridine/water (6:1:1, v/v/v) on Whatman 3MM paper) before dilution with carrier sucrose. [fructose-U-14C]sucrose was from New England Nuclear (Boston, MA).

Organism

Lyophils of Leuconostoc mesenteroides NRRL B-512F were obtained from the Northern Regional Research Center (formerly Northern Regional Research Laboratory, NRRL) (Peoria, IL). Cultures from these lyophils produced surprisingly little enzyme (0.02 U/ml in culture supernatant), in view of reported values a hundred times higher (e.g., by Jeanes [163]). Such cultures were treated with nitrosoguanidine [1], and colonies were selected for high polysaccharide production. The resulting organism, used for this study, typically gave 3-4 U dextranucrase per ml culture supernatant, with a range of 1.5-7 U/ml.
Dextranase Preparation

Dextranase (2.6 mg solids assayed at 1000 U (total) by Sigma, 1 U being the amount that releases 1 μmol isomaltose equivalent per minute from dextran at pH 6.0 and 37°C) was dissolved in 25 ml 0.02 M sodium acetate buffer (pH 5.2) containing 0.05 M NaCl and 40 mg/ml mannitol, and lyophilized. The resulting powder, nominally 1 IU/mg at pH 6 and 37°C, was assayed at 0.09 IU/mg at 4°C and pH 5.2, using dextran T2000 as substrate.

Enzyme Assays

Glycansucrase assays were performed at 25°C in 0.05 M sodium acetate, pH 5.2, containing 1 mM calcium and 1 mg/ml Tween 80. Levansucrase and total glycansucrase activity (levansucrase + dextran sucrase) were measured by following the incorporation of [1-14C]fructose and [U-14C]sucrose, respectively, into methanol-insoluble polysaccharide [118]. One unit of enzyme is defined as the amount that will incorporate 1 μmol of glucose or fructose into polysaccharide in one minute under these conditions.

Dextranase was assayed by following reducing value increase by alkaline ferricyanide [162] or alkaline copper [253] methods. Alternatively, for qualitative detection, dextran T2000 (10 mg/ml in glycansucrase assay buffer) was incubated with enzyme solution, and aliquots were analyzed by thin-layer chromatography [66] for the production of isomalt-oligosaccharides.

Protein and Carbohydrate Analysis

Protein was determined by the method of Lowry et al. [213], with bovine serum albumin as standard. The buffer component imidazole was found, like many amines [267], to raise the absorbance of the blank and decrease the slope of the standard curve. Precipitates caused by the
presence of detergent [267] were removed by centrifugation.

Carbohydrate was determined by the phenol-sulfuric acid method [76], using maltose as standard. Polysaccharide composition was determined by acid hydrolysis [286] followed by thin-layer chromatography [66]. $^{13}$C-n.m.r. spectra were recorded as described by Côté and Robyt [67].

Gel Electrophoresis

SDS gel electrophoresis was performed by the method of Laemmli [203] on 5 x 90 mm cylindrical gels (6% (w/v) acrylamide). Protein was stained with Coomassie Blue G-250 [144]. Dextran sucrase and levansucrase activities were detected by incubation with sucrose and subsequent staining for polysaccharide by a periodic acid-Schiff procedure [173]. Gels stained with a periodic acid-Schiff stain were bleached with 10 mg/ml sodium metaperiodate to reduce background and to resolve closely spaced, intensely stained bands.

Hydrophobic Column Preparation

O-(phenoxyacetyl)-cellulose was prepared from phenoxyacetyl chloride and microcrystalline cellulose by the method of Butler [39]. Octyl Bio-Gel was prepared by heating Bio-Gel P-4 for 12 h at 94°C in neat, freshly redistilled n-octylamine. Hexyl Bio-Gel was prepared similarly by Paula J. Martin.

Dextran sucrase Production and Purification

-Leuconostoc mesenteroides NRRL B-512F was grown in sucrose medium as previously described [286], with the addition of 0.5-1.0 mg/ml Tween 80 to stabilize the enzyme [237]. Buffers contained 1 mg/ml Tween 80, 2 mM CaCl$_2$, and 0.2 mg/ml sodium azide in addition to any other components stated. The procedure given here is for a 1-liter culture. Modifications for processing larger amounts are described under Results. Unless
stated otherwise, all steps were conducted at 4°C. Measurements of pH were always made on room-temperature solutions. No temperature corrections were applied. This is of particular relevance to Fig. 4, where the pH-overlap shown between buffers at 4°C may be off by up to half a pH unit.

Cells were removed by centrifugation, and sodium azide (0.2 mg/ml) was added to the culture supernatant. Dextranase (20 mg of a lyophilized sample assayed at 0.09 IU/mg solids at 4°C and pH 5.2, made from a pH 5.2 solution containing 40 mg/ml mannitol) was immediately added to the culture supernatant, which was then dialyzed overnight against 2 L of 0.02 M sodium acetate (pH 5.2), 0.05 M NaCl. The dialysate was loaded onto a 1.5 x 35 cm DEAE-cellulose column equilibrated with the same buffer. The column was washed with 0.25 L of this buffer, and then with 1.9 L of 0.02 M sodium acetate (pH 5.2), 0.2 M NaCl. This was followed by 0.5 L of 0.02 M imidazole-HCl (pH 6.7), 0.2 M NaCl, and subsequently by a linear NaCl gradient (1.6 L; 0.2-1.0 M) run over a period of 5 days.

Sephadex G-200 chromatography was performed at room temperature (20-23°C). The active fractions eluted from DEAE-cellulose were pooled, applied to a Sephadex G-200 column (4.2 x 14 cm), and washed with 0.1 L of 0.02 M imidazole-HCl (pH 6.7), 0.4 M NaCl. The column was then washed with 0.2 L of 0.05 M imidazole-HCl (pH 6.7) without NaCl, and finally eluted with the same buffer containing 3 M urea. Urea solutions were made up within a few hours of use to minimize cyanate formation [319]. The eluted fractions were dialyzed against 0.02 M imidazole-HCl (pH 6.7) containing 0.2 M NaCl.
RESULTS

Purification

All purification steps except Sephadex G-200 chromatography were normally conducted at 4°, but chromatography at room temperature did not reduce yields.

Choice of stabilizer

All procedures, including the bacterial culturing, were performed in the presence of calcium and of Tween 80 or PEG 20,000, surfactants that had earlier been found to stabilize dextranucrase when present at concentrations as low as 10 μg/ml [237]. Generally, hundred-fold levels higher (1 mg/ml) were used. In cultures, enzyme yield began to decline between 0.3 and 1 mg/ml Tween 80, and bacterial growth between 3 and 10 mg/ml (Fig. 1).

Tween 80, most used in this study, proved in retrospect not to be the best choice of surfactant. Structurally, it is extremely heterogeneous. It is commercially available only as a viscous, amber liquid of variable density and water content, and has a potentially labile ester linkage [140]. Its chief drawback, however, lies in its high contribution to background in carbohydrate determinations (Table I). When enzyme solutions containing Tween 80 were concentrated, Tween 80 partitioned unevenly between the concentrate and the filtrate, with the result that it was present in the concentrates at high but unknown levels. This increased the uncertainty in carbohydrate concentration. Turbidometric determination of Tween 80 concentration with phenol [358] proved to be of uncertain reliability, due to the instability of the turbidity. No other type of determination was tried.

PEG 20,000 contributed negligibly to carbohydrate background, but solutions containing it could not be concentrated far without precipitating the enzyme. Purification of PEG 20,000 by precipitation from acetone with ether, intended to remove a spectroscopic impurity in the
Figure 1. Effect of Tween 80 concentration on bacterial growth and dextranulcrose production.

Turbidity (○) of a ten-fold dilution of culture with water was measured 20 h after inoculation of 2 ml Tween 80-containing culture medium with 0.2 ml culture grown without Tween 80. Cells from undiluted culture were pelleted by centrifugation and the supernatants were assayed for dextranulcrose (plus levansucrase) activity (●). ▲, pH of culture supernatant.
Table I. Carbohydrate equivalents and absorptivities at 280 nm of several dextranucrase stabilizers

<table>
<thead>
<tr>
<th>Stabilizer</th>
<th>10 mg/ml at 280 nm</th>
<th>Maltose equivalent (µg maltose per mg stabilizer)</th>
<th>Relative maltose equivalent</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG 20,000 (unpurified)</td>
<td>2.94</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>PEG 20,000 (purified)</td>
<td>2.97</td>
<td>0.2</td>
<td>1</td>
</tr>
<tr>
<td>Tween 80</td>
<td>0.67</td>
<td>11.6</td>
<td>58</td>
</tr>
<tr>
<td>Lubrol PX</td>
<td>0.09</td>
<td>1.8</td>
<td>9</td>
</tr>
<tr>
<td>Lubrol WX</td>
<td>0.73b</td>
<td>---</td>
<td>---</td>
</tr>
</tbody>
</table>

*a* Twice precipitated from acetone by ether [2].

*b* Unlike Lubrol PX, Lubrol WX solutions are cloudy.
commercial product [2], and thus make direct protein determination by ultraviolet absorbance feasible, did not have this effect (Table I). The ultraviolet absorbance of Tween 80 and other polyoxyethylene detergents has been discussed by Helenius et al. [141].

The Lubrols, poly(oxyethylene ether) derivatives of fatty alcohols [140], represent a compromise choice. Lubrol PX is superior to Lubrol WX in that the latter forms cloudy solutions even at low concentration, possibly as a result of the association of micelles to form large aggregates [141], and so gives a higher u.v. absorbance (Table I).

Dextranase treatment

Without dextranase treatment, a sizable fraction of the activity failed to bind to DEAE-cellulose or eluted at low ionic strength ($I = 0.2$ or less), and the yield of the the fraction of the enzyme remaining bound after a low-salt wash was low. A small amount of dextranase action on the dialyzed culture supernatant prior to DEAE-cellulose chromatography caused all the activity to bind at $I = 0.2$. For very light dextranase treatments, the binding was tenacious, leading to broad peaks and immense peak tailing during elution, even for eluents containing 1-3 M NaCl. Somewhat greater dextranase treatments caused a second peak of dextran sucrase activity to appear in a position corresponding to the tail of the activity peak shown in Fig. 2. Increasing the dextranase treatment reduced the size of this second peak.

Because of a protease impurity in the dextranase (Fig. 3), the dextranase treatment was not made exhaustive, but was kept as light as was consistent with an acceptable yield on subsequent DEAE-cellulose chromatography. (Sigma grade I dextranase, used in this study, was found by Tim Walseth to contain the least protease of several commercial preparations.) Dextran was not completely hydrolyzed. As a result, elution had to be carried out over a period of days to prevent eluting the activity as a very broad peak. This was believed to result from the slow release of dextran-dextran sucrase aggregates from the support.

Dextranase was not completely separated from dextran sucrase on DEAE-
Figure 2. DEAE-cellulose chromatography of dextranase-treated culture supernatant

DEAE-cellulose chromatography of dextranase-treated culture supernatant. Before the first fraction shown in the figure was collected, the column was extensively washed with buffers of both pH 5.2 and pH 6.7 (20 mM sodium acetate or imidazole), containing enough NaCl to give ionic strengths of 0.00 and 0.2, respectively. Dextranucrase was then eluted in the course of a 5-day sodium chloride gradient in this buffer. All buffers contained 2 mM CaCl₂, 1 mg/ml Tween 80, and 0.2 mg/ml sodium azide. Symbols: △, protein; ▽, carbohydrate; ○, dextranucrase activity; □, NaCl.
Dextranucrase eluted from DEAE-Trisacryl M was incubated at 37° at pH 5.5–6.5 in the presence of Sigma dextranase at a nominal concentration of 8 U/ml (in actuality less than 4 U/ml; contributes 2 μg protein on gels containing dex­tranase (A, B, and E–H)). Aliquots were removed at inter­vals, frozen, and thawed just prior to SDS gel electrophore­sis. Gels were stained for protein. The tops of the gels, not shown here, are blank.

**Gels A–C**  Extensive dextranase treatment. (A) Dex­tranase only; (B) Dextranucrase (60 μg protein) after 18 h incubation with dextranase; (C) Same dextranucrase (70 μg protein) without dextranase treatment.

**Gels D–H**  Intermediate dextranase treatments. A dif­ferent preparation of purified dextranucrase was used than for gels (B) and (C). (D) Dextranucrase (100 μg protein) without added dextranase. (E)–(H) Dextranucrase (50 μg pro­tein) incubated with dextranase for 1 min (E), 1 h (F), 2 h (G), and 4 h (H).
If a pH 5.2 wash was not included in the protocol to elute most of the remaining dextranase, enough was often present during Sepha-
dex G-200 chromatography to cause the flow rate to deteriorate rapidly, often stopping it completely before elution was complete.

**DEAE-cellulose chromatography**

Most of the purification occurred at this step (Tables II and III). The specific activity increased 52-fold, and carbohydrate was washed out almost quantitatively before application of the salt gradient. Surfac­tant stabilizers did not increase the yield, but without them the eluted enzyme lost 30-40% of its activity within a week. Optimal yield was at 2 mM calcium, but the effect of calcium on yield was small as long as a surfactant was present. The profile of the carbohydrate coeluting with the dextranucrase activity (Fig. 2) showed that most of the carbohydrate was contributed by the shoulders of two peaks that did not correspond to the activity peak. The monosaccharide composition of the pooled, active fractions showed roughly equal amounts of mannose, glucose, and ribose, with a smaller amount of galactose.

**pH Elution pH** was found to be critical (Fig. 4). Below pH 6.6, the yield dropped rapidly to less than 10%, while above pH 6.8, the yield also dropped, but more slowly. The drop in yield above pH 6.8 was found to be due mostly to decreasing enzyme stability. The dramatic decrease below pH 6.6 was caused by tighter binding of the enzyme to the column. In a control experiment, only 8% of the bound activity could be eluted at pH 5.0. After increasing the pH to 6.7, an additional 74% was eluted. This behavior suggests the existence of different populations of dextran­ucrase (e.g., differing in degree of aggregation or in amount of bound dextran) or of non-equivalent binding sites on the DEAE-cellulose.

Lowering the pH normally weakens binding to DEAE-cellulose by de­creasing the negative charge on an enzyme. The opposite behavior of B-512F dextranucrase was exploited by conducting a wash at low pH (5.2) before eluting the enzyme at pH 6.7. This slightly increased the degree of purification and greatly reduced the residual dextranase content,
Table II. One-liter scale dextranase purification without DEAE-Trisacryl M chromatography

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume after step (ml)</th>
<th>Dextran-α-sucrase (Units)</th>
<th>Step Yield (%)</th>
<th>Cumulative Yield (%)</th>
<th>Levan-β-sucrase (Units)</th>
<th>Protein (mg)</th>
<th>Specific Activity (U/mg protein)</th>
<th>Carbohydrate (mg)</th>
<th>Ratio of mg/ml carbohydrate to mg/ml protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell removal</td>
<td>980</td>
<td>3600^b</td>
<td>100</td>
<td>100</td>
<td>78</td>
<td>2600</td>
<td>1.4</td>
<td>5100</td>
<td>2.0</td>
</tr>
<tr>
<td>Dextranase treatment and dialysis</td>
<td>1200</td>
<td>3200</td>
<td>89</td>
<td>89</td>
<td>52</td>
<td>1300</td>
<td>2.5</td>
<td>3400</td>
<td>2.6</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>480</td>
<td>2800</td>
<td>88</td>
<td>78</td>
<td>0</td>
<td>21</td>
<td>130.</td>
<td>15</td>
<td>0.71</td>
</tr>
<tr>
<td>Sephadex G-200</td>
<td>260</td>
<td>2000</td>
<td>71</td>
<td>56</td>
<td>0</td>
<td>12^c</td>
<td>170.</td>
<td>1.6^d</td>
<td>0.13</td>
</tr>
</tbody>
</table>

^a Levan-α-sucrase detection limit by use of [fructose-U-14C]sucrose is about 1% of dextranase present. This percentage is not much lowered by increasing the specific activity of the [fructose-U-14C]sucrose, due to increasing background and scatter in the methanol-wash assay procedure.

^b 3.7 U/ml.

^c 0.045 mg/ml.

^d 0.006 mg/ml.
Table III. One-liter scale dextranucrase purification with DEAE-Trisacryl M chromatography

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume after step (ml)</th>
<th>Dextranase (U/ml)</th>
<th>Total Dextranase after step (U)</th>
<th>Step Yield (%)</th>
<th>Cumulative Yield (%)</th>
<th>Protein (mg/ml)</th>
<th>Specific Activity (U/mg protein)</th>
<th>Carbohydrate (mg/ml)</th>
<th>Ratio of mg/ml carbohydrate to mg/ml protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell removal</td>
<td>970</td>
<td>4.5</td>
<td>4400</td>
<td>100</td>
<td>100</td>
<td>3.1</td>
<td>1.5</td>
<td>7.3</td>
<td>2.4</td>
</tr>
<tr>
<td>Dextranase treatment and dialysis</td>
<td>1100</td>
<td>2.9</td>
<td>3200</td>
<td>73</td>
<td>73</td>
<td>1.5</td>
<td>1.9</td>
<td>3.8</td>
<td>2.5</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>500</td>
<td>4.2</td>
<td>2100</td>
<td>66</td>
<td>48</td>
<td>0.043</td>
<td>98.</td>
<td>0.041</td>
<td>0.95</td>
</tr>
<tr>
<td>Sephadex G-200</td>
<td>400</td>
<td>3.9</td>
<td>1600</td>
<td>76</td>
<td>36</td>
<td>0.030</td>
<td>130</td>
<td>0.0010</td>
<td>0.033</td>
</tr>
<tr>
<td>DEAE-Trisacryl M*</td>
<td>53</td>
<td>26.</td>
<td>1400</td>
<td>88</td>
<td>32</td>
<td>0.20</td>
<td>130</td>
<td>0.0055</td>
<td>0.028</td>
</tr>
</tbody>
</table>

* 3 M urea in eluent.
Figure 4. Yield of dextranucrase from DEAE-cellulose as a function of pH.

Dextranucrase, as dialyzed culture supernatant (30 U), was bound to a 0.7 x 4 cm column at the indicated pH in 0.05 M buffer containing 2 mM CaCl$_2$ and 1 mg/ml PEG 20,000, and eluted with 150 ml of the same buffer with 1 M sodium chloride added. Activity peaks showed considerable tailing in imidazole buffers (●), but not in MES (▲) or sodium acetate (○) buffers. The dashed line is the pH activity profile of the enzyme taken from Fig. 13.
although washing with 0.2 M NaCl even at pH 6.7 removed most of the dextranase [46, 108].

Dextranase treatment If the dextranase treatment was insufficient, a second peak of dextranucrase activity appeared at higher salt concentration (in the tail of the first peak). Decreasing the dextranase treatment increased the size of this second peak. A second peak does not appear in the activity profile of Fig. 2, but the eluted enzyme is probably heterogeneous with respect to dextran content, with enzyme of higher dextran content eluting in the tail of the peak. If the same elution procedure was used, but a DEAE-Sephacel column (1.5 x 30 cm) was substituted for DEAE-cellulose, the peaks in Fig. 2 were sharpened, and the tail of the dextranucrase activity peak became a small second peak overlapping the much larger first one. (In resolving capacity, DEAE-Sephacel is closer to microcrystalline DEAE-cellulose than to fibrous DEAE-cellulose. DEAE-Sephacel was not usually used for purification, because yield was lower than with fibrous DEAE-cellulose, and because no greater increase in specific activity was realized.)

Analytical chromatography of dextranase-treated enzyme on DEAE-cellulose occasionally resolved the tail of the dextranucrase peak into as many as five or six additional peaks of decreasing size, the fraction of the activity in these peaks decreasing with dextranase treatment. Because for preparative chromatography, dextranase treatment was incomplete, very slow flow rates were required during elution to prevent the broad peaks caused by heterogeneity of dextran content.

Affinity chromatography on Sephadex

The polysaccharide containing mannose, ribose, and galactose was removed by chromatography on Sephadex G-200 (Fig. 5), as was any residual dextranase.

Capacity Significant amounts of dextranucrase bound only to Sephadex G-50 and to the more porous Sephadexes (Fig. 6). With a given volume of enzyme solution, the binding capacity of Sephadex was not linear with bed volume (Table IV), possibly because of the presence of
Figure 5. Affinity chromatography on Sephadex G-200

Eluate from DEAE-cellulose (2800 U dextranucrase in 460 ml) was washed into a 4.2 x 14 cm gel bed. The column was washed with 0.05 M imidazole (pH 6.7) containing 1 mg/ml Tween 80, 2 mM CaCl₂, and 0.2 mg/ml sodium azide, and the activity was then eluted with the same buffer with the addition of 3 M urea. Aliquots of each fraction were dialyzed and assayed. □, dextranucrase; +, protein; ◇, carbohydrate.
Figure 6. Binding capacity of different Sephadex types for dextransucrase.

Dextransucrase (14 U) eluted from DEAE-cellulose was loaded at 0.06 ml/min onto 0.7 x 4 cm (1.5 ml) Sephadex columns in 5 ml eluent (0.05 M imidazole (pH 6.7), containing 2 mM CaCl₂, 1 mg/ml PEG 20,000, and 1 M sodium chloride). A single fraction (10 ml) was collected at the same flow rate and assayed for nonbound enzyme. Chromatography was performed at 4°C.

The percentage of bound enzyme is plotted (top axis) against water regain (weight of water taken up per weight of dry gel) and, equivalently, against the G-designation of the gel (bottom axis). The number in Sephadex G-type names is defined as ten times the water regain of the gel.
Table IV. Nonlinearity of dextranucrase adsorption by Sephadex

<table>
<thead>
<tr>
<th>Gel</th>
<th>Smaller column</th>
<th>Larger column</th>
<th>Comparison of the two columns</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dimen-</td>
<td>Bed</td>
<td>% of activity bound</td>
</tr>
<tr>
<td></td>
<td>sions (cm)</td>
<td>Volume (ml)</td>
<td></td>
</tr>
<tr>
<td>Sephadex</td>
<td>0.7 x 4.0</td>
<td>1.5</td>
<td>22</td>
</tr>
<tr>
<td>G-50</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sephadex</td>
<td>0.7 x 4.0</td>
<td>1.5</td>
<td>59</td>
</tr>
<tr>
<td>G-75</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sephadex</td>
<td>0.7 x 4.0</td>
<td>1.5</td>
<td>74</td>
</tr>
<tr>
<td>G-100</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Using the same chromatographic conditions as for Figure 6, eluate from DEAE-cellulose (14 U dextranucrase) was loaded onto two Sephadex columns of different sizes. The effluent was assayed for nonbound dextranucrase.
small residual amounts of dextran in the enzyme eluted from DEAE-cellulose, as discussed above. Therefore, while the capacity of Sephadex G-200 was 90 U/ml gel, considerably more than 1 ml of gel was required to bind all the activity in a sample containing 90 U. The practical result was a small loss of enzyme at this step in the purification. For example, for Table II, 4.5% of the applied activity was not bound, although the loading was only 14 U/ml gel.

**Required pretreatments**  
Dextranase treatment to remove dextran (or more likely just to lower its molecular weight), followed by DEAE-cellulose chromatography to remove most of the dextranase, were both required before affinity chromatography on Sephadex G-200. If dialyzed culture supernatant untreated with dextranase was passed through Sephadex G-200, half or more of the enzyme failed to bind, presumably because endogenous dextran competes with Sephadex for binding to dextransucrase. More than the slightest trace of dextranase caused the flow rate to slow rapidly.

**Desorption agents**  
Of a series of compounds tested for ability to elute dextransucrase from Sephadex G-200 (Table V), urea and dextran proved to be best. Dextrans in the molecular weight ($M_w$) range $1 \times 10^4$ – $2 \times 10^5$ were equally good (Fig. 7), and urea was as effective as dextran. The addition of salt appeared to reduce the desorption yield (Table V). This was convenient, because it meant that the enzyme eluted from DEAE-cellulose could be applied directly to the Sephadex, and the relatively high ionic strength in the sample served, if anything, to enhance the binding of the dextransucrase, while at the same time prevent nonspecific binding of other proteins.

**Desorption pH**  
The effect of pH (measured as apparent pH after the addition of desorbing agent: without 8 M urea, pH was 0.2–0.3 units lower) on yield was examined for urea and guanidine hydrochloride (Fig. 8). Yield was constant with length of incubation in 8 M urea solution (0.5–48 h). At pH 6.5 (measured before addition of urea), the best yield was at 3–4 M urea (Fig. 9). Sephadex G-200 flow rates were approximately twice as high at 3 M urea as at 4 M.
Table V. Desorption of dextransucrase from Sephadex G-200 by various compounds

<table>
<thead>
<tr>
<th>Addition to buffer</th>
<th>Yield(^a) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8 M urea</td>
<td>89</td>
</tr>
<tr>
<td>8 M urea + 1 M NaCl</td>
<td>66</td>
</tr>
<tr>
<td>1 M thiourea</td>
<td>0</td>
</tr>
<tr>
<td>1 M thiourea + 1 M NaCl</td>
<td>1</td>
</tr>
<tr>
<td>6 M guanidine hydrochloride</td>
<td>72</td>
</tr>
<tr>
<td>10 mg/ml dextran ((M_\text{w} = 2 \times 10^6))</td>
<td>85</td>
</tr>
<tr>
<td>0.5 M maltose</td>
<td>2</td>
</tr>
<tr>
<td>none</td>
<td>2</td>
</tr>
</tbody>
</table>

\(^a\) Buffer (20 ml) containing 2 mM CaCl\(_2\), 1 mg/ml PEG 20000, 0.05 M imidazole (pH 6.7), and the additional components listed in the table, was incubated 12 h at 25\(^\circ\) with a washed slurry of Sephadex G-200 (1.9 g) having 3.6 U/g of bound dextransucrase. Aliquots of the supernatant were dialyzed and assayed.
Figure 7. Dependence of desorption yield from Sephadex G-200 on dextran molecular weight

Experimental conditions are the same as for Fig. 8, except that the buffer in all cases was 0.05 M imidazole (pH 6.7) containing 2 mM CaCl₂, 1 mg/ml PEG 20,000, and 10 mg/ml dextran.
The experiments in Fig. 8 performed with MES and acetate buffers were performed on the same Sephadex slurry as for the imidazole buffers, but a week later. Because of variability from experiment to experiment, even where efforts were made to reproduce conditions exactly, the difference between yields at pH 6.3 with imidazole and MES buffers is probably not significant.

Activity losses

Yield of adsorbed enzyme ranged from 16-89%, although 70% was most common. The reason for the lost activity has not been determined, but two possibilities will be discussed here.

Non-eluted enzyme After elution with urea, a large bed of Sephadex G-200 was hydrolyzed with a trace of dextranase, and the hydrolysate was concentrated over an Amicon PM10 membrane and assayed for protein. None was detected. The presence of protease in the dextranase prevented certainty about how much protein should have been found, but from the 30% of activity lost, about 65 mg was expected. Binding of protein by the PM10 membrane (approximate capacity 0.3 mg albumin/cm²), was too small to account for significant protein loss from the concentrate in this experiment.

Urea Urea was inhibitory to the enzyme eluted from DEAE-cellulose, but concentrations after routine pre-assay dialysis were too low for detectable inhibition. The effect of urea on enzyme stability is not clear. The enzyme could be kept in buffered 8 M urea at room temperature for two days without reducing the activity recovered on urea removal (up to 100%), but the amount of activity recovered often dropped rapidly with longer incubations. Most of the inhibition caused by dilute urea (<1 M) in the absence of detergent stabilizer was not recovered by lowering the urea concentration by dilution or dialysis, and extended incubations (1-14 days) with 2 M urea at 4°C caused a slow loss of recoverable activity.
Figure 8. pH dependence of dextran sucrose desorption from Sephadex G-200

Buffer (25 ml, 0.05 M) containing 8 M urea ( ■, ▲, ○) or 6 M guanidine hydrochloride ( ○) was incubated 12 h at 25°C with a Sephadex slurry (2.5 g) having an estimated 8 U bound dextranucrase per g. Supernatant pH was then measured and a sample of the supernatant was dialyzed and assayed. No activity was detected for the control without urea and guanidine hydrochloride. Error bars represent the S.E.M. for 3 concurrent trials using the same Sephadex-slurry enzyme. Buffers contained 2 mM CaCl_2 and 1 mg/ml PEG 20,000. Buffers (0.05 M); ■, sodium acetate; ▲, MES; ○, ○, imidazole.
Figure 9. Effect of urea concentration on dextranucrase desorption yield from Sephadex G-200

Dextranucrase (28 U) eluted from DEAE-cellulose was applied to 1.5-ml (0.7 x 4 cm) columns of Sephadex G-200 at room temperature (21°) in 5 ml 0.02 M imidazole (pH 6.7) containing 2 mM CaCl₂, 1 mg/ml PEG 20,000, and 1 M NaCl. Columns were washed with this buffer until a total of 10 ml eluate was collected, resulting in the recovery of 8 U nonbound dextranucrase. Columns were then washed with 15 ml 0.05 M imidazole (pH 6.5 without added urea) containing 2 mM CaCl₂, 1 mg/ml PEG 20,000, and 0–8 M urea. Eluates were dialyzed to remove urea and assayed.
Scale-up

The purification detailed in Table II gave 12 mg of purified dex-
transucrase from just a 1-liter culture. The workup of much larger
volumes has been investigated.

Enzyme production

Up to 240 l of culture have been grown over a two-day period by
adding 10% inocula to 20 l volumes of unsterilized medium in 25 l
carboys, giving as much as \( 1.7 \times 10^6 \) U (7 U/ml) dextransucrase. The
cultures were grown at 28° without stirring or aeration. After 20 h
growth (longer than required for maximum yield, but not long enough to
cause any activity loss), sodium azide was added to 0.2 mg/ml and the
cultures were cooled to 4°. Cells were removed by microfiltration (HVLP
membrane) with a Millipore (Bedford, MA) Pellicon membrane cassette
system. As the filtration proceeded, a decreasing fraction of the dex-
transucrase was filtered, the enzyme remaining increasingly in the vis­
cuous, high-dextran cell fraction. This was centrifuged, and the superna­
tant was pooled with the filtrate.

The cell-free fluid was concentrated and dialyzed in the Pellicon
system (PTGC membrane; nominal M.W. cutoff 10,000). The degree of con­
centration was limited primarily by the viscosity of the concentrate, and
could be greatly increased by adding a minute amount of dextranase.

The yield to this point ranged from 59% to 94%. The method of
concentration and dialysis (e.g., efficient cooling of the enzyme solu­
tion, choice of peristaltic or high-capacity centrifugal pump) seemed to
be important, probably because of the long processing times. Cell-free
concentrates prepared in this way (50–100 U/ml) have been stored frozen
(−20°) in 2-liter volumes for over a year with no loss of activity.

DEAE-cellulose chromatography

Gram quantities of dextransucrase with high specific activity and
low carbohydrate content have been made by applying samples containing
over 10^5 units of dextranucrase to large columns. For example, 1.3 x 10^5 units were purified in 95% yield on a 6 x 39 cm column. The capacity of the DEAE-cellulose was 200-300 U/ml packed bed. Yields were 70-100%, with 80-85% most common. These large columns could be run at room temperature, with only a small increase over the time required for small columns, and with no shift in the pH dependence of yield, which might have been expected from the large temperature shift of the buffer pK_a [316]. (pH measurements were always made on solutions at room temperature, and no temperature correction was ever applied.) Step gradients of NaCl were used, with most of the activity eluting at 0.4 M and none at 0.2 M.

The enzyme eluted from DEAE-cellulose could be concentrated over an Amicon PM10 membrane to 800-900 U/ml in 90% yield. No loss of activity from this concentrate occurred on storage for 6 months at 4°, although small amounts of a fine precipitate developed, which was only partly removed by centrifugation at 27,000 x g. Complete removal of the precipitate by filtration through clarifying or sterilizing membrane filters (0.2 um pore diameter) did not reduce the activity of the solution. The precipitate continued to form after the clarifying filtration.

Concentrates of this type have been stored frozen in 0.1-liter volumes for over a year with no loss of activity. Lyophilization of the concentrates resulted in 10% loss of activity on reconstitution. Some of the lyophil could not be dissolved in buffer, or in buffer plus 2 M urea (see ref. 44). The insoluble portion could be dissolved in buffer with 6 M urea, but this did not increase the activity of the solution when measured after urea removal.

**Affinity chromatography**

Scale-up of the affinity step (Sephadex G-200 column chromatography) was more difficult, because of bed compression at low pressure. By using wide, shallow columns, hundred milligram amounts of purified enzyme could be made. For example, 1.8 x 10^4 units from a DEAE-cellulose column was purified in 71% yield on a 12 x 4 cm (diameter x height) column in 71%
yield. The Sephadex could also be used batchwise. Sephacryl S-200, a less compressible but more highly modified dextran-based gel filtration matrix than Sephadex [21], bound no enzyme from dialyzed culture supernatant concentrate under conditions where Sephadex G-200 bound over 50%.

**Ion-exchange following affinity chromatography**

**Rationale** Reagent-grade urea contains water-insoluble fines that are only partly removed by filtration through sterilizing (0.2 µm pore) membranes. Clear enzyme concentrates required the removal of these fines. This could be done by ultrafiltering buffers before use, or alternatively, by binding the enzyme desorbed from Sephadex to an anion-exchanger, and then letting the fines be washed through the matrix or filtered out by it. An ion-exchange step at this point also had the advantages of concentrating the enzyme and of eliminating traces of uncharged carbohydrate still associated with the enzyme [228] or leached from the Sephadex matrix [6].

**Chromatography on DEAE-Trisacryl M** DEAE-Trisacryl M was chosen as the anion-exchanger because of its high mechanical rigidity, high exclusion limit (>10⁷ Da), and high capacity, but especially because its acrylic matrix contains no carbohydrate. All the dextranucrase activity in Sephadex G-200 eluates, which contained 3-8 M urea, bound completely to a DEAE-Trisacryl M column. The urea was then washed out, and the enzyme was eluted with 1 M NaCl in 0.05 M imidazole (pH 6.7). The best enzyme preparation produced in this way contained 28 mg protein (0.71 mg/ml) with a specific activity of 170 U/ml and marginally detectable carbohydrate (0.5 µg/ml, or 0.7 µg/mg protein). Overall activity yield, relative to culture supernatant, was 31%.

If the elution of DEAE-Trisacryl M was performed as described above for preparations from small (1 liter) cultures, the activity bound tightly, and was only partly eluted over several days after adding 1-2 M NaCl to the eluent. This did not appear to be due to any special property of the anion-exchanger, which behaved identically to DEAE-cellulose in the chromatography of dialyzed culture supernatant. For preparations
from 1-liter cultures, the enzyme could be eluted from DEAE-Trisacryl M as a sharp peak if 3 M urea was included in the eluent (Fig. 10). The enzyme in the Sephadex G-200 eluate could also be concentrated many-fold on DEAE-Trisacryl M (e.g., see Table III), but dialysis was then required to remove the urea, and the overall carbohydrate content was not reduced by this step (Table III).

Aging the enzyme preparation before any chromatographic purification changed its elution behavior on DEAE-cellulose and DEAE-Trisacryl M. In aged preparations, elution could be performed much more rapidly without causing peak broadening. The lowest carbohydrate contents in eluates from DEAE-cellulose and DEAE-Trisacryl M also resulted from aged samples. Aging was a significant factor only for large-scale preparations, which took longer to process to the stage where they could be applied to DEAE-cellulose.

Specific activity was usually unaffected (neither increased nor decreased) by adding a DEAE-Trisacryl M step to the procedure (e.g., see Table III). While it was nearly always used as the last step in a purification, chromatography on DEAE-Trisacryl M has not been included in the standard procedure given in Table II, because of the low yield when urea was not included in the eluent, and because of the failure to reduce the carbohydrate content if it were included.

Alternative Purification Procedures

A number of purification procedures were tried other than those already described. These fell into two groups, depending on whether they were used before or after DEAE-cellulose chromatography. Methods tried before DEAE-cellulose chromatography (i.e., on crude enzyme) were hydrophobic interaction chromatography, and various gel filtration procedures and precipitation with poly(ethylene glycol). Methods tried after DEAE-cellulose chromatography, as alternatives to affinity chromatography on Sephadex, were hydroxyapatite chromatography, hydrophobic interaction chromatography, and precipitation with organic solvents or with ammonium
Figure 10. DEAE-Trisacryl M chromatography

Eluate from a Sephadex G-200 column like that of Fig. 5 (400 ml containing 1600 U dextran sucrase) was washed into a 2.0 x 10. cm column of DEAE-Trisacryl M equilibrated with the same buffer used to elute the enzyme from Sephadex. Activity was eluted by applying a 3-6 M urea gradient at a constant NaCl concentration of 1 M. Fractions (20-25 ml) were dialyzed and assayed. (For this step, buffer with 6 M urea was filtered through an Amicon PM10 membrane to remove fines, with Tween 80 added to the ultrafiltrate to a concentration of 1 mg/ml.) □, dextran sucrase; +, protein; ◆, carbohydrate.
sulfate.

**Procedures used before DEAE-cellulose chromatography**

**Hydrophobic interaction chromatography**  The crude enzyme (dialyzed supernatant concentrate) completely failed to bind to hexyl and octyl Bio-Gels (in 0.05 M sodium acetate, 2 mM CaCl₂, pH 5.0). Chromatography on phenoxyacetyl cellulose [39, 65, 73] was then tried. While all the activity (15 U) bound to a column (0.5 x 1.9 cm) of this material equilibrated with the same buffer, only 26% was eluted on adding 10 mg/ml Tween 80 to the eluent buffer, and only an additional 8% eluted on changing the detergent to 10 mg/ml Triton X-100. The eluted enzyme was not characterized for specific activity or carbohydrate content, although Côté and Robyt [65] found that the soluble-glucan-producing glucansucrase from *L. mesenteroides* NRRL B-742 could be largely freed of carbohydrate by chromatography on phenoxyacetyl cellulose.

**Gel filtration and precipitation with poly(ethylene glycol)**  This is discussed in Part III of the dissertation.

**Procedures used after DEAE-cellulose chromatography**

**Hydroxyapatite chromatography**  Hydroxyapatite chromatography was first tried in the presence of 6 M urea (Fig. 11), as has been done for levansucrase [47]. Some characterization of the binding (pool I) and nonbinding (pool II) fractions in Fig. 11 are given in Table VI. Error in the numbers given in Table VI is significant because of low protein concentrations. The degree of error can be gauged from the line of the table listing total protein.

Enzyme activity was entirely in the nonbinding fraction (Table VI). Proteins generally bind more weakly to hydroxyapatite when they are denatured by urea [22]. The higher ratios in pool II and pool I of A₂₆₀ to A₂₈₀ and of carbohydrate to protein may reflect the presence of nucleic acids in pool II.

Hydroxyapatite chromatography in the absence of urea (Fig. 12) halved the carbohydrate content of the preparation, but only if all the
Figure 11. Hydroxyapatite (Ultrogel HA) chromatography in urea buffers

Pectransucrase eluted from DEAE-cellulose (120 U in 3 ml) was diluted to 12 ml with 9 M urea and dialyzed against 4 changes of buffer (10 mM potassium phosphate, pH 6.8) 6 M in urea at room temperature (27°C) over 24 h. The entire dialysate was applied to a 0.55 x 8.0 cm (1.9 ml) column of Ultrogel HA, with fraction collection beginning simultaneously with enzyme loading. A linear phosphate gradient (0.01-0.50 M, pH 6.8) was begun at the start of fraction 16. Eluents all contained 6 M urea. Fractions were pooled as indicated, dialyzed, and assayed. □, absorbance at 280 nm; X, molarity of potassium phosphate.
Table VI. Characterization of fractions in Fig. 11

<table>
<thead>
<tr>
<th>Quantity</th>
<th>Urea-treated control</th>
<th>Pool I</th>
<th>Pool II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activity yield</td>
<td>46%</td>
<td>44%</td>
<td>0.9%</td>
</tr>
<tr>
<td>(A_{280}) yield^b</td>
<td>100%</td>
<td>17%</td>
<td>71%</td>
</tr>
<tr>
<td>Total protein (mg)</td>
<td>0.50</td>
<td>0.61</td>
<td>0.10</td>
</tr>
<tr>
<td>Specific activity (U/mg protein)</td>
<td>92</td>
<td>71</td>
<td>7.3</td>
</tr>
<tr>
<td>(\mu g) Carbohydrate per (\mu g) protein</td>
<td>0.44</td>
<td>0.22</td>
<td>1.35</td>
</tr>
<tr>
<td>(A_{260}/A_{280})</td>
<td>1.67</td>
<td>1.17</td>
<td>1.74</td>
</tr>
</tbody>
</table>

^a Sample applied to column.

^b 100% = 6.0 \(A_{280}\) units (\(A_{280}\) x volume (ml)).
active fractions were discarded except for a central cut of the dextran-sucrase peak. If the entire peak was retained, the carbohydrate content was only reduced slightly.

Overall yield of glycansucrase activity in Fig. 12 was 60%. The best fraction (No. 15) had a specific activity of 83 U/mg protein and a carbohydrate to protein weight ratio of 0.19:1. This fraction contained 22% of the glycansucrase activity applied to the column. The sample applied had a specific activity of 85 U/mg and a carbohydrate to protein weight ratio of 0.39:1.

The absence of multiple peaks of dextran-sucrase activity in Fig. 12, as obtained by previous students in this laboratory, is probably due to the use of a continuous gradient. Step gradients often cause "false" peaks and strong tailing even with pure samples, if the adsorption isotherm is highly curved [22]. The same point can be made to a lesser degree for DEAE-cellulose chromatography.

Hydrophobic interaction chromatography Chromatography was done on a column of octyl Bio-Gel P-4 (9 x 0.7 cm, made from 1.0 g dry octyl Bio-Gel P-4), using a reverse ammonium sulfate gradient (1.0–0.0 M in 0.05 M imidazole, pH 7.0). Approximately one-third of the applied activity (53 U) failed to bind, and the remainder failed to elute at all, either during the gradient, after the gradient, or when the buffer was made 50% (v/v) in ethylene glycol. Overall recoveries were 34% for glycansucrase activity, 52% for protein, and 85% for carbohydrate. Thus, the bound fraction had a lower carbohydrate content than the eluted fraction. Detergents were not used in this experiment.

Precipitation with organic solvents and ammonium sulfate In preparing samples of enzyme eluted from DEAE-cellulose for chromatography on octyl Bio-Gel, it was found that no precipitate formed on dialysis against 1 M ammonium sulfate (in 0.05 M imidazole, pH 7.0), and that activity was fully retained (96%). Dialysis against 3 M ammonium sulfate (70–75% saturation) resulted in precipitate formation, and in the loss of all activity from solution, as well as 94% of the protein and 80% of the carbohydrate. Only enzyme solutions without Tween 80 could be used,
Figure 12. Hydroxyapatite chromatography of dextranulose eluted from DEAE-cellulose

An enzyme sample was dialyzed overnight against 0.01 M potassium phosphate (0.01 M), and the dialysate (2.2 ml) was applied to a column of hydroxyapatite (0.7 x 4 cm) and washed with a small volume of the same buffer. A linear gradient of potassium phosphate (pH 6.8) was then applied. (A) shows all profiles. (B)-(D) compare different pairs of the profiles in (A). (E) compares protein and carbohydrate on the same weight concentration scale.

Maximum values were 130 μg/ml for protein (◇), 26 μg/ml for carbohydrate (+), 11 U/ml for dextranulose activity (□), 0.33 for absorbance at 280 nm (△), and 0.30 M for potassium phosphate (measured by conductivity) (line without symbols).
since Tween 80 was not soluble at 1 mg/ml in 1 M ammonium sulfate, but oiled out unless glycerol were added. Tween 80 was soluble at 1 mg/ml in buffers containing 5 M NaCl, but 5 M NaCl did not cause any observable precipitate formation (at pH 6.7).

Ethanol and dioxane were tested as precipitants. The minimum tested concentrations causing any precipitation when enzyme was dialyzed at 4°C against solvent in buffer (0.05 M imidazole, pH 6.7, containing 0.2 M NaCl, 2 mM CaCl₂, and 1 mg/ml Tween 80) were 9% (v/v) dioxane and 23% (v/v) ethanol. In the latter case, 21% of initial activity was recovered from the pellet, and 60% from the supernatant, giving a 19% loss. The enzyme eluted from DEAE-cellulose, which was yellow or brown in color depending on the degree of concentration (by ultrafiltration) after elution, was completely decolorized by dialysis against buffered aqueous solutions of dioxane or ethanol.

**Enzyme properties**

**pH optimum**

The pH optimum of the purified enzyme was 5.0-5.5 (Fig. 13). While long-term stability of the purified enzyme over this pH range has not been studied, losses from pH instability were not significant for the experiment of Fig. 13 in the pH interval 4.0-7.5.

**Stability**

**pH** In the presence of stabilizer (1 mg/ml PEG 20,000), dialyzed culture supernatant was stable over 10 days at 4°C in the pH range 5.0-6.8 (Fig. 14), with calcium concentration having virtually no effect. Stability began to decrease sharply between pH 7.0 and 7.2 (Fig. 14). It is important to keep in mind that the pH values quoted were measured at room temperature, and that they are not the same at 4°C. The temperature dependence for the imidazole buffers is particularly high, so that pH at 4°C is substantially higher than at room temperature.

**Calcium** The effects of calcium have not been investigated in
Dextranase eluted from DEAE-Trisacryl M was diluted 1:50 with buffer of a given pH and assayed after a 20 min preincubation at 25°C. Assay concentrations were 1 mM CaCl₂, 0.1 mg/ml Tween 80, 0.15 M sucrose, and 0.025 M buffer. Buffers: A, sodium acetate; M, MES; P, PIPES; H, HEPES; B, Bicine.
Concentrated culture supernatant dialysate (in 7 mM sodium acetate, pH 5.0, with 3 mM CaCl$_2$) was diluted ten-fold with the buffers described in the legend to Fig. 4. Aliquots were stored at 4$^\circ$C and assayed at intervals after adjusting the pH to 5.2. The remainder was chromatographed (day 1) on DEAE-cellulose (Fig. 4).

(A) Acetate buffers: $\bullet$, pH 5.0; $\circ$, pH 5.5.
(B) MES buffers: $\circ$, pH 5.5; $\bullet$, pH 6.0; $\triangle$, pH 6.5.
(C) Imidazole buffers: $\circ$, pH 6.4; $\bullet$, pH 6.6; $\triangle$, pH 6.8; $\diamond$, pH 7.0; $\blacktriangle$, pH 7.2; $\square$, pH 7.4.
great detail, and should be regarded as preliminary; in particular, as being subject to artifacts causing only apparent stability, discussed below under the subheading "Partial stability". In the presence of 1 mM calcium but the absence of a surfactant or polysaccharide stabilizer, dilute enzyme from DEAE-cellulose (1.5-3. U/ml) was most stable at pH 6-7, retaining only 50% of the activity of the pH 7 control after 2 days at pH 5 or 5 days at pH 8. Increasing the calcium concentration to 5 mM or creating a reducing environment by the addition of 1% (v/v) 2-mercaptoethanol made the enzyme as stable at pH 5 as at pH 7.

Increasing the calcium concentration to 10 mM caused large activity losses at 40 within 2 days at all pH values. However, this loss was not seen in one experiment at an even lower concentration of enzyme (0.3 U/ml), conducted at high ionic strength (1 M NaCl). For this experiment, activity was constant for ten days at pH 6.7 and 40, irrespective of calcium concentration (<0.1-10 mM).

**Lyophilization**  
Culture supernatant could be lyophilized and reconstituted with distilled water with full recovery of activity. Lyophilization of dilute (2 U/ml) purified enzyme in sodium acetate buffer resulted in loss of most of the activity (Table VII), whether it was reconstituted with water or buffer. The loss was prevented by adding 20 mg/ml dextran T10 or dextran T2000 to the lyophilization buffer. Small apparent losses in the presence of dextran were attributed to inhibition by exogenous dextran, described later. The loss was also prevented without the addition of dextran if the enzyme was lyophilized in a nonvolatile buffer (MES, pH 5.5). The loss was not prevented by PEG 20,000 (Table VII), another stabilizer of the enzyme [237].

**Freezing**  
Freezing the purified enzyme resulted in considerable loss of activity. Preliminary results indicate that a polymeric stabilizer (dextran or poly(ethylene glycol)) prevents most of this loss. Limited attempts to freeze or lyophilize concentrated solutions (up to 1600 U/ml) of purified enzyme have given buffer-insoluble solids. Only part of the lost activity could be recovered by dissolving the solids in urea. In all cases, more activity was recovered from lyophils than from
Table VII. Lyophilization and reconstitution of purified dextranucrase

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Buffer pH</th>
<th>Stabilizer</th>
<th>Stabilizer concentration</th>
<th>pH and yield after reconstitution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Reconstitution with water</td>
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<tr>
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<td></td>
<td></td>
<td></td>
<td>pH</td>
</tr>
<tr>
<td>Sodium acetate</td>
<td>5.2</td>
<td>None</td>
<td>---</td>
<td>7.0</td>
</tr>
<tr>
<td>Sodium acetate</td>
<td>5.2</td>
<td>Dextran T10</td>
<td>20 mg/ml</td>
<td>6.5</td>
</tr>
<tr>
<td>Sodium acetate</td>
<td>5.2</td>
<td>PEG 20000</td>
<td>20 mg/ml</td>
<td>8.5</td>
</tr>
<tr>
<td>MES</td>
<td>5.5</td>
<td>PEG 20000</td>
<td>20 mg/ml</td>
<td>5.7</td>
</tr>
<tr>
<td>MES</td>
<td>5.5</td>
<td>Dextran T10</td>
<td>2 mg/ml</td>
<td>5.7</td>
</tr>
<tr>
<td>MES</td>
<td>5.5</td>
<td>None</td>
<td>---</td>
<td>5.7</td>
</tr>
</tbody>
</table>

^ Enzyme eluted from DEAE-Trisacryl M (0.05 ml) was mixed with the buffer described in the table (0.95 ml), lyophilized, and reconstituted with water or buffer (1 ml).

^ Buffers were 0.05 M and contained 2 mM CaCl$_2$, 0.2 mg/ml Tween 80, and 0.2 mg/ml sodium azide.

^ Buffer was 0.1 M sodium acetate (pH 5.2), containing 4 mM CaCl$_2$, 0.4 mg/ml Tween 80, and 0.4 mg/ml sodium azide.

^ pH after reconstitution.
frozen solutions.

Precipitate formation. A precipitate began to form in the purified enzyme preparation after about a week, and activity was concurrently lost from the solution. Loss of activity from solution was greater than loss of protein. All the precipitate that formed within 3 months at 4°C could be dissolved in 1-2 min by the addition of sucrose (to 5 mg/ml) or NaCl (to 1 M). The precipitate could also be dissolved, but incompletely, in 1 h by the addition of dextran T2000 (to 5 mg/ml). Longer incubations (15 h) did not increase the amount dissolved by dextran.

Precipitate formation occurred at any time after DEAE-cellulose chromatography. It was retarded by ionic strengths above 0.2 and was accelerated by refrigeration. High salt concentration did not prevent slow precipitation of the enzyme described here. Fig. 15 shows that even in 1 M NaCl, nearly all of the enzyme that elutes from DEAE-cellulose is aggregated or soon becomes aggregated. How much of this aggregation can be attributed to dextran, which is apparently still present to a small degree after DEAE-cellulose chromatography, is not known.

"Partial" stability. False observations of stability sometimes resulted when enzyme assays were delayed until one or two days after an enzyme fraction was prepared. For example, enzyme eluted from DEAE-cellulose in the absence of Tween 80 lost 30-40% of its activity within 2 days of elution, but the remaining activity was constant for at least 4 months, and with no precipitate formation, despite frequent handling, which had previously been found to cause activity losses [237]. Other examples of rapid but only partial loss of activity from B-512F enzyme preparations have been reported (e.g., see ref. 172 and Part VI of this dissertation).

Enzyme products

The purified enzyme preparation produced the same products with sucrose and with acceptors as did the crude enzyme [26, 282]. The dextran was a typical, lightly branched B-512F dextran, as shown by $^{13}$C-n.m.r. and by its endodextranase hydrolysis products (see Part IV of this dissertation).
Figure 15. Size distribution of dextranucrase eluted from DEAE-cellulose.

This dextranucrase preparation had 0.5 mg carbohydrate per mg protein and contained no precipitate. A 2.0 ml sample (46 U) was applied to a 2.5 x 72 cm column of Bio-Gel A-5m, which was eluted with 0.05 M sodium acetate (pH 5.2) containing 2 mM CaCl₂, 1 mg/ml PEG 20,000, and 1 M sodium chloride. □, 20-min assay; +, 200-min assay.
dissertation). The dextrans made separately by each of the two molecular-weight forms of the enzyme (see below) have not been characterized.

The physical properties of the dextran could be changed by varying the sucrose and enzyme concentrations. If high enzyme concentration (50–500 U/ml) and low sucrose concentration (3–50 mM) were used together, a dextran was made that precipitated from solution as it was synthesized. The heaviest precipitates were made at the highest ratios of enzyme to sucrose. This dextran was soluble in DMSO or in large volumes of water. Using low sucrose concentration alone produced high-molecular-weight dextran but no precipitate (see Part III of this dissertation), even when the dextran concentration became relatively high (e.g., 25 mg/ml of freshly-synthesized dextran for Fig. 1C of Part III. The structures of these dextrans were not investigated.

Effects of dextran

"Activation" by endogenous dextran In enzyme preparations with lowest carbohydrate content, reaction velocities were found to increase up to 75% (usually 20–40%) in the early part of the reaction (Fig. 16). The increase was difficult to detect except at low enzyme or sucrose concentrations, and did not take place in the presence of added dextran (Fig. 16). At the usual assay concentration (0.15 M sucrose and about 0.5 U/ml dextransucrase), this acceleration phase lasted approximately 1 min. The acceleration has been observed in numerous experiments on many independent enzyme preparations. It occurred with enzyme eluted from DEAE-cellulose and from DEAE-Trisacryl M, but not always after the intermediate step of elution from Sephadex G-200.

To test whether the acceleration was a result of dissolving the enzyme precipitate, the precipitate was dissolved beforehand by adding a small amount of sucrose (0.1 volume, 0.3 M) or sodium chloride (to 1 M). The acceleration was still observed (Fig. 16). Moreover, with either method of dissolving the precipitate, the acceleration phase did not begin immediately, but only after a linear phase. The point at which the acceleration started, in terms of cpm incorporated into methanol-insolu-
Figure 16. Nonlinear dextran synthesis by purified dextransucrase

(A) Pre- and post-acceleration phases of de novo dextran synthesis for enzyme eluted from DEAE-Trisacryl M. Reaction mixture contained 13 mU/ml dextransucrase and 150 mM sucrose. The initial and final rates are shown by the two lines, extrapolated (dotted portions) to show the difference more clearly.

(B) and (C): the early part of the reaction only. For (B) and (C), precipitate in the stock enzyme solution was dissolved with NaCl before dilution and assay. Reaction mixtures contained 34 mU/ml purified dextransucrase, 5 mM (▼) or 50 mM (▲) sucrose, and no dextran T10 (B) or 10 mg/ml dextran T10 (C).
New Dextran Synthesized (mg/ml)
ble dextran, was the same for all sucrose concentrations for a given experiment; i.e., it depended only on the concentration of dextran synthesized de novo. In the experiment of Figs. 16-17, this dextran concentration was about 0.1 mg/ml.

Velocities determined before or after the acceleration phase gave double-reciprocal plots (Fig. 17) that were linear in the range 3-50 mM sucrose, with positive deviation at higher concentration due to substrate inhibition. The substrate inhibition constant, \( K_{is} \), was approximately 0.9 M (Table VIII). The \( K_m \) for purified enzyme was not changed significantly by the addition of 10 mg/ml dextran (Table VIII), and was the same as at all earlier stages of purification (12-16 mM). \( K_m \) was the same, or nearly the same, both before and after the acceleration phase.

"Inhibition" by exogenous dextran Thin-layer chromatography (not shown) showed that glucose release by the purified enzyme was not significantly changed on adding dextran. The enzyme was inhibited a constant 10-15\% by 1-10 mg/ml of either dextran T10 or native B-512F dextran, independent of the length of preincubation (10 min-6 h) of the dextran with the enzyme. Table VIII shows that before the acceleration phase, there is little difference in velocity caused by the presence of added dextran T10. This means that the apparent inhibition by dextran T10 noted above is only an inhibition when compared to the enzyme "activated" by newly-synthesized dextran.

It should be noted that reagent-grade sucrose contains dextran [164], and that no effort was made in this work to remove it, so that minute concentrations of dextran were present in all assays. Extremely low levels of native B-512F dextran (less than 1 \( \mu \)g/ml) have been found to increase the stability of the enzyme [237], and may influence activity as well, masking a greater effect of added or newly-synthesized dextran on activity than was observed.

Purity

Carbohydrate concentrations in the fully purified enzyme were close to the detection limit, although this was relatively high in the
Figure 17. Effect of exogenous dextran on the $K_m$ of purified dextransucrase

Initial velocities for enzyme eluted from DEAE-Trisacryl M were determined in the presence ($\updownarrow$) or absence ($\Delta$) of 10 mg/ml dextran T10. Initial velocity in the absence of dextran T10 was taken to be the rate before activation (shown in Fig. 16) occurred. The curves plotted are fits to a rate equation that includes a term for substrate inhibition.
Table VIII. Kinetic parameters from Fig. 17 (double-reciprocal plots)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Without dextran T10</th>
<th>With 10 mg/ml dextran T10</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_m$ (mM)</td>
<td>$12.6 \pm 0.5$</td>
<td>$12.5 \pm 0.7$</td>
</tr>
<tr>
<td>$K_{is}$ (M)</td>
<td>$0.84 \pm 0.18$</td>
<td>$0.96 \pm 0.34$</td>
</tr>
<tr>
<td>$V_{max}^a$</td>
<td>$40.0 \pm 1.0$</td>
<td>$42.9 \pm 1.5$</td>
</tr>
</tbody>
</table>

$^a$ ug dextran per ml per min.
Tween 80-containing buffers used (Table I). Whether the residual carbohydrate is dextran has not been determined. Carbohydrate was detected in SDS gels of culture supernatant only at the top of the gel, and not at all in gels of purified enzyme, even at heavy loadings (Fig. 18).

SDS gel electrophoresis of culture supernatant (Fig. 18), followed by activity staining, showed two major and two minor bands with glycansucrase activity (gels B–D). The minor bands, at approximately 92 kDa and 116 kDa, produced polysaccharide from raffinose, demonstrating that they were levansucrase, since raffinose is a substrate for levansucrase but not for dextransucrase [43]. The major bands, at 177 ± 8 kDa and 158 ± 10 kDa, made no polysaccharide from raffinose, and were therefore both dextransucrase.

After DEAE-cellulose chromatography, the enzyme preparation still had considerable heterogeneity, as shown by Figs. 12, 15, and 18–20. The heterogeneity of the carbohydrate in the preparation, although not of the protein (Fig. 19), was initially reduced by affinity chromatography on Sephadex G–200. The peptide heterogeneity was reduced by ethanol precipitation (Fig. 20).

The purified enzyme (Fig. 18, gels E–H) lacked levansucrase, but had the same two dextransucrase bands, and frequently a minor one just below the 177 kDa form (clearest in gel F, which shows a protein stain). The proportion of the 158 kDa form of dextransucrase increased from merely a trace in fresh preparations to a large fraction of the total enzyme during storage of either the culture supernatant or the purified enzyme. An inactive band at 117 ± 4 kDa (gel F) also appeared in purified enzyme preparations during storage.

Fairly extensive dextranase treatments caused the disappearance of all protein bands migrating above the dextranase band (Fig. 19, gels A–C). Whether protease in the dextranase contributes to the conversion of the 177 kDa form of dextransucrase to the 158 kDa form is uncertain, but the conversion occurs even without the addition of dextranase.
Figure 18. SDS gel electrophoresis of dextranucrase fractions

Gels (A)-(D) are culture supernatant (400 μg protein) and gels (E)-(H) are purified enzyme (following DEAE-Trisacryl M chromatography). DS and LS mark the positions of the dextranucrase and levansucrase activities, respectively. The position of the inactive band (I) developing on storage of the purified enzyme is indicated by the dashes to the right of gels F–H.

**Culture supernatant**  
(A) Protein; (B)–(D) Dextranucrase and levansucrase activity:  
(B) gel soaked in 10% acetic acid, 25% 2-propanol after 6 h incubation with sucrose and 10 mg/ml dextran T10 (no bands could be seen against a dark background but the two visible here); (C) periodic acid–Schiff stain after 5 min incubation with sucrose; (D) periodic acid–Schiff stain after 3 h incubation with sucrose.

**Purified enzyme**  
(E) and (F): protein. (G) and (H): dextranucrase activity (periodic acid–Schiff stain):  
(E) after storage at 4°C for 1 week (100 μg protein); (F) after storage at 4°C for 8 months (70 μg protein); (G) after storage at 4°C for 8 months (70 μg protein; 5 min incubation with sucrose); (H) after storage at 4°C for 3 months (10 μg protein; 15 min incubation with sucrose).
Figure 19. SDS gels of dextranucrase at various stages of purification

Gels (A)–(C) were stained for protein and gel (D) for dextranucrase activity (periodic acid–Schiff stain after 15 min incubation with sucrose). The tops of the gels, not shown here, are blank. (A) Concentrated dialysate of culture supernatant (same sample as for gel A in Fig. 18; approximately 400 µg protein); (B) and (D) After DEAE-cellulose chromatography (approximately 11 µg protein; preparation had been stored at 4°C for over 6 months); (C) After DEAE-Trisacryl M chromatography (same sample as gel H in Fig. 18; approximately 13 µg protein; preparation had been stored at 4°C for 3 months).
Figure 20. Heterogeneity by nondenaturing polyacrylamide gel electrophoresis of dextran sucrase after various purification procedures

(A) Enzyme eluted from DEAE-cellulose.

(B) Enzyme eluted from DEAE-Trisacryl M (corresponding to SDS gel G in Fig. 13). (C) Solubilized pellet of ethanol precipitate of enzyme eluted from DEAE-cellulose. Gels were stained for protein. Gel (C) contains approximately 30% of the protein contained by gel (A). Gel (B) contains approximately 140 µg protein.

For each gel shown, an identical one (not shown) was incubated with sucrose. White polysaccharide zones formed corresponding to the smeared protein bands beginning about one-third of the way down the gels. The presence of surfactants in the samples may be partly responsible for the diffuse boundaries of the bands [98].
DISCUSSION

Choice of Enzyme Source (Leuconostoc vs. Streptococcus)

Current research is focused on streptococcal glucansucrases, especially those of S. mutans [133], because of their involvement in the etiology of dental caries [52, 127, 128, 133, 239, 255, 343]. While advances continue to be made in the purification of glucansucrases from Streptococcus, a problem that remains is the elaboration of undesirable enzymes (multiple glucansucrases, and high amounts of fructansucrase, dextranase, and invertase [52, 127, 128, 133, 239, 255, 343]), which are difficult to separate completely [52, 239, 255, 310, 343]. By contrast, Leuconostoc mesenteroides B-512F produces only one glucansucrase [312, 343], a dextranucrase, and produces it in high amounts (discussed below), with only small levels of related, contaminating enzymes [286, 343]. Some strains of S. sanguis have nearly the same advantages [343, 350], but culture yields are not as high [150].

A disadvantage frequently cited [312, 343] for Leuconostoc dextranucrases is difficulty of purification (said by Walker [343] to be in a "primitive" state), arising from the fact that the enzyme must be induced with sucrose, and that the dextran consequently formed associates strongly with the enzyme and is nearly impossible to remove, especially because the activity becomes unstable on treating with dextranase to this end [343]. Instability on dextranase treatment is not unique to Leuconostoc preparations, but has also been reported for S. mutans [54].

Glucansucrases are constitutive in Streptococcus [128], and the purified enzymes therefore have lower carbohydrate content, especially if precautions are taken to eliminate traces of sucrose and glucan in the growth medium [200, 301, 343]. These purified preparations are rarely if ever carbohydrate-free, however [52, 127, 128, 133, 150, 239, 255, 310], although carbohydrate is not always detected [226]. Carbohydrate contents (considered as weight ratio to protein) from 1% [310] (with 3.8% overall activity yield) to 38% [117] have been reported for purified
dextran sucrase from S. mutans 6715, while in this laboratory, extensive purification of this enzyme gave a preparation still containing 6% carbohydrate [68].

Compared to L. mesenteroides B-512F, strains of Streptococcus produce low levels of enzyme: frequently 0.1-0.2 U/ml culture [44, 97, 299, 311], rarely more [150, 311], and often far less [51, 179, 200, 229, 315, 341]. The specific activities of the purified glucansucrases [52, 127, 239, 255] are low, high values being in the neighborhood of 50 U/mg protein (e.g., [68]), although an exceptionally high value of 296 U/mg was reported by Mayer's group for a dextran sucrase fraction isolated in 1.3% yield from S. sanguis [150]. The higher specific activities of B-512F dextran sucrase preparations are in fact understated, because they are determined by assay at 25°, while for streptococcal glucansucrases assay temperature is 37°. B-512F dextran sucrase shows the expected activity increase with temperature [172, 247].

In summary, the chief advantages of B-512F dextran sucrase for use as a model glucansucrase are the large amounts produced in culture, the relatively high initial enzymatic purity (low amounts of levansucrase, etc.), and the high specific activity obtainable. The high solubility and the structural simplicity of B-512F dextran (it is among the least branched dextrans known [164]) also make this dextran sucrase attractive for some types of mechanistic studies. With the only real drawback to studying the B-512F enzyme being the high carbohydrate content, the principal motivation for this work was to reduce carbohydrate in purified B-512F dextran sucrase to a level comparable to the levels in purified glucansucrases from Streptococcus, without sacrificing the advantages cited above for the B-512F enzyme.

**Enzyme Production**

**Culture yield of dextran sucrase**

As described under the heading "Organism" in Materials and Methods, cultures from the original lyophils of L. mesenteroides NRRL B-512F
produced very little enzyme, as low as 0.02 U/ml in the culture superna­tant. In this laboratory, Walseth [345] found that different lyophils of *L. mesenteroides* B-512F gave enzyme levels from 0.5 to 5 U/ml, with the amount produced by a given lyophil unchanged by subculturing. However, values reported in the literature are not always even as high as the low end of the range found by Walseth (e.g., 0.09 U/ml found by Kobayashi and Matsuda [188]).

Based on limited observation, culture yield tends to decrease after long culture and many transfers. This may be due to culture degeneration of the sort that is frequently a problem for high-yielding microbial strains [12]. *Leuconostoc* has been reported by Pederson and Albury [265] to lose or gain the ability to synthesize dextran, depending in large part on the environment (medium and temperature) and maintenance (e.g., number of transfers). By periodically treating cultures with nitrosoguanidine and re-selecting colonies showing high polysaccharide production [237], average yields were kept above 3 U/ml.

The high producers that were selected by this procedure are probably very similar to the B-512F strain used in other laboratories where high culture yields are reported. At the Northern Regional Research Center, cell supernatants from this strain are reported usually to contain 2.5-3.5 U/ml [163]. Lopez and Monsan [212] found 1.5 to 9 U/ml, the higher levels being produced by the continuous addition of sucrose to the growing *Leuconostoc* cultures. Increasing the initial sucrose concentration in batch cultures was reported as early as 1952 [190] to increase the production, but also to make cell removal more difficult by increasing the dextran content of the culture.

**Detergents**

Tween 80 was included in the growth medium as a dextransucrase stabilizer [237]. It did not result in higher activities in the culture (Fig. 1), although it is known to increase the production of glucansu­crases in *Streptococcus* species [311, 341, 355] and of exocellular bacterial enzymes in general [93]. The increase is generally smaller for
organisms already selected for high production [93], which was the case for the strain of *L. mesenteroides* used here.

The use of Tween 80 to improve the purification yield of glucan-sucrases was first reported by Figures and Edwards [97], who also found that it gave a less aggregated form of glucansucrase [98]. We have not looked at the effect of detergents on aggregation in the B-512F system.

**Enzyme Purification**

**DEAE-cellulose chromatography**

Unlike other dextransucrases successfully purified on DEAE-cellulose [150, 158, 186, 299], dextranase from *L. mesenteroides* B-512F binds so tightly to this material that yields are normally very low [286]. In this laboratory, it was found that over a narrow range of conditions, DEAE-cellulose gave high yields and an excellent purification. Critical requirements were dextranase treatment to degrade the associated dextran prior to chromatography, and the use of an optimal elution pH (Fig. 4).

Most of the purification occurred at this step (Figs. 3 and 19). Nearly all the carbohydrate (99.6%) and protein (98.4%) was removed (Tables II and III), including nearly all of the added dextranase. The remaining carbohydrate, by its monosaccharide composition (mannose, ribose, galactose, and glucose) and by its retention on a DEAE-cellulose column, appeared to consist of heterogeneous, charged polysaccharides. Sources might include lipoteichoic acid [40, 128, 201, 294] and other *Leuconostoc* cell wall components [131], and various polysaccharides introduced with the yeast extract in the culture medium (e.g., RNA and phosphorylated mannans) [14, 313]. Some gram-positive bacteria produce exocellular mannan (p. 158 in Brock et al. [35]), but mannose-containing polymers are more typical of gram-negative bacteria [131].

Lawford et al. [205] also purified B-512F dextranase on DEAE-cellulose with apparent success. Adapting the procedure used by Itaya and Yamamoto [158] for dextranase from *L. mesenteroides* IAM 1046, these investigators [205] prepared the enzyme for DEAE-cellulose chroma-
...by adding egg albumin and then precipitating with ammonium sulfate. (Ammonium sulfate precipitation in the absence of a coprecipitant gives poor results [286].) Unfortunately, neither the yield, the specific activity, nor the carbohydrate content of the resulting enzyme was reported. The results of Itaya and Yamamoto [158] at this step, however, were inferior to those here in Tables II and III. The step yields reported by Itaya and Yamamoto for the precipitation and for the DEAE-cellulose chromatography were 51% and 55%, respectively, giving 28% for the two steps combined.

**Affinity chromatography on Sephadex**

The carbohydrate remaining after DEAE-cellulose chromatography was removed by affinity chromatography on Sephadex G-200 (Fig. 5), as was the remaining trace of dextranase. The use of affinity chromatography to remove dextranase from glucansucrase has been recommended by Walker [343], based on the work of McCabe and Smith [229].

Dextranucrase bound in significant amounts only to Sephadex G-50 and the more porous Sephadex gels (Fig. 6). Initially, it was thought that the curve in Fig. 6 might reflect the oligomer size distribution of the enzyme after elution from DEAE-cellulose, with dextranucrase monomers of about 30 kDa or less being able to enter the pores of Sephadex G-50, dimers being able to enter the pores of Sephadex G-75, etc. A gel filtration profile on Bio-Gel A-5m (nominal exclusion limit 5 x 10^6) showed, however, that the smallest active particle (the right-most shoulder in the activity peak of Fig. 15) had a molecular weight of over 10^5, with most of the activity in even larger aggregates.

In the binding of dextranucrase to Sephadex, then, the porosity of the gel is probably less important than a lower degree of modification; i.e., a lower degree of crosslinking. (Sephadex is made by crosslinking B-512F dextran with epichlorohydrin.) In support of this contention, the B-512F enzyme bound poorly if at all to Sephacryl S-200, a dextran-based gel filtration matrix that is very porous (nominal exclusion limit 250,000), but nevertheless highly modified, in chemical terms, relative
to soluble dextran. The possibility that dextranase oligomers of 100 kDa or more could be induced to dissociate to monomers of 30 kDa or less by the presence of Sephadex was not addressed experimentally.

As estimated from the desorption yield from Sephadex G-200 (Table V), high ionic strength enhanced the binding of dextranase to Sephadex. High ionic strength has been reported by Schachtele et al. [301] to enhance the interaction of glucansucrases with soluble glucan, implicating hydrophobic interactions in the binding. Hydrophobic interactions may be the driving force for nearly all binding of proteins to neutral carbohydrates [143, 208], although nonionic detergents alone did not elute dextranase from Sephadex. Ono et al. have recently reported [258] the finding that salt increased the yield of a glucansucrase from S. mutans 6715 from an affinity column when the column was eluted with clinical dextran.

Desorption from Sephadex

Use of urea. To avoid contamination with clinical dextran, which was used by Kobayashi and Matsuda [188] to elute bound B-512F dextranase from Sephadex G-100, urea was chosen as the eluting agent, as done previously by Figures and Edwards for the affinity chromatography of S. mutans glucansucrases [99]. Guanidine hydrochloride has been found to be better than urea for other glucansucrase systems (e.g., [258]).

As found by Smith and coworkers [156, 229] for a streptococcal glucansucrase bound to an insoluble-glucon column, B-512F dextranase was not eluted from Sephadex by salt, nonionic detergent, or maltose. Smith's group also found EDTA and methyl α-glucoside to be ineffective. As in these studies ([156, 229]), dextran size had little effect on the yield (Fig. 7), which was found in this laboratory to be consistently below the yield given by urea.

The effect of urea concentration in eluting dextranase from Sephadex, shown in Fig. 9, has a remarkable inverse correlation with a previously published plot [247] of dextranase activity vs. urea concentration.
Yield

Desorption yield from Sephadex was rarely over 75%. Part of the loss (0-10%) could be accounted for by failure of a fraction of the enzyme to bind to the gel (discussed above). Another explanation, partial inactivation by urea, was found for B-512F dextranucrase by Neely [247]. In contrast, Figures and Edwards [99] have reported that urea caused no irreversible denaturation of *S. mutans* glucansucrases, and also that a glucansucrase fraction could be eluted from an affinity column by low-molecular-weight B-512F dextran that was not eluted by 6 M urea.

Urea was quite inhibitory to the B-512F dextranucrase after elution from DEAE-cellulose, but urea concentrations were too low to cause detectable inhibition after the dialysis always performed before assaying. The effect of urea on enzyme stability is not clear from the few experiments conducted to examine this. A certain amount of irreversible denaturation appeared to occur, although it may have been only indirectly related to the presence of urea. Speculatively, it could have been due to the process causing inactivation in the absence of calcium [237], which would be a situation identical to the calcium-mediated effect of urea on the stability of $\alpha$-amylase from *Bacillus subtilis* [153]. Calcium stabilizes many types of enzymes [354], and has been shown to have a strong effect on the stability of B-512F dextranucrase [172, 212].

For the affinity chromatography of *S. mutans* glucansucrases, Smith and coworkers [156, 229] have reported yield averages and yield variability that were quantitatively identical to those found in this study, although their glucansucrases had different specificity and their glucan-containing column a different glucan structure. Interestingly, Russell [293], unlike Inoue and Smith [156], found little relation between the specificity of the glucansucrase (structure of the glucan product) and the ability of the enzyme to bind to immobilized glucans having variable degrees of $\alpha-(1+6)$ and $\alpha-(1+3)$ linkages. Inoue and Smith [156] found that after no more glucansucrase could be eluted with clinical dextran, the a glucan-based affinity column produced fructose from sucrose, showing that some glucansucrase was still bound.
Figures and Edwards [97] found that almost 90% elution of the S. mutans 6715 glucansucrase that was bound to a glucan-containing affinity column was eluted with clinical dextran. Eluting with 6 M urea and then with clinical dextran [99] gave only 68% recovery; again, almost identical to what was observed in this study. They presented evidence [99] that the difference between the yields of the two elution procedures was not due to irreversible denaturation or to irreversible binding of glucansucrase to the column, but to the separation of two synergistic glucansucrase activities.

Affinity chromatography has been used by other researchers for purifying streptococcal glucansucrases [97, 99, 228, 229, 293, 315], and by Kobayashi and Matsuda [188] in the purification of B-512F dextran sucrase; however, Kobayashi and Matsuda used only the fraction of the enzyme that failed to bind to Sephadex G-100 (about half the applied activity), because the clinical dextran used to elute the binding portion was an unacceptable impurity for their work.

Sephadex has been used effectively as the affinity matrix in some of the investigations on glucansucrase from Streptococcus [129, 228, 230, 293], but does not seem to bind dextran sucrases from Leuconostoc [186, 205, 321], poor binding being obtained even for B-512F dextran sucrase itself [188]. This is probably due to the endogenous dextran present in the Leuconostoc preparations, since removal of the dextran by dextranase treatment and DEAE-cellulose chromatography greatly increased the fraction of the activity that bound to Sephadex G-200. This conclusion is supported by recent work by Ono et al. [258], who found that very small amounts of dextran prevented the binding of glucansucrase from S. mutans 6715 to glucan-based affinity columns.

Additional purification

A final ion-exchange step on DEAE-Trisacryl M was usually employed to remove fines originating in reagent-grade urea, to eliminate traces of uncharged carbohydrate still associated with the enzyme or leached from
the Sephadex matrix, and to concentrate the enzyme. The enzyme could be eluted from DEAE-Trisacryl M with salt as a sharp peak only if 3 M urea was maintained in the eluent (Fig. 10). In the absence of urea, the enzyme usually bound so tightly to the matrix that elution over several days with 1-2 M NaCl washed off only part of the activity (10-30%), with more slowly bleeding from the column on longer washing. The enzyme in the Sephadex G-200 eluate could be concentrated many-fold on DEAE-Trisacryl M, but then dialysis was required to remove urea. The carbohydrate content was not reduced, and the specific activity was not increased (Table III).

Problems with the procedure

Protease impurity in dextranase Protease was present in the dextranase preparation (Fig. 3). This meant that exhaustive dextranase treatments could not be used. While partial hydrolysis of dextran in the culture supernatant greatly improved column performance, an even greater extent of hydrolysis resulted in a much shorter DEAE-cellulose run time and in a lower final carbohydrate content.

Ciardi et al. [54] found for the glucansucrases of a strain of S. mutans that dextranase treatment created a new glucansucrase band on non-denaturing gel electrophoresis, but then caused the loss of all glucansucrase activity. No new active glucansucrase species resulted from the dextranase treatment here, as judged by SDS gels (Fig. 18), but activity was lost from proteolysis, and inactive bands increased in quantity. Other reports of activity loss from dextranase treatment of Leuconostoc glucansucrases [343] could also be due in part to proteolysis.

Use of urea Another problem was the use of urea. In aqueous solutions, urea decomposes to give an equilibrium concentration of ammonium cyanate, which chemically modifies proteins and can partly or completely inactivate them [318, 319]. Guanidine hydrochloride, used by other investigators to desorb glucansucrases from insoluble glucans [293, 315], was not a good substitute for urea because of the lower yield.
obtained (Fig. 8, Table V), but many other potential substitutes for urea exist [260, 293, 342].

Other miscellaneous problems were the low yield from Sephadex, the slow elution from DEAE-Trisacryl M in the absence of urea, and the use of cellulosic dialysis tubing, which appeared to increase the carbohydrate content of the final dialysate. Most importantly, the lack of a reliable way to store concentrated, purified enzyme made large-scale purification beyond DEAE-cellulose chromatography unattractive.

Alternative Purification Procedures

After DEAE-cellulose chromatography was optimized, a way was sought to reduce carbohydrate content (usually 20-50% of protein by weight at this stage) even further. Hydroxyapatite chromatography, in the presence (Fig. 12) and absence (Fig. 11) of denaturing urea, and hydrophobic interaction chromatography were tried with limited success. While affinity chromatography on Sephadex was eventually adopted, precipitation by organic solvents may offer comparable purification (as measured by protein heterogeneity (Fig. 20)) and comparable yield, but with greater convenience. Carbohydrate content was not determined after precipitation by organic solvents.

Precipitation with ammonium sulfate, while its effect on protein heterogeneity was not examined, was found to be difficult in the presence of detergent, and to coprecipitate carbohydrate in its absence. Drawbacks of precipitation by ammonium sulfate or by organic solvents, which are related to coprecipitation of dextran and to "aggregation" of the precipitated enzyme, have been discussed by Robyt and Walseth [286] and by Monsan and Lopez [238].
Enzyme Properties

General

Most of the properties of the purified enzyme, such as pH optimum (Fig. 13), \( K_m \) (Table VIII), etc., were the same as for the unpurified enzyme (culture supernatant), which has been well characterized [172, 188, 286, 312]. The purified enzyme did not contain detectable levan-sucrase or dextranase, unlike the culture supernatant, and contained very low amounts of carbohydrate (Tables II and III). It produced a typical, lightly-branched B-512F dextran (see Part IV of this dissertation), and the usual low-molecular-weight products from sucrose [26, 282], both in the presence and absence of acceptors. Specific activity was usually at the upper end of the range 130-170 U/mg protein.

Stability

Calcium Intermediate concentrations of calcium (1-5 mM) were found to be most stabilizing, with higher concentrations required at lower pH. A strong effect of calcium on the stability of B-512F dextran-sucrase at pH 4.5 has been reported [172], as well as an increased requirement of purified dextran-sucrase for stabilizing calcium [212] relative to crude enzyme. Itaya and Yamamoto [158] have also found that calcium increases the pH stability range of dextran-sucrase from \( L. \) mesenteroides IAM 1046, from 5.0-5.5 to 5.0-6.5.

2-Mercaptoethanol Limited evidence was obtained that 2-mercaptoethanol contributes to the enzyme's stability under some conditions. Reports of non-specific stabilization of enzymes by various thiol agents are common [354]. For example, cysteine (also calcium and histidine) prevent inactivation of a cell-associated \( S. \) salivarius fructansucrase. This appears to be in large part by protecting it from proteolysis by a cell-associated protease [160].

Storage At any stage before affinity chromatography, the enzyme could be frozen or lyophilized with little or no loss of activity. Lengthy storage of the enzyme after affinity chromatography has not been
successful. On extended refrigeration, solutions of purified enzyme slowly lost activity (e.g., 20% in one month; 90% in 9 months), with the formation of a precipitate. Related to this activity loss, Ciardi [52] found irreversible inactivation of glucansucrase from S. mutans 6715 by precipitation in phosphate buffer, while over 65% of the activity remained after two years of storage at 4°C when a pH 6.5 imidazole buffer was used. These conditions are very close to the ones used for enzyme storage here.

Like precipitates of highly purified dextransucrase observed by others [44, 84, 286], the precipitates formed in this work were apparently active, since they could be dissolved by sucrose or dextran. Salt also dissolved all the precipitate forming within a few months at 4°C, but even at 1 M did not prevent precipitation indefinitely. Prevention of the precipitation of purified dextransucrase by high ionic strength [44, 286] and by urea [44] has been reported. Salt effects on glucansucrase aggregation have also been described in the literature [186, 301]. With respect to glucansucrase purification, Figures and Edwards [97] have found it vital to maintain a high ionic strength for good yields.

Dissolving the precipitate did not restore lost activity. This was to be expected, because the precipitate was sufficiently fine that assays were always conducted on uniform suspensions. The precipitate would have been dissolved rapidly at the usual assay concentration of sucrose, ten times the concentration that dissolved the precipitate within 2 min.

Freezing (-20°C) and lyophilization caused loss of activity from some solutions of purified enzyme but not from others. At present, only dilute solutions of purified enzyme can be reliably frozen or lyophilized without loss of activity (Table VII). As found by Scales et al. [299], lyophilization caused less loss than freezing. The optimal pH for the chromatographic steps, approximately 6.7, was used for storage of the purified enzyme. A lower pH might be better (see next paragraph).

pH Other investigators have found the B-512F enzyme to be unstable at pH 6.7, with maximum stability in the pH range 5.0-5.2 [190, 163] or 5.5-6 [172], and fair stability in the range 5.0-6.5 [172]. While a
A thorough study of the pH stability of the purified enzyme described here has not been done, experiments at other stages of purification (e.g., for concentrated dialysate of culture supernatant in Fig. 14) give results basically in accord with these reports, except for somewhat greater stability found at higher pH in this study, despite differences in enzyme purity and the inclusion of surfactant stabilizers.

**Purity**

**Carbohydrate content** Carbohydrate concentrations in the purified enzyme were close to the detection limit, although this was relatively high in the Tween 80-containing buffers used (Table I). Whether the residual carbohydrate is dextran has not been determined. Neither culture supernatant nor purified enzyme gave positive PAS stains for the dextranase species on gels (Fig. 18), even at heavy loadings (70 µg protein). This argues against the possibility [52, 127, 128, 133, 188, 239, 255, 286, 312] that the enzyme is a glycoprotein, in agreement with Shimamura et al. [310], and suggests that any covalently bound dextran must be small in size or attached to the enzyme through a labile linkage [188, 285, 287].

**Peptide heterogeneity** Like the culture supernatant, the purified preparation contained two forms of dextranase (Figs. 18 and 19), differing in molecular weight. Differences in properties other than molecular weight were not investigated. Aging the culture supernatant at 4°C for several weeks before carrying out the purification resulted in a purified preparation containing more of the lower-molecular-weight species, at the expense of the higher-molecular-weight form (Fig. 18). Similar results have been reported by Grahame and Mayer [123] and by Kenney and Cole [180]. Fig. 19 shows that the heterogeneity in the peptide molecular weight distribution does not decrease after DEAE-cellulose chromatography: Figs. 18 and 19 both show that over time, it actually increases. Evidence that the conversion is caused by endogenous protease has been considered elsewhere (see Part VI of this dissertation).
As was found by Grahame and Mayer [123], aging (in this case for several months at 4°C) did not reduce the activity of the culture supernatant. Aging of the purified enzyme did cause loss of activity, along with the production of an inactive peptide of 117 kDa, at the expense of the peptides with dextranucrase activity. Protease is therefore likely to be present, although as discussed under Results, it is not necessarily just the protease from the dextranase preparation.

Aging the culture supernatant enzyme also improved the chromatographic performance of the enzyme, and resulted in lower carbohydrate contents. Since increasing the dextranase treatment had the same effect, it is possible that the culture supernatant contained a small amount of endodextranase, as do supernatants from cultures of Streptococcus [343]. Other evidence for the presence of a dextranase in cultures of *L. mesenteroides* B-512F has been reported [163, 286, 336], but may in most cases [286, 336] have resulted from transferase reactions carried out by the dextranucrase itself [26].

In addition to the two dextranucrase peptides, there were small amounts of many peptides of lower molecular weight without dextranucrase or levansucrase activity (Fig. 18). Why these remained after affinity chromatography is not clear, although origin by proteolysis of active dextranucrase seems likely. None of the usual causes (see Scouen [306], pp. 99-102) of nonspecific adsorption on affinity matrices should be important for Sephadex with the procedure followed, although Sephadex does have some hydrophobic character [21, 356] and a small number of carboxyl groups [340], which can cause non-specific binding. This raises the question of whether some of the peptides bind to Sephadex specifically. Exocellular dextran-binding proteins without glucansucrase activity have been isolated from *S. mutans* by Russell [293] and McCabe et al. [230]. The latter investigators found considerable protein heterogeneity in the dextran-binding fraction [230].
Dextran synthesis

Dextran structure Changing the enzyme and sucrose concentrations apparently changed the resultant dextran structure in some way, as shown by the production of high-molecular-weight dextran at low sucrose and acceptor concentrations (see Fig. 3 in Part III of this dissertation), and more remarkably, by the production of a relatively water-insoluble dextran at high enzyme and low sucrose concentrations. Some literature exists on the effect of sucrose and enzyme concentrations on the structure of the dextrans produced by dextransucrase from \textit{L. mesenteroides} strains B-512F [34, 337] and IAM 1046 [339], but no synthesis of insoluble dextran has been reported for the B-512F enzyme, probably because enzyme concentrations have not remotely approached the high values used here. In the course of this work, purified dextransucrase preparations were made with activity concentrations as high as 1600 U/ml.

Reaction kinetics Added dextran decreased reaction rates slightly for the purified enzyme, as has been occasionally observed [176, 350, 355] for streptococcal glucansucrases, and prevented an increase otherwise observed early in the reaction (Fig. 16). The cause of the increase is unknown, although it was correlated with \textit{de novo} dextran synthesis. Part of it could also be caused by dissociating aggregates or by artifacts of the assay method, such as would be the case if the enzyme's sucrose hydrolysis (invertase) activity were high at the very low dextran concentrations in the earliest stage of the reaction, a phenomenon for which some evidence has been found for streptococcal glucansucrases [107, 214, 258]. Activation by dextran has been been reported for dextransucrase from some strains of \textit{Leuconostoc} [186, 189]. For B-512F dextransucrase, activation has been found only in some studies (e.g., in ref. 188), and not in others (e.g., in ref. 286).

Summary

Gram quantities of dextransucrase with high specific activity and low carbohydrate content can be made by carrying the purification de-
scribed here through DEAE-cellulose chromatography. This enzyme is satisfactory for most purposes. By adding an affinity chromatography step, hundred-milligram amounts of enzyme can be made with higher specific activity than yet reported for B-512F dextranucrase, and with a very low carbohydrate content. Finally, although lowering overall yield to about 30%, ten-milligram amounts of highly purified enzyme without detectable carbohydrate can be produced by an additional step of chromatography on DEAE-Trisacryl M.
"The horror! The horror!"

—Mr. Kurtz,

discussing dextranucrase purification

in Joseph Conrad's *Heart of Darkness*
PART III

PURIFICATION OF DEXTRANSUCRASE FROM LEUCONOSTOC MESENTEROIDES NRRL B-512F BY TWO-STAGE GEL FILTRATION CHROMATOGRAPHY
Dextran isomerizes the glucosyl moiety of sucrose to form dextran, an α-(1→6)-D-glucan with α-linked branches [343]. It is a primarily exocellular enzyme elaborated by a number of Leuconostoc and Streptococcus species. Unlike streptococcal glucansucrases, which are constitutive, Leuconostoc dextransucrases must be induced with sucrose. The resulting dextran impurity is usually considered a major deterrent to studying the enzyme from this genus [312, 343].

L. mesenteroides NRRL B-512F secretes dextranase in much higher amounts [238] and with much smaller levels of other sucrose- and dextran-utilizing enzymes [286] than do species of Streptococcus [179]. These advantages are largely nullified by a high carbohydrate content. We propose here the combination of two purification methods that are based on gel filtration chromatography [238, 286] to give a B-512F dextranase preparation with high specific activity and a much lower carbohydrate content than any previously published method of comparable simplicity.
Crude enzyme, described elsewhere [237], was a dialyzed concentrate of culture supernatant. Dextranulose activity was measured by following the incorporation of $^{14}$C from $[\text{U-}^{14}\text{C}]$sucrose into methanol-insoluble polysaccharide [118]. One unit of dextranulose is defined as the amount that will incorporate 1 umol of glucose into polysaccharide in one minute at pH 5.0-5.2 and 25°. Protein was measured by the dye-binding method of Bradford [32], and carbohydrate by the phenol-sulfuric acid method of Dubois et al. [76], using bovine serum albumin and maltose, respectively, as standards. Bio-Gels A-5m and A-50m were from Bio-Rad Laboratories (Richmond, CA).
RESULTS AND DISCUSSION

Gel Filtration

Robyt and Walseth [286] have described a simple purification procedure for B-512F dextranucrase that consists of treating crude enzyme with dextranase, followed by removing the added dextranase by gel filtration, which simultaneously separates the dextranucrase from levansucrase. Dextran is eliminated in this way, but there remain large amounts of a heterogeneous polysaccharide containing mannose, galactose, and glucose [286], and the purified activity is unstable, a common consequence of dextranase treatment [286, 343]. In contrast, two research groups have described methods that do not remove dextran, but instead use gel filtration to separate dextranucrase from other culture components as its high-molecular-weight dextran complex [172, 238]. Yields of up to 96\% and specific activities as high as 122 U/mg protein have been reported [238].

Most of the different advantages of gel filtration with and without prior dextranase treatment can be obtained by doing both in sequence. This is illustrated in Fig. 1. Fig. 1A shows chromatography on Bio-Gel A-5m (nominal exclusion limit 5 x 10^6 Da) of crude enzyme without a dextranase pretreatment. The dextranucrase peak is highly heterogeneous (left panel) and very high in carbohydrate (seen most easily in right panel).

Fig. 1B shows the corresponding profiles when the crude enzyme has first been treated with dextranase. This is the principal purification step of the method of Robyt and Walseth [286]. The carbohydrate in the dextranucrase purified by this method comes partly from the tail of a large void volume carbohydrate peak, but mostly from the leading edge of a broad peak of lower, but still large, molecular weight carbohydrate (Fig. 1B). Because the latter carbohydrate is of lower molecular weight than the dextran-dextranucrase complex before dextranase treatment, it could be almost entirely removed by using the void volume fraction from a
Figure 1. Gel filtration chromatography of dextranuclrase on Bio-Gel A-5m (2.5 x 70 cm column)

Righthand panels show the same carbohydrate and protein profiles as the corresponding lefthand panels, but drawn to different scale to emphasize their relative amounts.

(A) Crude enzyme (4.0 ml); (B) Crude enzyme (5.5 ml) after dialysis in the presence of dextranase; (C) Void volume peak (fractions 19-27) of (A) after concentration over an Amicon PM10 membrane and dextranase treatment as for (B) (applied sample 5.5 ml). ———, protein; ●●●, carbohydrate; ——, dextranuclrase activity; ——, absorbance at 280 nm.
Dextranase (U/ml)
Carbohydrate (mg/ml)
Protein (x 100) (mg/ml)

Dextranase (x 0.1) (U/ml)
Carbohydrate (mg/ml)
Protein (x 10) (mg/ml)

Dextranase (x 0.25) (mg/ml)
Carbohydrate (mg/ml)
Protein (x 30) (mg/ml)
Bio-Gel A-5m column as the substrate for dextranase digestion.

Gel filtration of the resulting dextranase digest is shown in Fig. 1C. The dextransucrase peak is much less heterogeneous than in the one-step procedure shown in Fig. 1B. The confusion of non-overlapping curves seen in Figs. 1B and 1C has vanished. The active fractions in Fig. 1C (Nos. 25-45) have an average carbohydrate-to-protein weight ratio of 2.4, with a minimum of 1.1 at No. 34, compared to average ratios as high as 30 for the unmodified method (Fig. 1B) [286]. The protein and activity curves in Fig. 1 are replotted in Fig. 2 to show the changes in enzyme samples as reflected in the specific activity profiles.

The trial procedure illustrated in Figs. 1A and 1C, where Bio-Gel A-5m was used for both gel filtration steps, and where intermediate concentration was performed by ultrafiltration over an Amicon PM10 membrane, gave 1.5 mg of purified dextransucrase with a specific activity of approximately 100 U/mg protein and an overall yield just under 30%. It may be possible to increase this yield by using PEG 400 precipitation instead of ultrafiltration, and by adding detergent [237] or extra calcium [238] to the eluent as enzyme stabilizers. Gel filtration yields for streptococcal glucansucrases have been improved by adding detergent and by performing the chromatography at high ionic strength [97, 258]. The addition of nonionic detergent also prevents most of the loss of activity that otherwise follows dextranase treatment [237].

A factor that contributed to the carbohydrate content of enzyme purified as in Fig. 1C was overlap at the first gel filtration step of the dextransucrase peak and the second carbohydrate peak (Fig. 1A). One reason for this overlap was the high molecular weight of the leading edge of the carbohydrate peak, but more important was a decrease in the average molecular weight of the dextran-dextransucrase complex, caused by aging of the crude enzyme. Freshly elaborated dextransucrase migrates in the void volume of a Bio-Gel A-50m column (nominal exclusion limit $5 \times 10^6$ Da) [286]. It is convenient, however, to store the enzyme for fairly long periods as a dialyzed concentrate ("crude enzyme"), since in this form the activity is stable indefinitely (years) at $4^\circ$ in the pres-
Figure 2. Specific activity of dextranucrase on Bio-Gel A-5m

Protein (—Δ—) and dextranucrase activity (—○—) curves from Fig. 1 (left panels) are replotted here, with specific activity (□) shown for the active fractions. Panels (A), (B), and (C) correspond to the same panels of Fig. 1.
ence of 0.2 mg/ml sodium azide. After a few months of storage, most of the activity no longer migrates in the Bio-Gel A-50m void volume (Fig. 3A), possibly because of the presence of a minute amount of endogenous endodextranase [163, 336].

The molecular weight of long-stored dextranucrase could be restored to high values by incubating the crude enzyme with sucrose, resulting in baseline separation on Bio-Gel A-50m from the heterogeneous polysaccharide of lower molecular weight (Fig. 3B). The method of incubation was critical. It is well-documented that low sucrose [30, 337] and acceptor [343] concentrations cause dextran of high molecular weight to be synthesized. The low sucrose and acceptor fructose concentrations present during dialysis of the crude enzyme against a buffered sucrose solution gave a dextran–dextranucrase complex of much higher molecular weight than when the enzyme was mixed directly with sucrose (Fig. 3C). Dextranase treatment of the pooled, active fractions from a Bio-Gel A-50m column run with a sample prepared by dialysis against sucrose resulted in dextranucrase preparations with less carbohydrate than protein.

Poly(ethylene glycol) Precipitation

A drawback to the use of consecutive gel filtration steps is sample dilution, a problem magnified by the relatively small amounts of protein that can be loaded onto the gel. Concentrating dextranucrase over an ultrafiltration membrane usually causes a large loss of activity. Concentration by poly(ethylene glycol) precipitation was tested as an alternative.

A fractional precipitation curve with PEG 400 is shown in Fig. 4 for dextranucrase eluted at the void volume of a Bio-Gel A-50m column. PEG 400 was chosen because it exhibits more selectivity in protein fractionations than poly(ethylene glycol)s of higher molecular weight [145, 292]. It has been claimed [145] that fractional precipitation with PEG 400 is comparable to gel filtration.

There are several features to note about the curve in Fig. 4. Most
Figure 3. Gel filtration chromatography of dextranucrase on Bio-Gel A-50m (1.8 x 60 cm column)

(A) A tenfold dilution of crude enzyme (2 ml) after 4 months of storage at 4°; (B) Same as (A), but after enzyme was dialyzed against buffered 10 mM sucrose at 4° for 22 h;
(C) A tenfold dilution of crude enzyme (6 ml), either dialyzed 5 h against buffered 10 mM sucrose at room temperature (-----) or made by mixing undiluted crude enzyme directly with 9 volumes of buffered 150 mM sucrose and allowing to react for 1 h at room temperature before chromatography (——).

(A) and (B): ●●●, carbohydrate; ———, dextranucrase activity.
importantly, activity is precipitated and re-solubilized quantitatively at PEG 400 concentrations of 26% (w/v) and higher. The high yield is probably related to the presence of dextran. Côté and Robyt [65] also obtained 100% recovery of activity for the precipitation of the glucansucrases of *L. mesenteroides* NRRL B-1355, which had high dextran content, with several sizes of poly(ethylene glycol). Fig. 5, on the other hand, shows that precipitation with PEG 6,000 results in considerable loss of activity from B-512F dextransucrase purified by DEAE-cellulose chromatography, a preparation with low polysaccharide content (50% or less of the protein by weight: see Part II of this dissertation), even at PEG 6,000 concentrations that do not fully precipitate the enzyme. (The experiment shown in Fig. 5 was performed to see if poly(ethylene glycol) precipitation would further reduce the amount of carbohydrate. Because of the overall loss of activity, the relative carbohydrate and protein concentrations of the pellet were never determined.)

A comparison of Figs. 4 and 5 with poly(ethylene glycol) precipitation curves published for glucansucrases from two other bacteria [65, 292] emphasizes the point that for preparations with a high dextran content, precipitation is complete at much lower poly(ethylene glycol) concentrations than for preparations with a low dextran content. The precipitation curve for PEG 400 shown in Fig. 4 closely matches the PEG 400 precipitation curve published for B-1355 glucansucrases by Côté and Robyt [65]. For PEG 6,000, Côté and Robyt [65] found complete precipitation of activity at 5–6% PEG (w/v). This contrasts with the 75% precipitation of activity at 20% (w/v) PEG 6,000 found for the low-dextran B-512F preparation in Fig. 5, and with the roughly 65% precipitation of activity by 50% (w/v) PEG 400 or by 25% (w/v) PEG 6,000 found by Russell [292] for the glucansucrases of *S. mutans* 3209, another preparation with low polysaccharide content. Two other useful features of poly(ethylene glycol) precipitation of dextransucrase with high dextran content are its speed (see legend to Fig. 4) and that it can be done at room temperature.

These observations above are in accord with studies showing that precipitation by poly(ethylene glycol) is promoted by high particle
Figure 4. Precipitation of partially purified dextranucrase by poly(ethylene glycol)

To 1.0 ml samples of the pooled void-volume fractions from the column of Fig. 4B (Nos. 27-41) were added various weights of PEG 400 (a liquid at room temperature). After 90 min at room temperature, the mixtures were centrifuged briefly in a Beckman Microfuge. The pellets were dissolved in buffer and assayed.
Dextranucrase purified by DEAE-cellulose chromatography (see Part II of this dissertation) (4.0 ml, containing 0.5 M NaCl, >1 mg/ml Tween 80, 2 mM CaCl₂, 0.2 mg/ml NaN₃, 20 mM imidazole (pH 6.7), and 350 U/ml dextranucrase) was mixed with an equal volume of unbuffered PEG 6,000 solution (in water at twice final concentration) and refrigerated for 15 h. Precipitates were pelleted in a clinical centrifuge and dissolved in 2.0 ml of the buffer just described, but containing only 1 mg/ml Tween 80. Assays of the supernatants and the dissolved pellets were at pH 5.2.
weight [232, 233] and by the presence of dextran or other polymers with which poly(ethylene glycol) has a thermodynamically-driven tendency to form a two-phase system [103].

Proposed Purification Procedure

The purification steps described above were never fully integrated into a single procedure, because they were superceded by a procedure giving a much lower carbohydrate content (see Part II of this dissertation). In practice, however, that other procedure was complex, and required experience to give high yields. Based on these results, a simpler method can be proposed to give a dextran-secrase preparation with less carbohydrate than protein and with a high specific activity (>100 U/mg protein).

The first step of this procedure would be dialysis of crude enzyme against a sucrose solution, to raise the effective molecular weight of the enzyme, in the form of a dextran-dextranase complex, to a very high value. The second step would be gel filtration of this complex on Bio-Gel A-50m, optionally followed by a concentration step of precipitating the enzyme with PEG 400. Next, the void-volume fraction from the Bio-Gel A-50m column would be treated with dextranase, and finally chromatographed on a Bio-Gel A-5m column.

This method would retain the simplicity of gel filtration chromatography while exploiting the association of dextran with dextranase. Normally considered detrimental, this association gives the enzyme stability [237] and high molecular weight [286]. The carbohydrate in the void-volume fraction from a Bio-Gel A-50m column consists almost entirely of dextran, which on dialysis in the presence of dextranase gives a dialysate with same or slightly less carbohydrate than protein. The final step proposed, chromatography on Bio-Gel A-5m, is intended to separate the dextranase from dextranase and from any remaining polysaccharide of high molecular weight. Even without this final step, the preparation had a carbohydrate content much lower than given by nearly
all other published purification methods (e.g., ref. 286), and as low as much more laborious and low-yield methods (e.g., ref. 188).
PART IV

ACTIVATION AND INHIBITION OF DEXTRANSUCRASE BY CALCIUM
Ca\textsuperscript{2+} is known in some cases to increase the rate of glucan synthesis by dextran sucrase (sucrose: 1,6-\(\alpha\)-D-glucan 6-\(\alpha\)-D-glucosyltransferase, EC 2.4.1.5) and related glucosyltransferases, and to increase the proportion of \(\alpha\)-(1\(\rightarrow\)3) branch linkages in the glucans synthesized. In this study, the effects of Ca\textsuperscript{2+} on dextran sucrase from \textit{L. mesenteroides} NRRL B-512F were investigated. Initial rate kinetics of polymer formation showed mixed-type nonessential activation by Ca\textsuperscript{2+} (i.e., Ca\textsuperscript{2+} increased \(V_{\text{max}}\) and decreased the \(K_m\) for sucrose, but was not required for polysaccharide synthesis) at concentrations below 1 mM, and linear competitive inhibition above 1 mM. These results suggest the existence of two kinetic pathways for glucan synthesis: one independent of Ca\textsuperscript{2+} and dominating in its absence, and the other considerably promoted by Ca\textsuperscript{2+}. Branching and acceptor reactions, as well as overall polymer production, occurred at appreciable rates at Ca\textsuperscript{2+} concentrations below 0.1 \(\mu\)M. Reactivation of the enzyme in the presence of EDTA after SDS gel electrophoresis shows that refolding to an active conformation can also take place at Ca\textsuperscript{2+} concentrations of no more than a few nanomolar. The effects of Ca\textsuperscript{2+} on this enzyme parallel some of the effects of exogenous dextran on other glucosyltransferases.
INTRODUCTION

Dextranucrase (sucrose: 1,6-α-D-glucan 6-α-D-glucosyltransferase, EC 2.4.1.5) is one of a family of glucosyltransferases and fructosyltransferases, also called glucansucrases and fructansucrases, elaborated by species of *Leuconostoc* and *Streptococcus* [312, 343]. Dextranucrase polymerizes the glucosyl moiety of sucrose to form dextran, an α-(1→6)-linked glucan with α-linked branches [343]. The single enzyme can make both main chain and branch linkages [280, 284].

Intuitively, the dextranucrases of *Leuconostoc* and *Streptococcus*, which are closely related genera, should be nearly identical in structure and mechanism. Several unexplained differences exist, however, among which are activation and stabilization by Ca$^{2+}$ of the *Leuconostoc* enzymes [158, 172, 188, 189, 205, 212, 237, 249, 286], but not of the *Streptococcus* enzymes [44, 50, 150, 255, 301]. The glucansucrases of both genera are known to be inhibited by a wide variety of other metal ions [188, 255, 286, 299, 339]. As a step towards understanding this metal ion behavior, we have investigated the effects of Ca$^{2+}$ on the dextranucrase from *L. mesenteroides* NRRL B-512F, primarily as they relate to the rate of dextran synthesis. Other Ca$^{2+}$ effects examined were those on the formation of glucose and acceptor products, on dextran structure, and on enzyme reactivation after SDS gel electrophoresis.
MATERIALS AND METHODS

Materials

Enzyme purification has been described elsewhere [26, 237]. Crude enzyme, a dialyzed concentrate of culture supernatant [237], had 98 U glycansucrase/mg protein, 0.02 U levansucrase/U dextran sucrase, and 77 mg/carbohydrate/mg protein. Purified enzyme [26] had 170 U dextran sucrase/mg protein, with no detectable carbohydrate (less than 0.7 μg/mg protein) or levansucrase (less than 0.01 U/U dextran sucrase). *Penicilium funiculosum* dextranase (Grade I, chromatographically purified) was from Sigma Chemical Co. (St. Louis, MO). Dextran T10 was from Pharmacia Fine Chemicals (Piscataway, NJ). [U-14C]sucrose was purified by paper chromatography (two ascents at 85° in 1-butanol/pyridine/water (6:1:1, v/v/v) on Whatman 3MM paper) before dilution with carrier sucrose. Iso-malto-oligosaccharide standards were prepared by partial acid hydrolysis of dextran. The EDTA used was the commercial disodium salt.

Enzyme Assays

Assays were conducted at 25° and pH 5.0 for crude enzyme (pH 5.2 for purified enzyme), and were buffered with 0.025–0.05 M sodium acetate, except for the experiment of Fig. 5, where calcium acetate was a significant additional buffer. Assays followed the incorporation of 14C from [U-14C]sucrose into methanol-insoluble polysaccharide [118], measuring the sum of dextran sucrase and levansucrase activities. Dextran sucrase activity was taken to be equal to this sum. This approximation had no effect on the numerical analysis: values in Table I were essentially unchanged by any assumptions about the kinetic behavior of a 2% levansucrase impurity, the highest amount ever present.

One unit of dextran sucrase is defined as the amount of enzyme that will incorporate 1 μmol of glucose into polysaccharide from 0.15 M sucrose in one minute. Initial rates of polymer formation were linear with
both crude and purified enzyme, and progress curves were linear under all conditions. Assays with purified enzyme contained 0.1–0.2 mg/ml Tween 80 and 10 mg/ml dextran T10 as stabilizers [237]. Denaturation was slight but usually detectable for crude enzyme, which was assayed in the absence of Tween 80. Corrections were made for this denaturation by means of a timed series of replicate assays [5].

\[ \text{Ca}^{2+} \text{ Concentrations} \]

Free $\text{Ca}^{2+}$ concentration ($[\text{Ca}^{2+}]_f$) in the presence of EDTA (a stronger chelator of $\text{Ca}^{2+}$ at pH 5 than EGTA) was calculated as described by Portzehl et al. [271], using stability constants from Martell and Smith [222] (pp. 75–76) and from ref. 49 (p. 624). Binding of $\text{Ca}^{2+}$ by EDTA$^{4-}$, EDTA$^{3-}$, and acetate (the last significant only in the absence of EDTA) were taken into account. Only 1:1 $\text{Ca}^{2+}$-ligand complexes were considered. It was assumed that uncontrolled sources of $\text{Ca}^{2+}$ (buffer salts, distilled-deionized water, glassware, etc.) contributed 10 ± 5 μM to total $\text{Ca}^{2+}$ concentration (Carter [45], pp. 61 and 103). No special precautions were taken to reduce this contribution, such as passing buffers through chelating resins or boiling glassware in EDTA solutions.

Dextran is known to bind divalent metal ions, and especially $\text{Ca}^{2+}$ [164]. The addition of dextran T10 to assays of crude enzyme performed at low $[\text{Ca}^{2+}]_f$ did not change initial velocities, so dextran was concluded not to be a significant source of $[\text{Ca}^{2+}]_f$ here.

*Abbreviations:

$[\text{Ca}^{2+}]_t$, total $\text{Ca}^{2+}$ concentration;

$[\text{Ca}^{2+}]_f$ and $[\text{EDTA}]_f$, free $\text{Ca}^{2+}$ and EDTA concentrations.
Kinetic Data Analysis

Kinetic parameters were estimated by fitting initial rate data to specific rate equations by weighted linear or nonlinear regression, using iteratively determined weights [220]. Where only part of the data for an experiment was fit, as in Fig. 5, weights were instead calculated for each point from a small number of replicate assays.

For linear regression, standard statistical tests were used for hypothesis-testing. For nonlinear regression, models were judged for reasonableness as described by Draper and Smith [75] (p. 282). Because of the statistical uncertainties associated with nonlinear regression, the numerical data by which models were compared have been presented as two lines of Table I (MS_R and MS_E). The best model was taken to be the one with the smallest residual mean square [219].

Dextran Synthesis and Purification

To 20 ml of 0.1 M sucrose in 0.025 M sodium acetate (pH 5.3) containing 0.1 mg/ml Tween 80, 0.2 mg/ml sodium azide, and either 1 mM CaCl_2 or 15 mM EDTA, 0.1 ml purified enzyme (5-6 U) was added at 0, 1.5, and 3 h. [Ca^{2+}]_f in the presence of EDTA was estimated as 0.04 µM. After 16 h at room temperature (21-23°), the enzyme solutions were diluted to 150 ml with water, and the polysaccharide was precipitated twice with 2 volumes ethanol, dehydrated under absolute ethanol, and dried under vacuum at 40°. By dry weight, yields of polysaccharide were 85% and 94% of theoretical for the dextrans made in the presence and absence of EDTA, respectively. Thin-layer chromatography of the ethanolic supernatants showed unreacted sucrose in the presence of EDTA, but none in its absence. The isolated dextrans, at 10 mg/ml in pH 5.0 solution, were hydrolyzed by dextranase over several days at room temperature, and the hydrolysis products were analyzed by thin-layer chromatography.
Thin-Layer Chromatography

As described by Côté and Robyt [66], multiple ascents were performed at 37° on Whatman K5 0.25 mm silica gel plates. Solvents are given in the figure legends. Carbohydrate was visualized by heating 10-20 min at 100-110° after spraying with methanol containing 20% (v/v) sulfuric acid [26], or for greater sensitivity, containing 3% (v/v) sulfuric acid and 2 g/l N-(1-naphthyl)ethylenediamine dihydrochloride [28].

Electrophoresis

SDS gel electrophoresis was performed by the method of Laemmli [203] on 5 x 90 mm rod gels (6% acrylamide). Protein was stained with Coomassie Blue G-250 [144]. Dextranucrase and levansucrase activity were detected by a periodic acid-Schiff stain [173] after reactivating the enzymes by overnight incubation with pH 5.2 buffer containing 10 mg/ml Tween 80 and 2 mM CaCl₂, incubating the gels in the same buffer with 50 mg/ml sucrose or raffinose, and washing out monosaccharides and unreacted sucrose in 10% (v/v) acetic acid, 25% (v/v) 2-propanol.
RESULTS

Initial Rate Kinetics

Ca\(^{2+}\) gave linear competitive inhibition at high concentration (Fig. 1), with \(K_i = 59 \pm 6 \text{ mM Ca}^{2+}\) (74 \pm 8\text{ mM if the binding of Ca}^{2+}\) to acetate is ignored). Because of concern about the variable ionic strength in this experiment, the effect of increasing ionic strength was examined with a different salt as well (NaCl). Fig. 2 shows that at 0.15 M sucrose, there was only about a 5% reduction in initial velocity at 0.18 M NaCl, which is equivalent to the highest ionic strength used in Fig. 1. Competitive inhibition at lower ionic strength by more strongly inhibitory metal ions, such as Zn\(^{2+}\) (data not shown), supported the conclusions that competitive inhibition by Ca\(^{2+}\) was linear and unrelated to ionic strength.

Ca\(^{2+}\) below 1 mM activated the enzyme (Figs. 3–4). (It has been previously established [158, 188, 205, 286, 339] that depletion of Ca\(^{2+}\) is the cause of EDTA inhibition.) At low [Ca\(^{2+}\)]\(_t\) (Fig. 3), assay of polysaccharide formation (used throughout this study) gave results identical to those of Neely and Hallmark [249], who used a reducing value assay. In particular, inhibition of crude dextran sucrase by EDTA was confirmed as being hyperbolic. This type of inhibition, also called partial inhibition, shows that polysaccharide is synthesized in the complete absence of [Ca\(^{2+}\)]\(_t\). By collecting data mostly near the 1/v-axis in Fig. 3, it was determined (see first two columns of Table I) that the inhibition was not competitive, as concluded by Neely and Hallmark, but mixed-type; i.e., that both slope and 1/v-intercept increased with EDTA.

For better characterization of the change in 1/v-intercept, the experiment of Fig. 3 was repeated at higher [Ca\(^{2+}\)]\(_t\) and with more EDTA concentrations (Figs. 4–6). Because double-reciprocal plots at 0 and 10 mM EDTA were linear between 10 and 100 mM sucrose for the purified enzyme, only these two sucrose concentrations were used (Fig. 4), to
Figure 1. Competitive inhibition of crude dextransucrase by Ca$^{2+}$

Vertical bars denote the S.E. of the mean for six determinations. (Half are omitted for clarity.)
Figure 2. Inhibition of crude dextranucrase by high ionic strength

Vertical bars denote the S.E. of the mean for three determinations. Reactions contained 1 mM Ca\(^{2+}\) and 0.15 M sucrose.
Figure 3. Hyperbolic mixed-type inhibition of crude dextranucrase by EDTA

Each point is the average of 3 determinations. The lines show the fit (third column of Table I) to eqn. 1.

Inset: slopes of the double-reciprocal plots as determined by separate linear regressions for each EDTA concentration.
estimate kinetic parameters most precisely from a given number of assays [90].

Slope and intercept replots from Fig. 4 were not linear (Fig. 5). Information about this nonlinearity was supplied by secondary replots (Fig. 6); specifically, the linearity of the secondary replots showed that the primary replots (Fig. 5) were segments of rectangular hyperbolas having asymptotes parallel or nearly parallel to the axes. The continued flattening of the slope replot at even lower \([\text{Ca}^{2+}]_f\) (Fig. 3 inset) is additional evidence for a horizontal asymptote.

Modifying the Michaelis-Menten equation to give this behavior (hyperbolic slope and intercept replots with horizontal and vertical asymptotes) results in eqn. 1 (Segel [307], pp. 833-841), where \(K_1-K_4\) are apparent dissociation constants. For an enzyme obeying eqn. 1, increasing \([\text{Ca}^{2+}]_f\) from zero to saturation increases the apparent \(V_{\text{max}}\) by a factor of \(K_3/K_4\) (1.8-fold for Fig. 4) and reduces the apparent \(K_m\) by a factor of \(K_1 K_4/K_2 K_3\) (from 70 to 15 mM for Fig. 4, or 4.8-fold).

\[
v = \frac{V_{\text{max}} x [\text{Sucrose}]}{\frac{1 + [\text{Ca}^{2+}]_f/K_1}{1 + [\text{Ca}^{2+}]_f/K_2} \times K_m + \frac{1 + [\text{Ca}^{2+}]_f/K_3}{1 + [\text{Ca}^{2+}]_f/K_4} \times [\text{Sucrose}]}
\]  

A complete description of the \(\text{Ca}^{2+}\) kinetics requires that eqn. 1 be modified to include \(\text{Ca}^{2+}\) inhibition, resulting in eqn. 2 [307]. Because \(K_1\) was very high (59 mM), estimates of the other constants were the same with either equation.

\[
v = \frac{V_{\text{max}} x [\text{Sucrose}]}{\frac{1 + [\text{Ca}^{2+}]_f/K_1 + [\text{Ca}^{2+}]_f^2/K_1 K_3}{1 + [\text{Ca}^{2+}]_f/K_2} \times K_m + \frac{1 + [\text{Ca}^{2+}]_f/K_3}{1 + [\text{Ca}^{2+}]_f/K_4} \times [\text{Sucrose}]}
\]  

(2)
Figure 4. Hyperbolic mixed-type inhibition of purified dextranucrase by EDTA.

The same data are plotted in both (A) and (B). Vertical bars denote S.E. of the mean for four determinations. The lines in (A) and curves in (B) show the fit (last column of Table I) to eqn. 1.
Figure 5. Nonlinearity of slope and intercept replots for Fig. 4A

Slopes (○) and intercepts (●) were determined by separate linear regressions for each EDTA concentration. Vertical bars denote the S.D.
Figure 6. Apparent linearity of \(1/\Delta\) slope (○) and \(1/\Delta\) intercept (●) plots derived from Fig. 4A.

(\(\Delta\) slope and \(\Delta\) intercept are defined as difference from the slope and intercept values in the absence of EDTA.) Vertical bars denote the S.D.
The numerical analysis of the initial rate experiments at low $[\text{Ca}^{2+}]_f$ is summarized in Table I. Residuals for all fits showed that eqns. 1 and 2 were missing additional terms with very small coefficients, such as could arise from substrate inhibition, product inhibition, the presence of multiple activities, or cooperativity. The data were not sufficiently precise to obtain meaningful numerical estimates of these coefficients.

Values cited for $[\text{Ca}^{2+}]_f$ may differ from the true values by several-fold, because of uncertainty in the apparent stability constants of the $\text{Ca}^{2+}$-ligand complexes [132]. This source of error is probably much greater than that caused by error in $[\text{Ca}^{2+}]_t$, and is why Figs. 5 and 6 are in terms of $[\text{EDTA}]_f$, which is more accurately known than $[\text{Ca}^{2+}]_f$, and which gives plots more easily tested for linearity. Because of the uncertainty in $[\text{Ca}^{2+}]_f$, it was not considered useful to determine $[\text{Ca}^{2+}]_t$ with great accuracy, and the differences among the experiments in Table I in the absolute values of the apparent kinetic constants is of questionable significance.

Kinetic Mechanism

Mechanistic interpretation of the initial rate data was not straightforward. This is best seen from Fig. 6. Using the fact that $[\text{Ca}^{2+}]_f$ is inversely proportional to $[\text{EDTA}]_f$, it can be shown (Segel [307], pp. 161-272) that the $1/[\text{EDTA}]_f$-axis intercept is proportional to $K_2$ for the $1/A$ slope plot and to $K_4$ for the $1/A$ intercept plot. For a rapid equilibrium model of mixed-type nonessential activation (Fig. 7), the two intercepts are the same ($K_2 = K_4$). This is clearly not the case in Fig. 6, where the difference in intercepts is significant at $P < 0.005$. The last two columns of Table I also show that imposing the condition $K_2 = K_4$ gives an inferior fit for this experiment. The less precisely determined intercept variation for the other two experiments in Table I did not allow a conclusion about whether $K_2 = K_4$; e.g., the second and third columns in Table I show that neither model is clearly
Table I. Comparison of kinetic models of dextranucrase activation by calcium

<table>
<thead>
<tr>
<th>Parameter or Statistic</th>
<th>Model (type of activation)</th>
<th>Expt. 1</th>
<th>Expt. 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hyperbolic competitive ⁰</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hyperbolic mixed-type</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rapid equilibrium ¹</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>Non-rapid equilibrium ²</td>
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<tr>
<td></td>
<td>Hyperbolic mixed-type</td>
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<tr>
<td></td>
<td>Rapid equilibrium ¹</td>
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</tr>
<tr>
<td></td>
<td>Non-rapid equilibrium ²</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M₅₀₉</td>
<td>250. (92)</td>
<td>184. (91)</td>
<td>185. (90)</td>
</tr>
<tr>
<td>M₅₀₉</td>
<td>197. (64)</td>
<td>197. (64)</td>
<td>197. (64)</td>
</tr>
<tr>
<td>Kₚ (µM)</td>
<td>96. ± 3.</td>
<td>67. ± 4.</td>
<td>64. ± 6.</td>
</tr>
<tr>
<td>K₁ (µM)</td>
<td>19. ± 3.</td>
<td>14. ± 2.</td>
<td>15. ± 3.</td>
</tr>
<tr>
<td>K₂ (µM)</td>
<td>1.11 ± 0.08</td>
<td>1.2 ± 0.2</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td>K₃ (µM)</td>
<td></td>
<td>1.6 ± 0.1</td>
<td>1.0 ± 0.7</td>
</tr>
<tr>
<td>K₄ (µM)</td>
<td></td>
<td>1.2 ± 0.8</td>
<td>0.7 ± 0.5</td>
</tr>
<tr>
<td>Vₘₐₓ (cpm/min)</td>
<td>720 ± 10</td>
<td>560 ± 20</td>
<td>550 ± 40</td>
</tr>
</tbody>
</table>

For each experiment, [ZD7A] was varied from 0 to 10 mM at constant [Ca²⁺]ₜ. Initial velocities were fit to different models, produced by forcing different pairs of constant eqn. 1 to be equal. The best model for a given experiment (see Methods) is the one with the smallest residual mean square (MS₉). Apparent kinetic constants are given ± asymptotic standard errors. The corresponding degrees of freedom are listed in parentheses.

Crude enzyme, [Ca²⁺]ₜ = 25 µM; data shown in Fig. 3.
Crude enzyme, [Ca²⁺]ₜ = 150 µM; data not shown.
Purified enzyme, [Ca²⁺]ₜ = 130 µM; data shown in Fig. 4.
Eqn. 1 when K₃ = K₄.
Eqn. 1 when K₂ = K₄.
Weighted residual mean square. The corresponding degrees of freedom are listed in parentheses.
Weighted mean square for pure error calculated from replicate assays. The corresponding degrees of freedom are listed in parentheses.
Regression failed to converge with this model for Expt. 2, because experimental errors too large to estimate K₃ or K₄, and consequently K₅ or Vₘₐₓ with reasonable precision.
Not determinable; cancels out of rate equation.
E is enzyme, S is sucrose, $k_p$ and $\beta k_p$ are catalytic rate constants, and the remaining symbols represent dissociation constants. Eqn. 1 becomes the rate equation for this mechanism when the equalities listed beneath the binding scheme hold.
The deviation just described from a rapid equilibrium model of mixed-type nonessential activation could not be eliminated by any assumptions about errors in the calculation of $[\text{Ca}^{2+}]_f$, because the double-reciprocal plots in Fig. 4 did not intersect at a common point (not obvious except at relatively high $[\text{Ca}^{2+}]_f$). This occurs for eqn. 1 only when $K_2$ is not the same as $K_4$, and does not depend on knowing the true values of $[\text{Ca}^{2+}]_f$.

Hyperbolic and mixed-type inhibition, as well as failure of the double-reciprocal plots to intersect at a common point, would be caused by the presence of two glucansucrase activities, one strongly inhibited by EDTA and the other weakly or not at all inhibited. Double-reciprocal plots would be concave-downward, although the curvature might be difficult to detect [307]. Residual plots (not shown) for the fit illustrated in Fig. 3 showed that the plots were concave-downward, but to a degree too small to draw useful conclusions. More importantly, two dextransucrase species were in fact found, one at 159 kDa and one at 176 kDa (Fig. 8B).

It has not been determined whether both dextransucrase forms are equally inhibited by EDTA. However, by comparing the intensity of the major levansucrase band in gel A with the intensities of the dextransucrase bands in gel B, and by assuming that levansucrase represents about the same fraction of the total activity on gel A as it did in the applied sample (2%), it can be concluded that EDTA does not dramatically inhibit either of the two dextransucrase species. This contrasts with levansucrase (the polysaccharide-forming bands at 93 kDa and 119 kDa in Fig. 8A), which apparently either is inhibited more strongly by EDTA or renatures less well in its presence.

The fraction of dextransucrase in the 159 kDa form increased with the age of the preparation at the expense of the 176 kDa form, a pattern also found for different molecular-weight forms of $S. \text{sanguis}$ dextransucrase [123] and $S. \text{mutans}$ glucansucrases [180, 294]. The crude enzyme used for Table I was mostly in the 159 kDa form, while the purified en-
Figure 8. Effect of EDTA on reactivation of dextranucrase and levansucrase following SDS gel electrophoresis

An equal amount of culture supernatant (40 μg protein) was applied to each gel. The positions and molecular weights of the two dextranucrase (dm) and the two levansucrase (ls) species are marked.

**Left:** Gels A-D were stained for glycansucrase activity and gel E for protein. Gel A; 2 mM Ca$^{2+}$ and no EDTA during both reactivation and 2-h incubation with sucrose. Gel B; 10 mM EDTA and 10 μM Ca$^{2+}$ ($[Ca^{2+}]_f = 0.02$ μM) during reactivation and 2-h incubation with sucrose. Gel C; 2 mM Ca$^{2+}$ and no EDTA during reactivation and 9-h incubation with raffinose (a substrate for levansucrase but not for dextranucrase [43]). Gel D; no exposure to substrate (stain control). If SDS was not diluted by diffusion or displaced by Tween 80, no polysaccharide formed on overnight incubation with sucrose.

**Right:** The two molecular-weight forms of dextranucrase. The top portions of gels A-C are shown after being re-photographed to reduce band intensity, revealing detail in the dextranucrase region.
zyme was approximately half in each form; yet the decrease in intercept with increasing \([Ca^{2+}]_f\) (numerically defined by the ratio of \(K_2\) to \(K_3\) in the columns of Table I where \(K_2 = K_4\)) was the same for both preparations. It is therefore unlikely that the presence of the two molecular-weight forms of dextransucrase fully accounts for the mixed-type inhibition by EDTA.

**Enzyme Products**

Thin-layer chromatography (not shown) of the reaction products at both 10 mM and 100 mM sucrose under the reaction conditions used for Fig. 4 showed only a small amount of glucose and acceptor products produced by the time that sucrose had disappeared. No difference of any kind could be seen between reactions performed in the presence and absence of EDTA at either sucrose concentration. Fig. 9 shows that the effect on acceptor reactions with added glucose was also small.

The products observed in these experiments, where sucrose was completely consumed, had higher amounts of low-molecular-weight products than those made under initial velocity conditions. This was because disproportionation reactions catalyzed by dextransucrase \([26]\) continue to take place after the exhaustion of sucrose. In agreement with the expectation that disproportionation reactions should be promoted by a high ratio of enzyme to disproportionation substrates, the fraction of acceptor products for 10 mM sucrose was slightly larger than for 100 mM sucrose.

Dextrins were synthesized from purified enzyme in the presence and absence of EDTA, and were isolated by precipitation with ethanol. The dextran made in the presence of EDTA precipitated at lower ethanol concentration than the dextran made in the absence of EDTA, and formed much more viscous solutions at a given weight concentration. During dehydration under absolute ethanol, the dextran made in the presence of EDTA was an easily worked, finely granular material. This contrasted with the dextran made in the absence of EDTA, which was the usual sticky gum, dif-
Figure 9. Effect of EDTA on acceptor reactions with glucose

Reactions were conducted with purified enzyme at 30 μM Ca$^{2+}$ in the combined presence of 1.5 M sucrose and 1.5 M glucose. The plate was given 1 ascent in 1-propanol/nitromethane/acetonitrile/water (5:2:4:4, v/v/v/v). Lane A; reaction containing no EDTA. Lane B; isomalto-oligosaccharide standards. Lane C; reaction containing 27 mM EDTA ([Ca$^{2+}]_f = 0.02$ μM). Compound designations: glc, glucose; fruc, fructose; im, im$_3$, etc., isomaltose, isomaltotriose, etc.
icult to manipulate. The dextran made in the presence of EDTA dried to a much less dense solid and dissolved in water more easily than the dextran made in the absence of EDTA. Removal of Ca$^{2+}$ from the dextran made in the absence of EDTA (by autoclaving a solution of it in the presence of EDTA and by subsequent re-precipitation) did not change these physical properties; i.e., did not make them more like those of the dextran made in the presence of EDTA.

While these differences in physical properties suggested that the dextran made in the presence of EDTA was less branched than the dextran made in its absence, thin-layer chromatography of the endodextranase hydrolysis products of the two dextrans (Fig. 10) showed at most a slight reduction in the degree of branching for the dextran made in the presence of EDTA. $^{13}$C-n.m.r. of the two dextrans (Fig. 11) was uninformative about structural differences, because the degree of branching for both was too small to see a peak from the $\alpha$-(1-3) linkage.
Figure 10. Effect of EDTA on dextran structure (branching)

Dextrans synthesized from purified enzyme were hydrolyzed by endodextranase. Equal weights of hydrolysis products were spotted on the thin-layer plate shown, which was given 2 ascents in 1-propanol/nitromethane/acetonitrile/water (5:2:4:4, v/v/v/v). Lane A; hydrolysis products of dextran synthesized in the presence of 1 mM Ca$^{2+}$ and absence of EDTA. Lane B; isomalto-oligosaccharide standards. Lane C; hydrolysis products of dextran synthesized in the presence of 15 mM EDTA and 30 μM Ca$^{2+}$ ([Ca$^{2+}$]$_f$ = 0.04 μM). Compound designations: $b_4$, $b_5$, $b_6$, branched limit dextrans containing four, five, and six glucose residues; other designations as in Fig. 9.
$\text{glc}$

$\text{im}$

$\text{im}_3$

$\text{im}_4$

$\text{im}_5$

$\text{im}_6$

A  B  C
Figure 11. $^{13}$C-n.m.r of dextran made by purified enzyme in the presence (B) and absence (A) of EDTA

The $^1$H-decoupled spectrum of 30 mg/ml polysaccharide was measured at 80°. The leftmost peak, labeled 'C', is due to the α-(1→6) anomeric linkage.
DISCUSSION

Ca\(^{2+}\) has been reported to activate and to stabilize dextran sucrases in three strains of *Leuconostoc mesenteroides* (NRRL B-512F [172, 188, 205, 212, 237, 249, 286], NRRL B-1299 [186], and IAM 1046 [158, 339]). Ca\(^{2+}\) has been reported both to activate [242] and not to activate [50, 286] streptococcal glucansucrases. While the effect on stability found for the *Leuconostoc* enzymes has not been explained, it has been suggested [249] that the activation occurs through enhancement of sucrose binding. The experiments in this study (Figs. 3-4, Table I) show that an increase in V\(_{\text{max}}\) also takes place.

Above 1 mM, Ca\(^{2+}\) inhibited dextran sucrase competitively (Fig. 1). This has not been observed previously, and suggests that other inhibitory metal ions may also inhibit competitively. By contrast, Ca\(^{2+}\) below 1 mM activated the enzyme. The kinetics of activation was complex, but similar to rapid equilibrium mixed-type nonessential activation (Fig. 7), in which the binding of activator increases V\(_{\text{max}}\) by some factor \(B\) and decreases K\(_{m}\) by some factor 1/\(\alpha\). For the dextran sucrase studied here, V\(_{\text{max}}\) was nearly doubled at maximum activation (\(B = 1.8\)), and the K\(_{m}\) for sucrose was lowered from 70 mM to 15 mM (\(\alpha = 4.8\)).

The kinetics of activation by Ca\(^{2+}\) deviated slightly but significantly from rapid equilibrium mixed-type nonessential activation. Reasons for the deviation might be non-rapid equilibrium kinetics, the binding of more than one equivalent of sucrose or Ca\(^{2+}\), or the presence of multiple enzymes with different kinetics. Of several artifacts (change in product ratios, use of an assay measuring polysaccharide synthesis only) that might have caused the deviation, or otherwise have caused simple underlying kinetics to appear complex, only the presence of multiple enzymes (two forms of dextran sucrase with different molecular weight) could be demonstrated. Results do not exclude the possibility that these forms have different Ca\(^{2+}\) kinetics, but make it unlikely (Fig. 8B) that either has an absolute requirement for Ca\(^{2+}\).

The mechanism of activation remains unknown. Nonessential activa-
tion is a sign of the operation of alternative kinetic pathways for product formation (Segel [307], pp. 833-841). To test the possibility that these might be pathways to alternative products, rather than alternative pathways to the same products, the effect of EDTA on the identity and distribution of the reaction products was investigated. EDTA sufficient to reduce $[\text{Ca}^{2+}]_f$ to less than 0.1 mM had little effect on dextran branching (Fig. 10), acceptor reactions with glucose (Fig. 9), or the proportions of low-molecular-weight products made from sucrose.

Activation by metal ions at low concentration and inhibition by the same metal ions at high concentration is often found for enzymes whose true substrate is a metal-ion complex of the apparent substrate (Dixon and Webb [74], pp. 381-399). While an inhibitory Ca$^{2+}$-sucrose complex may be the cause of Ca$^{2+}$ inhibition (Fig. 1), Ca$^{2+}$ complexes with sucrose or dextran seem unlikely to be involved in the activation (Figs. 3 and 4). Maximum activation occurs below 1 mM Ca$^{2+}$. The low affinity of neutral carbohydrates for Ca$^{2+}$ [7], taken together with the weakness of the Ca$^{2+}$ inhibition, should cause maximum activation by such a complex to take place at a Ca$^{2+}$ concentration far above 1 mM.

The activity exhibited by the enzyme after SDS gel electrophoresis was not reduced in major degree by reducing $[\text{Ca}^{2+}]_f$ to less than 0.1 mM during the removal of SDS and subsequent incubation with sucrose (Fig. 8). There does not, therefore, appear to be a strict conformational requirement for Ca$^{2+}$, since it was not required for renaturation. This does not exclude the possibility that Ca$^{2+}$ induces a more stable and catalytically efficient conformation.

Increasing the rate of branching could increase the overall reaction rate [281, 343], as do the related acceptor reactions of most mono- and oligosaccharide acceptors [51, 246]. Relevant kinetic studies are absent, but both monovalent and divalent cations (Na$^+$, K$^+$, NH$_4^+$, Mg$^{2+}$, Ca$^{2+}$, Ba$^{2+}$, Mn$^{2+}$, Fe$^{2+}$) have been reported to increase the fraction of α-(1→3) linkages in glucans synthesized by glucansucrase preparations from S. mutans [68, 242, 256] or from L. mesenteroides IAM 1046 [158, 339]. For a mixture of soluble- and insoluble-glucan-forming glucansu-
crases from S. mutans 6715, most of these cations have also been reported [242] to activate glucan synthesis and to increase the amount of activation produced by the addition of soluble dextran, primarily by increasing the synthesis of water-insoluble glucan. However, no absolute requirement for metal ions in the synthesis of branch linkages has ever been demonstrated.

A comparison can be made between the activation of B-512F dextran-sucrase by Ca^2+ and the activation of other glucansucrases by dextran. B-512F dextran-sucrase is markedly activated by low concentrations of Ca^2+, while glucansucrases from Streptococcus are not. (More specifically, they are not inhibited by EDTA [44, 50, 150]. One report of inhibition by EDTA [299] may be due to inhibition of levansucrase in the preparation [112, 159].) In contrast, it has not so far been shown convincingly that B-512F dextran-sucrase is activated by dextran [286], while this activation is easily demonstrated for many glucansucrases [281]. In several cases [51, 189, 226], glucansucrase activation by dextran has been found to be hyperbolic mixed-type, as is the activation of B-512F dextran-sucrase by Ca^2+. Competition of Ca^2+ with sucrose (Fig. 1) makes it very likely that Ca^2+ binds to the sucrose site. The parallels of activation by Ca^2+ and dextran suggest that Ca^2+ may also bind to a dextran site.

Failure to find a function of the enzyme with an absolute requirement for Ca^2+ is consistent with the work of other investigators of Leuconostoc enzymes. (Complete inhibition by EDTA has not been found except after lengthy incubation with the enzyme [158, 186, 188, 205, 237, 249, 286, 339]. This appears to be the result of slow, irreversible inactivation, a separate although probably related phenomenon from the initial, reversible inhibition studied here.) It is also consistent with a basic similarity between Streptococcus and Leuconostoc dextran-sucrases.
Progress does not consist in replacing a theory that is wrong with one that is right. It consists in replacing a theory that is wrong with one that is more subtly wrong.

—Hawkin's Theory of Progress
PART V

INHIBITION OF DEXTRANUCRASE BY ZINC, NICKEL, AND COBALT
ABSTRACT

It has long been known that low concentrations of Ca^{2+} stabilize and activate dextranucrase (sucrose: 1,6-α-D-glucan 6-α-D-glucosyltransferase, EC 2.4.1.5) from Leuconostoc mesenteroides NRRL B-512F and from a few related strains, but only recently has Ca^{2+} at high concentrations been found to be a competitive inhibitor of this enzyme. Initial rate kinetics of polysaccharide formation show that Zn^{2+}, Ni^{2+}, and Co^{2+} inhibit dextranucrase by binding at two types of metal ion sites. At one site, which has a low affinity for Ca^{2+}, these metal ions are competitive with sucrose. The remaining site(s) have a high affinity for Ca^{2+}, and lesser affinity for Zn^{2+}, Ni^{2+}, or Co^{2+}. The binding of Zn^{2+} to these high-affinity Ca^{2+} site(s) increases both \( V_{\text{max}} \) and the \( K_m \) for sucrose. This contrasts with Ca^{2+}, whose binding at these site(s) also increases \( V_{\text{max}} \), but decreases the \( K_m \).
Dextran (sucrose: 1,6-α-D-glucan 6-α-D-glucosyltransferase, EC 2.4.1.5), secreted by species of *Leuconostoc* and *Streptococcus* [312, 343], polymerizes the glucosyl moiety of sucrose to form dextran, an α-(1→6)-linked glucan with α-linked branches [343]. Zn\(^{2+}\) and other divalent metal ions, with the exception of Ca\(^{2+}\) and other members of the alkaline earth series, have usually but not universally (e.g., not in ref. 138) been found to inhibit dextranucrase [184, 186, 188, 286, 339]. With the exception of a handful of papers [158, 242, 249, 256, 339], most of which are concerned primarily with the effects of Ca\(^{2+}\) and other weakly inhibiting or non-inhibiting metal ions on enzyme stability and on the degree of branching in the glucan product, only passing attention has been paid to the interaction of metal ions with dextranucrase and other glucansucrases. This lack of attention is probably because no metal ion requirement has been demonstrated for glucansucrases, and because metal ions are not strong inhibitors. The molecular basis for inhibition by divalent metal ions is not known, and the kinetics of the inhibition has not been described.

The recent discovery (see Part IV of this dissertation) that Ca\(^{2+}\), which at low concentration activates dextranucrase from *L. mesenteroides* NRRL B-512F, at high concentration competitively inhibits dextran formation by this enzyme, led us to investigate in detail the inhibition by other metal ions. For many enzymes activated by Ca\(^{2+}\), inhibition by Zn\(^{2+}\) and similar metal ions can be fully explained by binding at sites where Ca\(^{2+}\) is the native metal ion [15, 197]. We were interested to know if this were the case for B-512F dextranucrase.
MATERIALS AND METHODS

Materials

Enzyme purification has been described elsewhere [26, 237]. Enzyme was a dialyzed concentrate of culture supernatant [237] having 98 U glycansucrase/mg protein, 0.02 U levansucrase/U dextran sucrase, and 77 mg carbohydrate/mg protein. [U-^14C]sucrose was purified by paper chromatography (two ascents at 85°C in 1-butanol/pyridine/water (6:1:1, v/v/v) on Whatman 3MM paper) before dilution with carrier sucrose.

Enzyme Assays

Assays were conducted at 25°C and were buffered with 0.05-0.1 M sodium acetate, pH 5.0, with some additional acetate present due to the use of acetates as the sources of Zn^{2+}, Ni^{2+}, and Co^{2+}. Ionic strength was not kept strictly constant, but varied over a small range for a given experiment. Assays followed the incorporation of ^14C from [U-^14C]sucrose into methanol-insoluble polysaccharide [118], measuring the sum of dextran sucrase and levansucrase activities. Dextran sucrase activity was taken to be equal to this sum. One unit of dextran sucrase is defined as the amount of enzyme that will incorporate 1 μmol of glucose into polysaccharide from 0.15 M sucrose in one minute.

Metal Ion Concentrations

Free metal ion concentrations were calculated by subtracting from total metal ion concentrations the concentrations of the acetate complexes formed between the metal ion and one to four acetates. For Zn^{2+}, Ni^{2+}, and Co^{2+}, both mono- and diacetates formed in significant amounts; e.g., about 40% of the Zn^{2+} in solution was typically in the form of acetate complexes.

Stability constants for metal ion-acetate complexes were taken pref-
erentially from Martell and Smith [222] (Vol.3, pp. 3-7 (1977)), and otherwise from ref. 157. For Ca\(^{2+}\), only the stability constant for the monoacetate was given in either of these sources.

As discussed in Part IV, it was assumed that uncontrolled sources of Ca\(^{2+}\) (buffer salts, distilled-deionized water, glassware, etc.) contributed 10 ± 5 μM to total Ca\(^{2+}\) concentration.

**Kinetic Data Analysis**

Kinetic parameters were estimated by fitting initial rate data to specific rate equations by weighted linear or nonlinear regression, using iteratively determined weights [220]. Rapid-equilibrium rate equations were derived as described on pp. 22-25 of Segel [307]. Rate equations are given in the text in double-reciprocal form because the data are displayed this way in the figures, but values quoted for apparent kinetic constants were always determined by nonlinear regression from the non-reciprocal form of the equations.
RESULTS

Initial Considerations

Zn\(^{2+}\) causes a slow denaturation of dextransucrase from which dextran has been removed [237]. It was found that if 10 mg/ml dextran T10 were added to the dextran-free enzyme, the denaturation did not occur. The presence of dextran did not change the initial inhibition caused by Zn\(^{2+}\), only the rate of denaturation. No dextran T10 was present in the experiments shown in this Part, but the concentration of native B-512F dextran was approximately 1 mg/ml. Under the conditions used for these experiments, Zn\(^{2+}\) did not cause significant denaturation of dextransucrase over a 1 h period, even at concentrations 50% higher than used in the experiments shown, in which exposure of the enzyme to Zn\(^{2+}\) was limited to 2-20 min.

Inspection of thin-layer chromatograms of dextransucrase reaction mixtures containing Zn\(^{2+}\) showed no obvious changes in the relative amounts of glucose and other low-molecular-weight products from the amounts formed in the absence of Zn\(^{2+}\). These relative amounts were not quantitated.

Inhibition by Zn\(^{2+}\)

In the rate equations to follow, [Zn\(^{2+}\)]\(_f\), [Ca\(^{2+}\)]\(_f\), and [sucrose] are given the same one-letter designations (Z, C, and S) as in Fig. 1. The one-letter abbreviations are frequently used in the text when referring to the equations.

Two-site model

The data are interpreted primarily in terms of the two-site model shown in Fig. 1. In this model, sucrose binds to one of the sites, and metal ions bind to both of the sites. All of the enzyme forms existing in the combined presence of Ca\(^{2+}\), Zn\(^{2+}\), and sucrose are shown in Fig. 1A.
Figure 1. Two-site model for metal-ion interactions with dextranu- 
crase

"E" is enzyme, "Z" is Zn$^{2+}$, "C" is Ca$^{2+}$, and "S" is sucrose. 
Lines connect forms of the enzyme which are interconverted by 
the binding of a single ligand. (A) All enzyme forms. Let-
ters to the right of "E" represent ligands bound to the su-
crose site, and letters to its left represent ligands bound 
to the other site. (B) Same as (A), after grouping together 
enzyme forms whose amounts cannot be estimated independently 
under the rapid-equilibrium assumption; e.g., "ZE" and "EZ" 
in (A) have become the single enzyme form "ZE" in (B). The 
symbols $k_p$, $\beta_1k_p$, and $\beta_2k_p$ are catalytic rate constants, and 
symbols of the form $\alpha_kK_y$ are apparent dissociation constants.
In deriving a rate equation for this model or any other, it is necessary to define expressions for the relative amounts of each enzyme form. With the rapid-equilibrium assumption, it is not possible to determine independently the amounts of forms that have the same set of bound ligands. Therefore, duplicate forms in Fig. 1A were combined, resulting in the simpler scheme shown in Fig. 1B.

**Development of the model**

Fig. 2 shows Dixon plots (1/v vs. effector concentration) for the inhibition of dextranucrase by Zn\(^{2+}\) at low [Ca\(^{2+}\)] (Fig. 2A) and at high [Ca\(^{2+}\)] (Fig. 2B). A positive slope shows inhibition: the greater the slope, the greater the inhibition. The shape of the Dixon plot reflects a power series in which, for rapid-equilibrium mechanisms, the binding of a single equivalent of inhibitor to the enzyme gives a straight line, the binding of two equivalents gives a parabola, and the binding of more than two equivalents gives a curve with higher order terms. The curvature in both Figs. 2A and 2B at the lowest [Ca\(^{2+}\)] therefore shows that at least two inhibitory Zn\(^{2+}\) sites exist.

Fig. 2A shows that increasing [Ca\(^{2+}\)] to just a few hundred micromolar reverses most of the inhibition caused by 6-7 mM Zn\(^{2+}\). The positive slope that remains above 1 mM Ca\(^{2+}\) (Fig. 2B), and the near linearity of the plots above 1 mM Ca\(^{2+}\) show that Ca\(^{2+}\) binds poorly at one (and only one) of the inhibitory Zn\(^{2+}\) sites. If Ca\(^{2+}\) had an affinity comparable to that of Zn\(^{2+}\) at this site, the lower line in Fig. 2B would shift up or down with increasing [Ca\(^{2+}\)], depending on whether it inhibited or activated the enzyme at that site. At the remaining site or sites, Ca\(^{2+}\) binds more strongly than Zn\(^{2+}\), preventing Zn\(^{2+}\) inhibition.

\[
\frac{1}{v} = b_0 + b_1 \left[ \frac{1}{s} \right] + b_2 \left[ \frac{1}{s^2} \right]
\]

The kinetics of the Zn\(^{2+}\) inhibition were defined in much greater detail by comparing double-reciprocal plots at two fixed concentrations
Figure 2. Dixon plots showing inhibition of dextranucrase by Zn$^{2+}$ (A) at low Ca$^{2+}$ concentration and (B) at high Ca$^{2+}$ concentration. Sucrose concentration was 75 mM. Each point represents a single determination.

(A) [Ca$^{2+}$]$_{f}$ (mM): ◇, 0.06; ⊕, 0.20; ◆, 0.34; ▲, 0.48; ×, 0.71; ▽, 0.77.

(B) [Ca$^{2+}$]$_{f}$ (mM): ◆, 0.06; ⊕, 1.4; ◆, 2.8; ▲, 4.2; ×, 5.6; ▽, 7.1.
of Ca\(^{2+}\) (Fig. 3), low and high. First, eqn. 1 was fit separately for each Zn\(^{2+}\) concentration for both experiments shown in Fig. 3. Plots of \(b_0\), \(b_1\), and \(b_2\) (empirical constants in eqn. 1) against [Zn\(^{2+}\)]\(_f\) are shown in Figs. 4-6.

Significance levels* for the existence (non-zero value) and [Zn\(^{2+}\)]\(_f\)-dependence of \(b_0\), \(b_1\), and \(b_2\) were determined. This was not done from the data shown in Figs. 4-6, which are meant for illustration only, but by including appropriate terms in linear regression analyses of the primary data shown in Fig. 3. Using the primary data gave a more consistent set of weights for the velocities. Significance levels determined from the primary data were generally different than when the derivative data in Figs. 4-6 were used instead.

The change in \(b_0\) with [Zn\(^{2+}\)]\(_f\) was significant at low [Ca\(^{2+}\)] \((P < 0.0001)\), but not at high [Ca\(^{2+}\)] \((P = 0.4)\). At high [Ca\(^{2+}\)], \(b_2\) decreased significantly with [Zn\(^{2+}\)]\(_f\) \((P < 0.0001)\), but at low [Ca\(^{2+}\)], \(b_2\) had little significance. The highest significance level, \(P = 0.08\), was found for a decrease in \(b_2\) proportional to [Zn\(^{2+}\)]\(_f\)\(^2\). At high [Ca\(^{2+}\)], \(b_1\) (Fig. 5B) increased linearly with [Zn\(^{2+}\)]\(_f\), but at low [Ca\(^{2+}\)], after a short region of linear increase, it curved upward (Fig. 5A).

Based on these results, the data for high [Ca\(^{2+}\)] (Fig. 3A) were fit to eqn. 2, in which the quantity [Zn\(^{2+}\)]\(_f\)/{(K_{ZnS})} compensates for curvature. This curvature term, although significant at \(P < 0.0001\), had a negative value and no direct physical meaning. Using it in eqn. 2 allowed a more accurate estimate of the apparent inhibition constant for

* The "significance" of a parameter is defined in terms of \(P\), the probability of exceeding the absolute value of the t statistic calculated assuming that the true value of the parameter is zero. The quantity \(1 - P\) can be thought of as the probability that the parameter is "real," in the sense of accounting for a nonrandom trend in the data; i.e., a trend not caused by indeterminate experimental error.
Figure 3. Double-reciprocal plots for the inhibition of dextransucrase by Zn$^{2+}$ at high (A) and low (B) Ca$^{2+}$ concentrations.

The data were fit separately to eqn. 1 for each [Zn$^{2+}$]$_f$.

(A) [Ca$^{2+}$]$_f$ = 9 mM. Each point represents the mean of four determinations. [Zn$^{2+}$]$_f$ (mM): □, 0; ⊕, 1.5; ◊, 3.0; △, 4.6; ×, 6.1.

(B) [Ca$^{2+}$]$_f$ = 20 μM. Each point represents the mean of two determinations. [Zn$^{2+}$]$_f$ (mM): □, 0; ⊕, 0.7; ◊, 1.4; △, 2.0; ×, 2.7. Three Zn$_f^{2+}$ concentrations (0.3, 1.0, and 3.4 mM) have been omitted for clarity.
Figure 4. Replot of $1/v$-intercept parameter $b_0$ for the experiments shown in Fig. 3

The double-reciprocal plot data in Fig. 3 were fit separately to eqn. 1 at each $[\text{Zn}^{2+}]_f$. The resulting $1/v$-axis intercepts ($b_0$ in eqn. 1) are plotted against $[\text{Zn}^{2+}]_f$. Vertical bars show the standard error.

(A) Low $[\text{Ca}^{2+}]_f$ (replot for Fig. 3B).
(B) High $[\text{Ca}^{2+}]_f$ (replot for Fig. 3A).
Figure 5. Replot of linear slope parameter $b_1$ for the experiments shown in Fig. 3

The double-reciprocal plot data in Fig. 3 were fit separately to eqn. 1 at each $[\text{Zn}^{2+}]_f$. The resulting linear slope coefficients ($b_1$ in eqn. 1) are plotted against $[\text{Zn}^{2+}]_f$. Vertical bars show the standard error.

(A) Low $[\text{Ca}^{2+}]_f$ (replot for Fig. 3B).

(B) High $[\text{Ca}^{2+}]_f$ (replot for Fig. 3A).
The double-reciprocal plot data in Fig. 3 were fit separately to eqn. 1 at each \([\text{Zn}^{2+}]_f\). The resulting coefficients of slope curvature (b_2 in eqn. 1) are plotted against \([\text{Zn}^{2+}]_f\). Vertical bars show the standard error.

(A) Low \([\text{Ca}^{2+}]_f\) (replot for Fig. 3B).

(B) High \([\text{Ca}^{2+}]_f\) (replot for Fig. 3A).
Coefficient in $\frac{1}{[\text{Sucrose}]}^2$ (min cpm$^{-1}$ mM sucrose$^{-2}$)
Zn\(^{2+}\) at saturating Ca\(^{2+}\) concentration (\(a_3 K_z\) in Fig. 1B) by separating the linear and quadratic components in 1/S. Fig. 7, showing the data in Fig. 3A as fit to eqn. 2, illustrates Zn\(^{2+}\) inhibition at a [Ca\(^{2+}\)] high enough to exclude Zn\(^{2+}\) from the high-affinity Ca\(^{2+}\) site(s). It shows that Zn\(^{2+}\) is competitive with sucrose at the site with low affinity for Ca\(^{2+}\).

\[
\frac{1}{v} = \frac{1}{V_{max}} + \frac{K_s}{V_{max}S} \left[ \frac{1}{1 + \frac{Z}{a_3 K_z}} + \frac{Z}{K_z S} \right]
\]  

(2)

The low-[Ca\(^{2+}\)] data (Fig. 3B) were fit to 4 trial equations, which were defined by setting \(m\) equal to 1 or 2 and \(n\) equal to 2 or 3 in eqn. 3. All terms in each of the 4 equations were significant at \(P < 0.0001\). Terms in 1/S\(^2\) were omitted because there were not good theoretical grounds for extrapolating from the highest experimental sucrose concentration to the 1/v-axis using equations containing such terms. The same problem did not exist in applying eqn. 2 to the high-[Ca\(^{2+}\)] data, because the 1/v-intercept was constant.

\[
\frac{1}{v} = \frac{1}{V_{max}} \left[ \frac{1}{1 + \frac{Z^m}{K_1^m}} \right] + \frac{K_s}{V_{max}S} \left[ \frac{1}{1 + \frac{Z}{K_2}} + \frac{Z^n}{K_3^n} \right]
\]  

(3)

With either value of \(n\), eqn. 3 fit the data better with \(m = 2\). Likewise, with either value of \(m\), the fit was better with \(n = 3\). This led to the obvious question of whether eqn. 4 would be a better expression than eqn. 3. However, the additional terms in eqn. 4 were not statistically significant; that is, the data contained only enough information to fit one power of \(Z\) in both the 1/v-slope and 1/v-intercept parts of the rate equation.
Figure 7. Competitive inhibition of dextranucrase by Zn$^{2+}$ at high Ca$^{2+}$ concentration

The data shown in Fig. 3A were fit to eqn. 2. $[\text{Ca}^{2+}]_f = 9$ mM. Each point represents the mean of four determinations. $[\text{Zn}^{2+}]_f$ (mM): $\square$, 0; $+$, 1.5; $\Diamond$, 3.0; $\triangle$, 4.6; $\times$, 6.1.
Because the binding of Zn$^{2+}$ at the site with low Ca$^{2+}$ affinity did not change the $1/v$-intercept, the change in intercept shown in Fig. 4A had to be caused by the binding of Zn$^{2+}$ to the high-affinity Ca$^{2+}$ site(s). The data in Fig. 4A, where the negative slope corresponds to the physically meaningless negative values that were determined for the apparent kinetic constants $K_1$ or $K_1^2$ in eqn. 3, could not be fit well by a kinetically plausible function simpler than eqn. 5, where $\phi_1-\phi_6$ are constants at fixed [Ca$^{2+}$]$_f$, and $\phi_5$ may be zero.

\[
\frac{1}{v} = \frac{1}{v_{\text{max}}} \left[ 1 + \frac{Z}{K_1} + \frac{Z^2}{K_2^2} \right] + \frac{K_g}{v_{\text{max}} S} \left[ 1 + \frac{Z}{K_3} + \frac{Z^2}{K_4^2} + \frac{Z^3}{K_5^3} \right]
\]  

(4)

Under rapid-equilibrium assumptions, this relationship requires at least two high-affinity Ca$^{2+}$ sites at which Zn$^{2+}$ could bind. However, a single Zn$^{2+}$ site would also give this equation if Zn$^{2+}$ bound in rapid equilibrium, but not sucrose (Segel [307], pp. 833-841). Similarly, the presence of $Z^3$ in the $1/v$-slope part of the best fits to eqn. 3 would require a total of three Zn$^{2+}$ sites in a rapid-equilibrium model, but not more than two sites in a steady-state model.

The question of whether the rate equation should contain a $Z^3$ term in the expression for $1/v$-slope was not settled conclusively by the data of Fig. 3A. The reason for this is illustrated in Fig. 8, the important feature of which is the dotted line showing the upward curvature that developed in the double-reciprocal plots at [Zn$^{2+}$]$_f$ above 3 mM. This happened because progress curves at low velocity were not linear; the apparent velocity increased during the course of the assay (Fig. 9). Values
Figure 8. Inhibition of dextran sucrase by Zn^{2+} at low Ca^{2+} concentration

Complete data for the experiment shown in Fig. 3B are shown. [Ca^{2+}]_f = 20 μM. Each point represents the mean of two determinations. The velocities at the highest Zn^{2+} concentration (dotted line) were not included in the analysis.

[Zn^{2+}]_f (mM): ▲, 0; ○, 0.3; ◊, 0.7; △, 1.0; X, 1.4; ▽, 2.0; □, 2.7; +, 3.4.
Figure 9. Nonlinearity of progress curves at low velocity for the methanol-wash assay for polysaccharide synthesis

Progress curves are shown for an experiment done at 13 mM Zn$^{2+}$$_f$. Sucrose concentrations were 100 mM (A) or 33 mM (B). The solid lines show the linear-regression fit.
of initial velocity determined by assuming that these progress curves were linear were too low, and so values of 1/v were too high.

It was not determined whether the nonlinearity of these progress curves was an inherent artifact of the assay method at low velocities, whether it was related to the increase in dextran concentration during the assay, or whether it was from some other cause. Whatever the cause, the net effect, in experiments with low velocities resulting from low sucrose concentrations, was to limit usable $[\text{Zn}^{2+}]_f$ at low $[\text{Ca}^{2+}]$ to less than 3 mM, where the question of whether to include a $Z^3$ term in the expression for $1/v$-slope could not be answered. Therefore, another type of experiment was performed, using a fixed, high sucrose concentration, where progress curves were all found to be linear.

This experiment is shown in Fig. 10. $\text{Zn}^{2+}$ was varied at three widely different, fixed concentrations of $\text{Ca}^{2+}$. The plot at the highest $[\text{Ca}^{2+}]$ (Figs. 10A and 10C) could be successfully fit by an equation whose highest power of $[\text{Zn}^{2+}]_f$ was $[\text{Zn}^{2+}]_f^2$, but the plots at the two lower $\text{Ca}^{2+}$ concentrations (Figs. 10A-10B) required $[\text{Zn}^{2+}]_f^3$, and were fit better by equations containing even higher powers of $[\text{Zn}^{2+}]_f$.

A clear alternative existed to the hypothesis that these many-term polynomials in $[\text{Zn}^{2+}]_f$ represented the binding of many equivalents of $\text{Zn}^{2+}$ to the enzyme, although this would not be unreasonable at such high $[\text{Zn}^{2+}]_f$. This alternative was that these polynomials were only approximations to a hyperbolic function (linear at both low and high $[\text{Zn}^{2+}]_f$, but curved at some intermediate $[\text{Zn}^{2+}]_f$).

The general form of a hyperbola in the X-Y plane is eqn. 6 [92], where A-F are arbitrary constants. The lines in Fig. 10 show the hyperbolas given by eqn. 6, with $X = [\text{Zn}^{2+}]_f$ and $Y = 1/v$.

$$AX^2 + BXY + CY^2 + DX + EY + F = 0$$ (6)

The rapid-equilibrium rate equation for the model in Fig. 1B is eqn. 7. At constant $[\text{Ca}^{2+}]_f$, eqn. 7 predicts hyperbolic slope and intercept replots (vs. $[\text{Zn}^{2+}]_f$) for the primary (double-reciprocal) plots.
Figure 10. Dixon plots showing inhibition of dextran sucrase by Zn$^{2+}$ at three widely spaced Ca$^{2+}$ concentrations.

Sucrose concentration was 75 mM. Each point represents a single determination. The solid lines show the fits to hyperbolas determined from eqn. 6.

(A) All data for the experiment. [Ca$^{2+}$]$_f$ (mM):

☐, 0.06; ⊕, 0.6; ◇, 7.

(B) 0.6 mM Ca$^{2+}$ only. (C) 7 mM Ca$^{2+}$ only.
$l/v \text{ (min} \cdot \text{ml/mg dextran)}$
The hyperbolicity is caused by the term $B Z/\alpha Z K_2$ in the multiplier of $V_{\text{max}}$, a term resulting from the presence of a dextran-forming enzyme species having bound Zn$^{2+}$ (ZES in Fig. 1B).

$$\frac{1}{v} = \frac{1}{V_{\text{max}}} \left[ 1 + \frac{B_1 C}{\alpha_1 K_c} + \frac{B_2 Z}{\alpha_2 K_z} \right] \left( 1 + \frac{C}{\alpha_1 K_c} + \frac{Z}{\alpha_2 K_z} \right)$$

$$+ \frac{K_s}{S} \left( \left[ \frac{1 + C}{K_c} + \frac{C^2}{\alpha_3 K_c} \right] + \left[ \frac{Z}{K_z} + \frac{CZ}{\alpha_3 K_c K_z} \right] + \left[ \frac{Z^2}{\alpha_4 K_z^2} \right] \right)$$

(7)

No reasonable fit to eqn. 7 could be made for the data at the lower two Ca$^{2+}$ concentrations in Fig. 10, even when the two concentrations were considered separately. For these two Ca$^{2+}$ concentrations, the slope of the fit to the data above 8 mM Zn$^{2+}$ could not be matched without displacing the high-[Zn$^{2+}$]$_f$ part of the curve toward the 1/v-axis, causing a large gap between the data and the fit at all [Zn$^{2+}$]$_f$ higher than 1-2 mM. The data below 8 mM Zn$^{2+}$ could be fit only by moving the rest of the curve far to the right of the data. An excellent fit could be obtained, however, if $Z^2$ in eqn. 7 were replaced by $Z^3/K_z$, and $Z/\alpha_2$ by $Z^2/\alpha_2 K_z'$. These substitutions also gave an equation more consistent with the 1/v-intercept relationship expressed by eqn. 5.

In another experiment, shown in Fig. 11, the Ca$^{2+}$ concentration was varied at two fixed concentrations of Zn$^{2+}_f$. The data at zero Zn$^{2+}$ concentration from Fig. 2A have been replotted in Fig. 1 to show that in the absence of Zn$^{2+}$, a broad range of Ca$^{2+}$ concentration had no effect on initial velocity. At high [Zn$^{2+}$], however, there was complex curvature at the high-[Ca$^{2+}$] end of the plot. This curvature showed that the rate
Figure 11. Dixon plots showing the effect of Ca\(^{2+}\) at free Zn\(^{2+}\) concentrations of 0 (●) and 13 (□) mM.

Sucrose concentration was 75 mM. Each point represents a single determination. The data at 0 mM Zn\(_f\)\(^{2+}\) are taken from Fig. 2A, and represents a separate experiment from the data at 13 mM Zn\(_f\)\(^{2+}\).
equation should also contain term(s) in \([\text{Ca}^{2+}]_f^3\).

**Inhibition by \text{Ni}^{2+} \text{ and Co}^{2+}\)**

Inhibition by \text{Ni}^{2+} \text{ and Co}^{2+} was examined in less detail than inhibition by \text{Zn}^{2+}, because \text{Ni}^{2+} \text{ and Co}^{2+} were weaker inhibitors, but no qualitative differences from the results with \text{Zn}^{2+} were found. Both \text{Ni}^{2+} \text{ and Co}^{2+} were competitive with sucrose at high \([\text{Ca}^{2+}]\), and gave a more complex type of inhibition at low \([\text{Ca}^{2+}]\). From Fig. 7, the apparent inhibition constant for \text{Zn}^{2+} at high \text{Ca}^{2+} concentration was calculated to be 0.95 ± 0.05 mM. The comparable inhibition constant for \text{Ni}^{2+} was 6.5 ± 0.4 mM, and for \text{Co}^{2+} was 5.8 ± 0.5 mM.
DISCUSSION

Inhibition Kinetics

The partial reversal of Zn$^{2+}$ inhibition by Ca$^{2+}$ that is seen in Fig. 2A shows the existence of an inhibitory Zn$^{2+}$ site (or sites) at which Ca$^{2+}$ binds more strongly than Zn$^{2+}$, and does not inhibit dextran synthesis. The linearity of the lower curve in Fig. 2B indicates that there is also a single inhibitory Zn$^{2+}$ site with negligible affinity for Ca$^{2+}$.

At the site with low Ca$^{2+}$ affinity, Zn$^{2+}$ was competitive with sucrose (Fig. 7). This was also true for Ni$^{2+}$ and Co$^{2+}$, although they were weaker inhibitors than Zn$^{2+}$. Ca$^{2+}$ has also been found to be a competitive inhibitor of B-512F dextranucrase (see Part IV of this dissertation), although one with a very high inhibition constant (59 mM).

Because Ca$^{2+}$ binds so strongly to the remaining site(s), which have high affinity for Ca$^{2+}$ (apparent $K_{diss}$ of < 10 μM (Part IV)), the kinetic effects of Zn$^{2+}$ at these site(s) were difficult to isolate from its effects at the sucrose site; i.e., [Ca$^{2+}$] could not easily be reduced to a value so low that Zn$^{2+}$ would bind almost exclusively to the high-affinity Ca$^{2+}$ site(s). In addition, the nonlinearity of progress curves at low velocity, of which an example is shown in Fig. 9, prevented the use, in experiments of the design shown in Fig. 8, of Zn$^{2+}$ concentrations high enough to draw firm conclusions about the algebraic form of the changes with [Zn$^{2+}$]$_f$ in the slope and intercept of double-reciprocal plots. However, the binding of Zn$^{2+}$ at the high-affinity Ca$^{2+}$ site increased the slope (Fig. 5A) and decreased the intercept (Fig. 4A) of double-reciprocal plots, showing that both the apparent $V_{max}$ and the apparent $K_m$ were increased.

Attempts to find a rate equation fitting the low-[Ca$^{2+}$] double-reciprocal plot data led to the finding that the 1/v-intercept part of the equation should contain [Zn$^{2+}$]$_f^2$ terms in both numerator and denominator, and that the 1/v-slope part of the equation must contain [Zn$^{2+}$]$_f^2$ or...
[Zn^{2+}]_f^3 terms, though not necessarily both. Analysis of the data in Figs. 10 and 11 showed that to explain observations under the combined conditions of high [Zn^{2+}]_f and low [Ca^{2+}]_f, the rate equation should contain terms in both [Zn^{2+}]_f^3 and [Ca^{2+}]_f^3, or terms that could simulate them.

Two-Site Model

A model for the interactions of Zn^{2+}, Ca^{2+}, and sucrose with dex-transucrase is given in Fig. 1. In this model, there are two divalent metal-ion sites, one of which is also the sucrose site. Divalent metal ions are competitive inhibitors at this site. For the four metal ions examined, the inhibition constant for this site was lowest for Zn^{2+} and highest for Ca^{2+}.

The binding of either Ca^{2+} or Zn^{2+} to the other site increases V_{max}. At this site, Ca^{2+} also decreases the K_m for sucrose (see Part IV of this dissertation), but Zn^{2+} increased it (Fig. 5A). The increase in V_{max} caused by Zn^{2+} at this site appeared to be greater than the increase given by Ca^{2+}; i.e., \beta_2 > \beta_1 in eqn. 7. \beta_1 is approximately 2 (Part IV).

The rapid-equilibrium rate equation for the two-site model shown in Fig. 1 (eqn. 7) did not contain terms of high enough order in Z and C to fit the data in Figs. 10 and 11, or to fit the data in Fig. 8 well. To add to eqn. 7 the terms required to obtain a good fit, and at the same time to retain the rapid-equilibrium assumption, it would be necessary to assume the existence of two inhibitory Zn^{2+} sites at which Zn^{2+} was excluded by the binding of Ca^{2+} to the high-affinity Ca^{2+} site(s). The analysis suggested that these two zinc ions would bind cooperatively.

It is more likely that rapid equilibrium does not exist between all kinetically important forms of the enzyme. If free sucrose were not in equilibrium with enzyme-bound sucrose, then the (steady-state) rate equation for the two-site model shown in Fig. 1 would probably be adequate to explain the data. Steady-state rate equations for this model, with either none of the enzyme forms in equilibrium or with some of them in
equilibrium, have not been derived.

This study addresses only the kinetic mechanism of inhibition by
divalent metal ions, and not the molecular mechanism. Metal ions inhibit
dextranucrase weakly compared to their inhibition of many other enzymes,
which necessitated the use of high metal ion concentrations in many of
the experiments. Even the strongest inhibitor, Zn\(^{2+}\), had a high inhibi-
tion constant (0.95 mM) in the presence of small amounts (less than 1 mM)
of Ca\(^{2+}\), relative to the micromolar inhibition constants for Zn\(^{2+}\) with
many other enzymes [15]. This may be why metal ion effects are not more
widely reported for glucansucrases, where the metal ion concentrations
tested when screening for inhibition are most commonly 1 mM. This is not
unreasonable, since Zn\(^{2+}\) can form fairly strong complexes with amino
acids (dissociation constants of 10\(^{-4}\) M [15]), and above 1 mM might be
expected to bind to the enzyme at what would not usually be considered as
specific metal ion sites.

Potential Artifacts

Changes in product distribution

As found for the activation of B-512F dextranucrase by Ca\(^{2+}\)
(Part IV of this dissertation), no major changes in the distribution of
low- and high-molecular weight products were caused by Zn\(^{2+}\). Therefore,
it was appropriate to use dextran synthesis as a measure of total enzyme
activity. Zn\(^{2+}\) also causes denaturation of dextranucrase, but this
could be prevented by the presence of dextran (1 mg/ml in these experi-
ments).

Nonlinearity of double-reciprocal plots

Double-reciprocal plots were not linear, as shown by the non-zero
curvature parameters (b\(_2\)) in Fig. 6. The curvature was not in the direc-
tion necessarily to suggest failure of the rapid equilibrium assumption
(i.e., not concave-upward; see p. 360 of Dixon and Webb [74]), although
rapid-equilibrium in such a complex system seems unlikely. The curvature
could be caused by negative cooperativity resulting from allosteric sucrose sites, but is more likely to result from the presence of a minor glycansucrase species with different kinetic constants than the major glycansucrase species. Streptococcal levansucrases are inhibited by divalent metal ions \([112, 160]\) and activated by \(\text{Ca}^{2+}\) \([159]\). Enough levansucrase was present in the enzyme preparation used here (2% of the amount of dextran sucrase activity) to give the curvature seen. The minor glycansucrase species could also be one of the different molecular-weight forms of 8-512F dextran sucrase (see Parts IV and VI of this dissertation); however, the preparation used for these experiments consisted almost completely of the 158 kDa form of dextran sucrase.

**Presence of dextran**

Another complication was the presence of dextran in the enzyme assays (about 1 mg/ml at time zero), which was required to prevent inactivation by \(\text{Zn}^{2+}\). The presence of product in initial rate experiments can be expected to complicate the analysis \([289]\). For other dextran-sucrases than the one studied here (e.g., that of \(S. \text{sanguis} [226]\)), dextran has been found to increase both \(V_{\text{max}}\) and the \(K_m\) for sucrose, the same pattern found here for \(\text{Zn}^{2+}\).

**Free vs. total metal ion concentrations**

The use of acetate as a buffer resulted in as much as 40% of some of the metal ions being bound in the form of acetate complexes. While the numerical analysis assumed no interaction of any of these metal ion-acetate complexes with the enzyme, there is no reason to believe this is true; however, none of the results were qualitatively changed by substituting total metal ion concentrations for free metal ion concentrations.

**Inconstant ionic strength**

Another factor that could compromise the interpretation to some degree was the absence of a strictly constant ionic strength. The increase in ionic strength with metal ion concentration was small in most
of the experiments, though, and B-512F dextranucrase is only slightly inhibited by fairly large increases in ionic strength, at least at high sucrose concentration (Part IV of this dissertation), at which condition the experiment that would be most affected was done (Fig. 10).
PART VI

MOLECULAR WEIGHT OF DEXTRANSUCRASE BY RADIATION INACTIVATION AND SDS POLYACRYLAMIDE GEL ELECTROPHORESIS
Two major forms of dextran sucrase from Leuconostoc mesenteroides NRRL B-512F were found on SDS polyacrylamide gel electrophoresis, of 177 and 158 kDa. A minor form of 168 kDa was sometimes seen as well. No form of dextran sucrase smaller than 158 kDa was found, although levansucrase was detected at 92 and 116 kDa. Radiation inactivation by $^{60}$Co indicated a single glycan sucrase species of 201 kDa, corresponding to the 158 kDa form seen on gels. It is concluded that no peptide association is required for dextran synthesis. For a proposed dextran sucrase mechanism in which dextran is synthesized by the cooperative action of two equivalent nucleophiles [J. F. Robyt, B. K. Kimble, and T. F. Walseth. Arch. Biochem. Biophys. 165, 634–640 (1974)], the ability of a single peptide to make dextran requires that both nucleophiles be located on the same peptide, rather than one on each subunit of a dimer.
INTRODUCTION

Dextranucrase (sucrase: 1,6-α-D-glucan 6-α-D-glucosyltransferase, EC 2.4.1.5), secreted by species of *Leuconostoc* and *Streptococcus* [312, 343], polymerizes the glucosyl moiety of sucrose to form dextran, an α-(1→6)-linked glucan with α-linked branches [343]. Robyt et al. [287] have proposed a mechanism for dextran synthesis having two nucleophiles at the dextranucrase active site, each of which alternately carries a glucosyl residue and a dextranosyl chain. Polysaccharide growth occurs when a dextranosyl group, bound by its reducing end to one of the nucleophiles, is transferred to the C-6 hydroxyl group of a glucosyl residue bound to the other nucleophile, forming a new α(1→6) linkage. After transferring the dextranosyl group, the first nucleophile, now free, is glucosylated from sucrose. The cycle then repeats, but with the roles of the nucleophiles reversed.

The presence of two equivalent nucleophiles in this model led us to speculate that the enzyme might consist of a dimer of identical subunits, each bearing one of the two nucleophiles. The question of whether dextranucrase is a dimer could not easily be answered by the conventional techniques of gel filtration, non-denaturing gel electrophoresis, or ultracentrifugation, because the enzyme aggregates readily under non-denaturing conditions to give particle sizes ranging up into the millions of daltons [98, 215, 239, 286, 290].

A less often used technique, radiation inactivation, measures the size of the minimal enzyme unit required to express activity [19, 178]. Because the size determined is for an enzymatically functional unit rather than a structural unit [19, 178], it does not reflect non-functional aggregation. By comparing the results of radiation inactivation and SDS gel electrophoresis, we show that dextranucrase is functionally a monomer consisting of a single peptide. Dimerization or higher association (aggregation) of dextranucrase peptides, while possibly having some biological role, is not needed for dextran synthesis.
MATERIALS AND METHODS

Materials

Leuconostoc mesenteroides NRRL B-512F was obtained from the Northern Regional Research Center (formerly Northern Regional Research Laboratory, NRRL) (Peoria, IL). Penicillium funiculosum dextranase (Grade I, chromatographically purified) and Schiff’s reagent were from Sigma Chemical Co. (St. Louis MO). Sodium dodecyl sulfate (>99%) was from Bio-Rad Laboratories (Richmond, CA). Ultrafiltration membranes were from Amicon Corp. (Danvers, MA). [U-¹⁴C]sucrose was from New England Nuclear (Boston, MA) and ICN (Irvine, CA), and [fructose-U-¹⁴C]sucrose was from New England Nuclear. [U-¹⁴C]sucrose was purified by paper chromatography (two ascents at 85° in 1-butanol/pyridine/water (6:1:1, v/v/v) on Whatman 3MM paper) before dilution with carrier sucrose.

Enzyme Preparations

For irradiation experiments, a dialyzed concentrate of the culture supernatant was used, whose preparation has been described elsewhere [237]. The concentrate had an activity of 150 U/ml dextransucrase, 3 U/ml levansucrase, and contained no Tween 80. For experiments on enzyme reactivation after SDS treatment, culture supernatant was used after dialysis against 0.063 M Tris-HCl (pH 6.8) or against 0.1 M sodium acetate (pH 5.2) containing 0.2 mM CaCl₂ and 0.2 mg/ml sodium azide. This dialysate contained approximately 1 mg/ml Tween 80.

For SDS gel electrophoresis, two enzyme preparations were used. One consisted of culture supernatant that had been concentrated five-fold over an Amicon YM30 ultrafiltration membrane, and then dialyzed overnight against 0.063 M Tris-HCl (pH 6.8). The other was a purified preparation from which levansucrase and dextran had been removed [26].
Enzyme Assays

Assays were performed at 25°C in 0.02 M sodium acetate buffer (pH 5.0-5.2), containing 1 mM CaCl₂, by following the incorporation of ¹⁴C from 0.15 M [U-¹⁴C]sucrose (for total glycansucrase = dextran sucrase + levansucrase) or [fructose-U-¹⁴C]sucrose (for levansucrase only) into methanol-insoluble polymer [118]. One unit of dextran sucrase or levansucrase is defined as the amount that will incorporate 1 µmol of glucose or fructose, respectively, into polysaccharide in one minute under these conditions.

Electrophoresis

SDS gel electrophoresis was performed by the method of Laemmli [203] on 5 x 90 mm cylindrical gels (6% (w/v) acrylamide). Protein samples were incubated for 1.5-3 min in a boiling water bath in a sample buffer modified from that of Laemmli [203], containing final concentrations of 0.063 M Tris-HCl (pH 6.8), 20 mg/ml SDS, 100 mg/ml glycerol, 0.05 M dithiothreitol, 0.01 M disodium EDTA, and 0.01 mg/ml bromophenol blue. Electrophoresis was conducted at room temperature with a current of 1 mA/gel. After electrophoresis, gels were cut at the position of the tracking dye.

Gel Staining

Gels to be stained for protein or carbohydrate were washed overnight in 10% (v/v) acetic acid, 25% (v/v) 2-propanol [94] to fix proteins and remove SDS. Protein was stained overnight in a solution of 0.4 mg/ml Coomassie Blue G-250 in 35 mg/ml perchloric acid and destained in 7.5% (v/v) acetic acid [144], with the exception of gel B in the righthand panel of Fig. 2, which was stained overnight in 2.5 mg/ml Coomassie Blue R-250 in acetic acid-methanol-water (10:40:50, v/v/v) [353] and destained in 10% (v/v) 2-propanol, 10% (v/v) acetic acid, containing 1 mg/l Coo-
massie Blue R-250 [94].

Dextranasesucrase and levansucrase activities were detected by a periodic acid−Schiff (PAS) stain. After electrophoresis, the enzymes were reactivated by overnight incubation of the gels at 25° in 10−15 volumes of pH 5.2 buffer (0.05 M sodium acetate) containing 10 mg/ml Tween 80, 2 mM CaCl₂, 0.2 mg/ml sodium azide, and in some cases, 10 mg/ml dextran T10. The gels were then incubated in the same buffer with 50 mg/ml sucrose (to detect both dextranasesucrase and levansucrase) or 50 mg/ml raffinose (to detect only levansucrase [43]). Gels incubated with raffinose were added immediately afterwards to ten volumes of the same buffer, lacking raffinose but containing 100 units of dextranase, and incubated 5 h at 37°. Dextranase was added after incubation with raffinose rather than during the incubation because of a protease impurity in the dextranase. Enzyme reactions were stopped by immersing the gels in 10 % (v/v) acetic acid, 25% (v/v) 2-propanol. After washing overnight in this solution to remove monosaccharides and unreacted sucrose, polysaccharide was stained by a periodic acid−Schiff procedure [173].

All gels were photographed through a green filter.

Enzyme Irradiation

Aliquots (0.1 ml) of dialyzed culture supernatant concentrate were placed in 2 ml ampules and lyophilized, and the ampules were sealed under vacuum. The ampules were irradiated at room temperature inside a rotating cannister symmetrically surrounded by six ⁶⁰Co sources. The dose rate was 0.34 Mrad/h, based on ferrous sulfate calibration [102]. Following irradiation, lyophils were reconstituted with 1 ml double strength assay buffer. Enzyme assays were performed 1 h after adding the reconstituting buffer. Glycansucrase activity was quantitatively recovered from non-irradiated lyophils.
Carboxymethylation

Extensive carboxymethylation of the culture supernatant concentrate, following denaturation and reduction, was performed by Method 2 of Weber et al. [349], with the addition of 10 mM disodium EDTA to the guanidine hydrochloride solution.
RESULTS

Radiation Inactivation

The rate of loss of enzyme activity during room-temperature irradiation is empirically related to enzyme size by eqn. 1 [221], where \( u \) is the slope of a plot of radiation dose (in Mrad) vs. the logarithm (\( \log_{10} \)) of remaining enzyme activity.

\[
\text{Molecular Weight} = (6.4 \times 10^5)(\ln 10)u \tag{1}
\]

Fig. 1 is a plot of radiation dose vs. dextransucrase (polysaccharide-synthesizing) activity. The plot is linear down to its end point, where only 4% of the initial activity remains. From Fig. 1, eqn. 1 gave a molecular weight of \( 201,000 \pm 3000 \).

SDS Gel Electrophoresis

Fig. 2 shows SDS gels of two dextransucrase preparations. A purified preparation, from which levansucrase and dextran had been removed (gels at left), shows two active dextransucrase bands at 177 kDa and 158 kDa (gel C) corresponding to two major protein bands at the same positions. A minor band at 168 kDa was sometimes observed as well. This band is visible in gel A at the lower edge of the major, 177 kDa protein band. Reduction and carboxymethylation reduced the mobility of the protein bands, but otherwise did not change the protein band pattern (gel B).

SDS gels of a dialyzed concentrate of culture supernatant (gels at right) show that by far the major glycansucrase activity in this preparation is that of the 177 kDa dextransucrase. The intense polysaccharide band in both gels C and F corresponds to the protein band at this position in gel A. A weaker band at 158 kDa can also be seen in gels C and F. The addition of 10 mg/ml dextran T10 to Tween 80- and sucrose-
Figure 1. Radiation inactivation of dextranucrase

Enzyme lyophils were irradiated with $^{60}$Co and reconstituted with buffer. The residual enzyme activity, measured by rate of polysaccharide formation, was then determined. Each point on the curve represents 5-6 enzyme lyophils. Error bars are 95% confidence intervals for the mean.
Figure 2. SDS gel electrophoresis of crude and purified dextranucrase preparations

The molecular weights and positions of standards are shown to the left of the gels.

**Left**: purified enzyme  
(A) Protein stain.  
(B) Protein stain on reduced and carboxymethylated sample.  
(C) Glycansucrase activity, detected by a periodic acid-Schiff stain after a short incubation with sucrose.

**Right**: crude enzyme (dialyzed concentrate of culture supernatant)  
(A)-(C) Same as (A)-(C) for gels of purified enzyme at left.  
(D) Glycansucrase activity; PAS stain after long incubation with sucrose.  
(E) Levansucrase activity; PAS stain after long incubation with raffinose and dextranase treatment.  
(F) Glycansucrase activity; polysaccharide visible by reflected light after long incubation with sucrose and dextran T10 (gel is in 25% 2-propanol, 10% acetic acid to enhance polysaccharide bands [215]).
containing buffers caused no additional bands to appear (gel F). As with the purified enzyme, reduction followed by carboxymethylation caused no change in the protein band pattern except for a general reduction in mobility (gel B).

Gels D and E show the presence of levansucrase at 92 kDa and 116 kDa. Gel E, where the substrate was raffinose (a substrate for levansucrase but not dextranucrase [43]), shows that the 92 and 116 kDa bands, undiminished in intensity, are levansucrase, and that the 158 and 177 kDa bands, which have virtually disappeared, are dextranucrase. A dextranase treatment was given to gel E after incubation with raffinose, because otherwise faint polysaccharide bands occurred at the 158 and 177 kDa positions, even though sucrose was not detected in the raffinose by thin-layer chromatography [26].

Unlike dextran, levan was never visible as white zones of polysaccharide (gel F), unlike other systems where larger amounts of levansucrase are present [256, 291]. Even with the sensitive periodic acid-Schiff (PAS) stain, bands of levan were clearly visible only after long incubations with sucrose or raffinose. (In gel D, where the substrate was sucrose, the top third of the gel is completely opaque due to the much higher amount of dextran.)

**Control Experiments**

Below are considered several factors that might cause SDS gel electrophoresis to overestimate dextranucrase molecular weight. The question of the effect of SDS on dextranucrase activity is considered separately in a subsequent section.

**Loss of small peptides or cofactors** The substitution of unconcentrated culture supernatant, even if undialyzed, had no effect on the staining patterns except to give fainter bands. (Dialysis lowers the ionic strength, accelerates stacking, and removes potassium that otherwise precipitated SDS from the electrophoretic sample buffer [141].)

When long incubations with sucrose failed to show any form of dextranucrase smaller than 158 kDa, the gels were checked for retention of
low-molecular-weight proteins by staining with Coomassie Blue. The low-
molecular-weight protein bands (those in the region of 50 kDa) could
easily be detected even two days after electrophoresis, although they
were diffuse. Protein is difficult to remove completely from SDS gels,
because part of it becomes physically attached to the gel [202].

Non-dissociation of peptides To be certain that neither of the
two dextranucrase peptides dissociated into smaller ones, the length and
temperature of the denaturing pretreatment were varied [349]. Pretreat-
ment in a boiling water bath for 20 min or at 37°C for 90 min gave the
same activity band patterns as the standard pretreatment in a boiling
water bath for 1.5 min, although the polysaccharide bands produced with
sucrose incubation were fainter after the 20 min treatment, and there was
a small shift in peptide distribution (from higher to lower molecular
weight). Peptide cleavage by hot SDS [196, 202] has not been ruled out
as the cause for this. The substitution of 5% (v/v) 2-mercaptoethanol
for 0.05 M dithiothreitol or the elimination of EDTA from the pretreat-
ment did not affect the patterns. The addition of 4 M urea, with or
without 10 mM EDTA, to the pretreatment had no effect on the protein band
pattern, except to decrease the resolution slightly. More convincingly,
reduction and extensive carboxymethylation (both gels labeled "B" in
Fig. 2) did not change the protein band pattern, other than to reduce
resolution and to decrease the mobilities of all the protein bands.

On the other hand, the PAS-positive region above these peptides that
developed on long incubation of the gels with sucrose argues for the pre-
sence of small numbers of aggregates of the 158 kDa and 177 kDa peptides;
small, because no protein was detected in this region with Coomassie
Blue. That no discrete bands occurred in this region, and that no gradi-
ent was seen in the intensity of the color, implies slow or reversible
formation of these aggregates. Alternative explanations for the active
protein seen in this region include incomplete exchange of Tween 80 with
SDS (which would reduce peptide mobility [199, 257]) and physical attach-
ment of non-aggregated peptides to the polyacrylamide matrix [202].

No activity was detected on the gels if SDS (1 mg/ml) was substi-
tuted for Tween 80 during the incubation with sucrose.

Presence of dextran  The presence of dextran in the sample, which causes the nondenatured enzyme to aggregate strongly, could be proposed as a factor preventing dissociation even under rigorous conditions. In control experiments, however, the presence of dextran could not be demonstrated to cause aggregation on SDS gel electrophoresis. Dextran T10 and native dextran were preincubated overnight at 10 mg/ml with the enzyme solution (at pH 5.2), or the enzyme was allowed to react with 50 mg/ml sucrose (at pH 5.2) shortly before electrophoresis. The migration of the dextranucrase peptides was not changed in any case, and no dextran could be detected at their positions by a PAS stain. Pretreatment of the purified enzyme with hydroxylamine (0.2 M hydroxylamine hydrochloride for 6 h at 4°C and pH 6.8), to remove carbohydrate that might be attached to the enzyme in ester linkage, changed neither the protein nor the activity band patterns.

No effort was made to remove endogenous dextran with dextranase, because all commercial dextranases examined were found to contain enough protease to alter the protein band pattern to give greater amounts of low-molecular-weight peptides (all inactive).

Artifacts of Laemmli gel system  The conclusion reached in this study depends on obtaining an accurate molecular weight for the dextran-sucrase monomer by SDS gel electrophoresis. In particular, it is important that electrophoresis not grossly overestimate the molecular weight. The only likely reason for such an overestimate would be anomalously low migration caused by the presence of a large amount of covalently bound carbohydrate [308]. Results described earlier in this section do not support the presence of a large amount of covalently bound carbohydrate at the time the dextranucrase peptides enter the separating gel. This is in agreement with the findings of Koga et al. [193] and of Shimamura et al. [310], who both concluded that the glucansucrases they were investigating were not glycoproteins.

Most of the other common causes of anomalous migration, which include large hydrophobic [140, 257] or strongly acidic [175] regions in a
protein, and larger-than-average pore size in the top half-centimeter of polyacrylamide gels [217], lead to higher than expected mobility, and so cause molecular weight to be underestimated, not overestimated.

Additional questions on the accuracy of the molecular weight determined by SDS gel electrophoresis arise from the use of a discontinuous buffer system [257, 322]. The apparent molecular weight of even moderate-sized proteins (e.g., 50-80 kDa) can change by 10,000 or more on switching from the Laemmli to the Weber-Osborn [348] system. For dextran sucrase, Weber-Osborn electrophoresis, with the use of 4 M urea in the protein-denaturing buffer, gave a single molecular weight for dextran sucrase of 156,000 for the aged enzyme concentrate used for the irradiation experiments. This is in good agreement with the 158,000 found with the Laemmli buffer system for the same dextran sucrase form.

Enzyme Reactivation after SDS Treatment

To conclude from the SDS gel experiments that no form of dextran sucrase exists smaller than 158 kDa depends on recovering the enzyme activity quantitatively after electrophoresis. Therefore, the method adapted from Russell [290, 291] for reactivating dextran sucrase and levansucrase activity in the presence of SDS, which consists of the addition of a ten-fold excess of nonionic detergent, was checked to see how nearly this was true. In the experiments described below, nonionic detergent was added to a concentration of 10 mg/ml. Tween 80 was used for most of the experiments.

Fig. 3 shows the effect of treating dextran sucrase with SDS at room temperature. If Tween 80 were added before SDS, no activity was lost. If SDS were added first, all activity was lost, but not all of the activity returned on adding Tween 80. In the presence of 1 mg/ml SDS, more than 2 h from the addition of Tween 80 was required for maximal recovery of activity. The addition of dextran T10, which could conceivably cause higher rates or yields for reactivation (Ghelis and Yon [120], pp. 493-496), did not affect the rate or the extent of reactivation.
Figure 3. Reactivation of dextranucrase by Tween 80 after preincubation with SDS at 25oC.

In all cases but O and ▲, buffered detergent solutions were added at time zero to aliquots of a single stock enzyme solution that had been preincubated for 30 min with buffered SDS (1 mg/ml). For these two cases, Tween 80 (10 mg/ml) was substituted for SDS in the 30-min preincubation, and buffered SDS (▲) or buffer only (O) was added at time zero. Buffer was 0.1 M sodium acetate, 0.2 mM calcium chloride, 0.2 mg/ml sodium azide, pH 5.2. Enzyme concentration was the same in all cases. Detergent concentrations after time zero:
- ●, 1 mg/ml SDS, no Tween 80; O, no SDS, 10 mg/ml Tween 80;
- □, 0.5 mg/ml SDS, 10 mg/ml Tween 80; △, ▲, and ▼, 1 mg/ml SDS, 10 mg/ml Tween 80. ▼, dextran T10 (10 mg/ml) also added at time zero.
If SDS were diluted to a lower concentration (0.5 mg/ml in Fig. 3 and as low as 0.05 mg/ml in experiments not shown), reactivation was faster but no more complete. Incomplete recoveries of activity were also obtained when the reactivating detergent was Triton X-100 or Lubrol PX, with the amount of activity recovered identical to that recovered with Tween 80. Ca^{2+} was present at 2 mM in the added solutions of the first two detergents, a tenfold higher concentration than for most other experiments of this section, as discussed below. Ca^{2+} is necessary to prevent inactivation of dextranucrase by Triton X-100 [237].

The fraction of activity recovered was variable, but independent of the length of the preincubation with SDS (1 min to 2 h) and of the SDS concentration in the precincubation (0.1-20 mg/ml). The fraction of activity recovered did appear to depend on prior loss of activity from other causes. The culture supernatant used had an activity of 3.5-4 U/ml initially. Overnight dialysis at 4° to lower Ca^{2+} concentration did not reduce this activity, whether the dialysis was at pH 5.2 or 6.8. However, a third of the activity was lost over the next 2 days, whether the dialysate was stored at 4° or -20°. The resulting enzyme was used in the experiment of Figs. 3-4.

In the following weeks, the remaining activity in the dialysates slowly decreased to about half the original activity (i.e., to about 2 U/ml). After preincubating with SDS, this residual activity, which was the same amount of activity recovered after SDS treatment in Fig. 3, was recovered completely on adding Tween 80. It is therefore unlikely that when failure to recover full activity took place, it was due to a too-rapid removal of bound SDS (by rapid dilution with nonionic detergent solution) [202, 209].

The preincubation buffers for the experiments of Fig. 3 contained 0.2 mM Ca^{2+}, one-tenth the concentration used for the gel experiments. In the experiments of Figs. 2 and 4, Ca^{2+} in the enzyme solution had been reduced to even lower levels by dialyzing against a buffer without added Ca^{2+}. Higher Ca^{2+} concentrations precipitated the dodecyl sulfate anion as the Ca^{2+} salt [198, 217], especially when the SDS concentration was
low [209]. This precipitated Ca$^{2+}$ salt could be dissolved by heating, by adding large amounts of sodium, or by adding small amounts of Tween 80. Higher concentrations of Ca$^{2+}$ during the preincubation with SDS did not change the activity recovered, despite reduction of SDS concentration by precipitation. This is probably because of the low residual concentration of SDS. Increasing the Ca$^{2+}$ concentration in the renaturing Tween 80 solution added to enzyme solutions that had been precincubated with SDS did not increase the amount of enzyme activity recovered (range of final Ca$^{2+}$ concentrations was 0.1–5.0 mM).

Fig. 4 shows that increasing the preincubation time at boiling water bath temperature decreased the activity recovered. The loss of activity without heating (time zero in Fig. 4) was apparently caused by SDS, as described above. None of the other components of the solubilizing buffer gave measurable inhibition at the assay concentrations.
Figure 4. Effect of incubation in a boiling water bath on reactivation of dextranucrase by Tween 80

Aliquots (0.2 ml) of culture supernatant dialyzed against the Tris buffer used for electrophoresis were mixed with an equal volume of double-strength SDS sample buffer and were held in a boiling water bath in tightly stoppered tubes for various lengths of time. These solutions were diluted 50-fold with buffer containing 10 mg/ml Tween 80 (the same reactivating buffer as for Fig. 1) and assayed after 2 h.
The question of whether dextran sucrase is active as a monomer, or only as a dimer or larger oligomer, is an issue that has not previously been explicitly addressed, largely because under nondenaturing conditions the enzyme aggregates strongly, with particle sizes often ranging up into millions of daltons [98, 215, 239, 286, 290]. The aggregation is mediated by dextran [116, 200, 286], but also occurs in its absence [116, 201, 286, 301]. The usual methods of determining the molecular weight of an active oligomer (i.e., ultracentrifugation, nondenaturing gel electrophoresis, and gel filtration) give questionable results, and as a consequence, no consensus exists on the molecular weight of the active species of dextran sucrase from either *Streptococcus* or *Leuconostoc*, although dextran sucrase has been known since 1941 [137], and a molecular weight of 284,000 for B-512F dextran sucrase was obtained by ultracentrifugation as early as 1962 [84].

To circumvent the aggregation problem, we have made use of radiation inactivation, a technique that measures the size of the minimal unit required to express enzyme activity. This unit may be either a monomer or an oligomer [178], but because the molecular weight found is that of a functioning unit of enzyme [19, 178], it should not reflect the nonfunctional aggregation that occurs with dextran sucrase. SDS gel electrophoresis has been used to determine the single-peptide molecular weights of dextran sucrase. The results of these two methods have been compared to see whether the peptides are functionally associated as subunits.

**Radiation Inactivation vs. SDS Gel Electrophoresis**

The molecular mass of 201 kDa determined by radiation inactivation is 14% above the 177 kDa of the dextran sucrase species which SDS gels indicate is responsible for the majority of dextran synthesis in fresh
culture supernatant, and 27% above the 158 kDa of the form whose amount increases in aged preparations. (For the effects of aging, see Part II of this dissertation.)

In relation to this discrepancy, some questions have been raised [151] about the validity of the empirical formula expressed in eqn. 1. More specifically, it has been shown [18] that at the low dose rates typically delivered by $^{60}$Co irradiation equipment, the effective dose rate for enzyme inactivation experiments is larger than the value determined by ferrous sulfate calibration, probably due to enzyme inactivation by secondary mechanisms, such as chemical attack by radiation-generated radicals. This results in some overestimation of the molecular weight unless enzyme standards are used to construct a molecular-weight calibration curve [18]. The degree of overestimation can be even higher in the presence of nonionic detergents [19].

The $^{60}$Co irradiation for this study was performed before the usefulness of such enzyme standards was described by Beauregard and Potier [18], but based on their report, the 201 kDa determined here by radiation inactivation probably corresponds to the 158 kDa form of dextransucrase seen on SDS gels, which predominated strongly in the aged preparation used for the irradiation experiments. Beauregard and Potier found [18] that a dose rate of 0.44 Mrad/h measured by the ferrous sulfate method was equivalent to 0.64 Mrad/h when measured by the loss of activity of irradiated enzyme standards. This caused eqn. 1 to overestimate molecular weights by 45%. In comparison, the 201 kDa determined here by radiation inactivation at a dose rate of 0.34 Mrad/h (ferrous sulfate calibration) is 27% above 158 kDa.

For the purposes of this study, where the important question was whether monomeric dextransucrase is competent to synthesize dextran, the sizes of 158-177 kDa determined by electrophoresis are not significantly different from the 201 kDa determined by radiation inactivation, which can be considered to represent a monomer. (To represent a dimer, radiation inactivation would have to underestimate the molecular weight by at least 57% (= 100% x $2 \times 158,000/201,000$).) This leads to the conclu-
sion that where dimer formation or higher aggregation of these peptides occurs, only one member of the aggregate must be active for dextran to be synthesized. From the perspective of dextran synthesis, the enzyme must be considered to be a monomer, and mechanistic models [287] should reflect this.

Another important conclusion is that the vast majority of dextran synthesis in the culture supernatant is carried out by these two peptides and not by substantially smaller ones. The large size of the active peptides is surprising, considering their bacterial source and their extracellular location, but is close to the size often found by SDS gel electrophoresis for glucansucrases from Streptococcus species [106, 123, 180, 193, 294]. There are reports of much smaller dextransucrases; among them, 45 kDa for S. mutans GS-5 [200], 48 kDa for L. mesenteroides B-1299 [186], and most critically for this work, 64 kDa for L. mesenteroides B-512F [188], the same enzyme source used here.

Because the culture supernatant used here was not purified, except by ultrafiltration and dialysis (and not always by either), all forms of the enzyme should have been present. No dextransucrase smaller than 158 kDa was detected on SDS gels, even after long incubations with sucrose or with sucrose and dextran T10 (e.g., gel F in Fig. 2), added in case a low-molecular-weight form of the enzyme depended strongly on dextran for activity, as do streptococcal glucansucrases [239, 281, 343]. Russell [290] has commented on his failure to detect a low-molecular-weight glucansucrase in S. mutans (such as was observed by Kuramitsu [200]), as have Grahame and Mayer [123] for S. sanguis dextransucrase.

The relationship between the different molecular-weight forms of dextransucrase were not investigated here. Other research groups have reported the formation of a smaller form of dextransucrase from a larger one in cell-free preparations from S. mutans [180] and S. sanguis [123]. In the latter case, a 172 kDa peptide was converted into a 155 kDa peptide. These peptide sizes are very close to those found here for L. mesenteroides B-512F dextransucrase. Evidence was given for proteolysis of S. mutans [180] and S. sanguis [123] glucansucrases, which is also likely
to be taking place in the B-512F system here. Russell has found that adding a protease inhibitor, phenylmethylsulfonylfluoride, to crude preparations of \textit{S. mutans} results in the detection of a 150 kDa glucansucrase in addition to the 132 kDa and 140 kDa forms observed in its absence. Ciardi et al. [54] found dextranase treatment of glucansucrases from \textit{S. mutans} OMZ 176 resulted in a new, rapidly migrating glucansucrase band on nondenaturing gels. While limited evidence was presented that this was not formed by protease action, incubation with dextranase caused increasing loss of activity.

An alternative to proteolysis for the origin of the multiple molecular weight forms is that the 158 kDa and 177 kDa forms consist of more than one peptide, and only partially dissociate on SDS electrophoresis. This is unlikely, as discussed under Results, based on the failure of rigorous dissociating conditions, such as carboxymethylation following denaturation by hot, concentrated guanidine hydrochloride and reduction by 2-mercaptoethanol, to reduce the amounts of these forms (compare gels A and B in either set of gels shown in Fig. 2).

Recovery of Activity Following Electrophoresis

The strength of any conclusion on the absence of a low-molecular-weight form of dextranucrase (i.e., in the neighborhood of 50,000) depends on the ability to recover dextranucrase activity quantitatively in SDS gels. Otherwise, an argument can be made that a low-molecular-weight form of the enzyme exists that is not reactivated. Russell [290] found that any of a variety of nonionic detergents at 10 mg/ml concentration would more than reverse the inhibition, caused by 1 mg/ml SDS, of the mixed glucansucrases of \textit{S. mutans} culture supernatant, actually producing a small net activation, with no change in the proportion of dextran, mutan, and levan synthesized.

For B-512F dextranucrase, however, inactivation was not fully reversed (Fig. 3), even for mild SDS treatments. Harsher treatments decreased the activity recovered further (Fig. 4). For the SDS gels of
Fig. 2, a 3-min incubation in a boiling water bath was used, in order to dissociate all but covalent bonds [140], despite the resulting decrease in recoverable activity occurring at this temperature. Failure to detect glucansucrase activity on SDS gels after pre-electrophoretic incubation in a boiling water bath for more than 5 min has been reported by Newman et al. [256] for S. mutans. In this study, activity was found even after 20 min, but this may be due to the much larger amount of activity applied to the gels.

Russell does not mention any lag in recovery of activity on adding nonionic detergent, as was observed here (Fig. 3). Whether the failure to recover full activity is caused by incorrect refolding of some of the enzyme molecules (see pp. 289-296, 366, and 471-484 in Ghelis and Yon [120]), by specific inhibition of the enzyme by tightly bound SDS [155, 209, 333], or by some other cause was not determined.

A quantitative recovery of the remaining activity was obtained from enzyme preparations whose activity had been substantially lost before SDS treatment (see Results). It is not known whether this loss was related to instability of the B-512F dextranulase at low Ca^{2+} concentrations [237], or if it is significant for streptococcal glucansucrases. The existence of subpopulations of enzyme molecules, differing in some as yet undetermined way, seems likely.

Summary

SDS gel electrophoresis of fresh culture supernatant showed that the major active form of B-512F dextranulase had a size of 177 kDa, and that on aging a second form of 158 kDa became prominent. Radiation inactivation of aged dextranulase gave a single size of 201 kDa, which probably corresponds to the 158 kDa form seen on SDS gels. No evidence for any dextranulase species smaller than 158 kDa was found, either in the presence or absence of dextran T10. These results show that dextranulase is active as a large, single peptide, and that subunit association is not required for dextran synthesis.
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PART VII

A PROTEIN-STAINING ARTIFACT IN THE PERIODIC ACID–SCHIFF REACTION FOR DETECTION OF DEXTRANSUCRASE ON SDS POLYACRYLAMIDE GELS
ABSTRACT

One use of the periodic acid-Schiff (PAS) stain is to detect glycan-sucrase activity on polyacrylamide gels. The products of glycansucrases, are polysaccharides that, unless they contain primarily α-(1→3) linkages, stain strongly with the PAS stain. When gels containing heavy bands of glycansucrase-synthesized polysaccharide were stained, however, a dark background developed, and proteins other than the active glycansucrase proteins became colored during the destaining washes of the PAS-staining procedure or during gel storage. Proteins on gels that were not PAS-stained became stained if the gels were placed in the destaining or storage solvents next to PAS-stained gels. This showed that the staining of proteins was being caused by reaction with stained material that had diffused from its original location on the PAS-stained gels. The diffusion could be slowed an order of magnitude or more by following the PAS stain by treatment with formaldehyde or other crosslinking reagent. The reaction of stained material with proteins could be prevented by using chymotrypsin to remove the proteins from the gels at the stage after polysaccharide synthesis but before using the PAS stain.
The periodic acid–Schiff (PAS) stain is widely used to detect glyco­
proteins on polyacrylamide gels [195, 334]. The PAS stain can also be
used as an activity stain for glycansucrases, enzymes that polymerize
either the fructose or glucose moiety of sucrose to form polysaccharide.
On SDS gels, this is done by reactivating the enzymes with a nonionic
detergent and then incubating the gels with sucrose [53, 123, 180]. Un-
reacted sucrose and low-molecular-weight products are washed away, and
polysaccharide, which remains at the enzyme position, is stained by a PAS
procedure.

Dextran sucrase, the glycansucrase that synthesizes the polysaccha-
ride dextran, has more than one molecular-weight form [123]. In looking
for minor, low-molecular-weight forms of dextran sucrase in cultures of
Leuconostoc mesenteroides (see Part VI of this dissertation), SDS gels
were PAS-stained after long incubations with sucrose, resulting in dense
staining of the major dextranucrase bands.

In gels with intensely stained bands, regions of the gel which were
colorless immediately after destaining developed strong background color
during storage. This background was not reduced by extended washing in
the dilute acetic acid used for the original destaining. More impor-
tantly, the presence of densely stained bands in the upper part of the
gels caused all the protein bands in the gel to become stained during
diffusion destaining. Even with moderately intense bands, which caused
minimal increases in background on storage, protein bands became stained
after one or two weeks. This raised special concern about whether pro-
tein bands that gave positive PAS stains after incubation with sucrose,
but that were barely resolved from much more strongly staining bands,
were truly active or were artifactually stained.

Proteins that are not glycoproteins sometimes give a positive PAS-
stain, for reasons that include the binding of dye to SDS or to unoxi-
dized protein [16, 121, 268, 348], and the reaction of Schiff's reagent
with periodate-oxidized protein [16]. The staining described here has a
different cause, related to the presence of large amounts of carbohydrate in the stained gels; much more carbohydrate than is present in the glycoprotein samples on which the PAS stain is normally used. To avoid the staining of proteins, protease was used to remove the protein in the gels after polysaccharide formation by the glycansucrases, but before using the PAS-stain.
MATERIALS AND METHODS

Materials

Schiff's reagent, vinyl sulfone (divinylsulfone), 1,4-butanediol diglycidyl ether, pararosaniline hydrochloride, α-chymotrypsin (Type II, crystalline, from bovine pancreas), trypsin (Type IX, crystalline, from porcine pancreas), and pepsin (crystalline, chromatographically purified, from porcine stomach mucosa) were from Sigma Chemical Co. (St. Louis, MO). Sodium cyanoborohydride was obtained from Aldrich Chemical Co. (Milwaukee, WI) and purified by precipitation from acetonitrile with methylene chloride [167]. Activated charcoal (6-14 mesh) was from Fisher Scientific Co. (Pittsburgh, PA). Amberlites IR-120, IRA-400, and MB-3 were from Mallinkrodt, Inc. (Paris, KY). Dextran T40 was from Pharmacia Fine Chemicals (Piscataway, NJ).

Electrophoresis

SDS gel electrophoresis was performed by the method of Laemmli [203] on 5 x 90 cm cylindrical gels (6% acrylamide). Electrophoresis was conducted at room temperature at 1 mA/gel. After electrophoresis, gels were cut at the position of the tracking dye.

Protease Treatment

Trypsin and chymotrypsin

After washing with 25% 2-propanol or alcoholic fixative, gels were incubated for at least 2 h in 20 volumes or more of 0.05 M Tris (pH 7.8) [100] containing 5 mM CaCl₂ [346, 352]. The buffer was changed, and protease solution (1-2 mg/ml trypsin or chymotrypsin in the same buffer) amounting to 1% of the total buffer volume was added. After approximately 12 h incubation at 37°, the gels were washed overnight in alcoholic fixative and PAS-stained.
The same procedure was followed as with trypsin and chymotrypsin, but using dilute HCl, pH 1.7 [295], instead of pH 7.8 Tris buffer.

**Gel Staining**

All staining and destaining procedures were done in the dark and at room temperature, except for incubations with Schiff's reagent, which were done at 4°C. Before staining, gels were washed overnight in 10% acetic acid, 25% 2-propanol [94], to immobilize proteins and remove SDS. Destaining was always done by diffusion. Electrophoretic destaining was not tried. The final treatment for all gels was washing and storage in 7.5% acetic acid.

**Protein stain**

Protein was stained overnight in a solution of 0.4 mg/ml Coomassie Blue G-250 in 35 mg/ml perchloric acid and destained in 7.5% acetic acid [144].

**Activity stain**

Gels to be stained for glycansucrase activity were incubated overnight at 25°C in 10 to 15 volumes of 0.05 M sodium acetate buffer (pH 5.2), containing 10 mg/ml Tween 80, 2 mM CaCl₂, and 0.2 mg/ml sodium azide. The gels were then incubated in the same buffer with the addition of 50 mg/ml sucrose. Enzyme reactions were stopped by immersing the gels in 10% acetic acid, 25% 2-propanol, in which gels were washed overnight to remove monosaccharides and unreacted sucrose. Carbohydrate was then stained by a PAS stain (procedure of Kapitany and Zebrowski [173] unless stated otherwise), by a periodic acid-Alcian Blue stain [347], or by a reductive dye-coupling procedure.

For reductive dye-coupling, periodate oxidation and post-oxidation washes were done as for PAS-staining. The oxidized gel was added to a
solution of 1 part 2-propanol to 2 parts 0.05 M sodium phosphate (pH 7), and sufficient solid pararosaniline hydrochloride and sodium cyanoborohydride were added to bring solution concentrations to 1 mg/ml and 20 mM, respectively. The high propanol concentration was required to prevent precipitation of the reduction product, pararosaniline hydride.

After overnight incubation, the gels showed reddish bands on an amber background the same color as the surrounding solution. Excess reagents were washed out with the same propanolic solvent, and the coupled dye was recolorized by incubating overnight (color did not increase after 2-3 h) in 20 mg/ml sodium iodate in 7.5% acetic acid. The stained bands were slightly more bluish in color than those of PAS-stained gels.

Post-staining treatments

Some of treatments described below caused the gels to swell, either during the treatment itself or, more commonly, during the subsequent washes. Gels could be returned to their original dimensions by washing in alcohol- or salt-containing solvents, and then in 7.5% acetic acid.

Formaldehyde Gels were treated with formaldehyde by the method of Burk et al. [37].

Divinylsulfone and 1,4-butanediol diglycidyl ether Gels were treated with basic, aqueous emulsions of divinylsulfone or 1,4-butanediol diglycidyl ether as described by Porath [269] or by Porath and Axén [270].

Quinone Gels were treated with quinone by a modification of the method of Porath and Axén [270]. If unmodified, this method gave a brown background, resulting mostly from the high pH of the treatment. Gels were given two 1 h washes in a freshly mixed solution of 4 parts 0.05 M sodium phosphate (pH 7.0) to 1 part 0.1 M quinone in 2-propanol, and then were washed overnight with the same solvent lacking quinone.

Bisulfite Gels were washed three times for 30 min in a fresh solution of 0.2-5% sodium metabisulfite, in the presence or absence of 7.5% acetic acid.
RESULTS

Background Increase

In the method of Kapitany and Zebrowski, background color was insignificant immediately after the initial destaining in 7.5% acetic acid, but developed on storage if heavily-stained bands were present in the gel (gel C). The rate of background increase varied with the batch of Schiff's Reagent for unknown reasons. This background was not visibly reduced by washing in dilute acetic acid.

Washing or storage in metabisulfite has been used to reduce or prevent similar background increases [268] (gels D-F). A variety of reductive and oxidative treatments were found to be equally or more rapid and complete. Storage for several days in 10 mg/ml periodic acid, or briefer storage in 10 mg/ml sodium periodate at pH 7, completely eliminated even the heaviest background color, giving sharp, dark bands on a colorless field.

Background could also be reduced by incubation with sodium cyanoboroxydride at pH 7, or more rapidly at lower pH. Washing with 0.1 M sodium hydroxide also reduced the background, but rapid diffusion from the stained bands subsequently took place, resulting in a faster increase in background than occurred before the hydroxide treatment. This faster diffusion might have been caused by the generation of small molecules by the alkaline degradation of periodate-oxidized dextran [351]. Sodium borohydride was no more effective at lightening the background or lightening the stained bands than the 0.1 M NaOH in which it was used, probably because the dye in the gels was in the nonreducible carbinol form.

All of these methods, including metabisulfite treatment, were essentially bleaching procedures, and decreased band intensity and therefore sensitivity. To avoid using these methods, ways of reducing the rate of background increase were investigated. Treating the freshly destained gels with crosslinking agents (divinylsulfone, formaldehyde, quinone, or 1,4-butanediol diglycidyl ether) slowed the background increase by at
least an order of magnitude. Divinylsulfone treatments, applied both before and after PAS-staining, were only effective after staining, implying derivatization of amino groups rather than hydroxyl groups. Derivatization of the amino groups of the dye by 1,4-butanediol diglycidyl ether was also suggested by a color change in the stained bands, which turned from magenta to bright blue.

Washing the stained gels with the solvent 10% acetic acid, 25% 2-propanol slowed the diffusion, and the gels could then be stored in 7.5% acetic acid alone, with the diffusion staying at the same low rate. This may be related to the report [240] that dextran oxidized by periodate in aqueous ethanol is insoluble in water after this treatment. A longer oxidation period (e.g., overnight) also slowed background development in the resulting stained gel, but a longer incubation in Schiff's reagent did not. The commonly used bisulfite or metabisulfite washes immediately after immersion in Schiff's reagent were also effective.

A different procedure, one that did not rely on the Schiff reaction, was the reductive coupling of pararosaniline to the oxidized gel. Pararosaniline and sodium cyanoborohydride were mixed to produce the leuco-base, pararosaniline hydride. This was reductively coupled with excess sodium cyanoborohydride to oxidized polysaccharide in the gels. The coupled leucobase was reoxidized to its intensely colored form with sodium iodate. Identical gels stained by reductive dye-coupling (gel G) and the standard PAS stain (gel H) are compared in Fig. 1. A high degree of crosslinking in gel G is suggested by the constriction at the region of highest stain density.

Oxidants that gave low background during reoxidation of the coupled leucobase, in decreasing order of effectiveness in recolorizing the dye, included periodic acid and ammonium persulfate, sodium iodate, potassium triiodide, ferric chloride, and hydrogen peroxide. This series was determined from oxidations in dilute acetic acid. At neutral or basic pH, weaker oxidants were also effective.
Protein Staining Artifact

The artifactual staining of nonglycosylated proteins can be seen by comparing gels A and B. Gel A was destained in dilute acetic acid in which a heavily-stained gel was also being destained. Gel B was destained with no other gels in the destaining solution. Other gels in Fig. 1 where artifactually stained protein bands can be seen are gels D-F (the band below the dense, heavy band), and gels H, L, and M (lower halves of the gels).

Alcian Blue

An alternative carbohydrate stain to the PAS stain, also developed for staining glycoproteins in polyacrylamide gels, is based on the binding of the strongly cationic, tetravalent dye Alcian Blue to the bisulfite adducts of periodic acid-oxidized carbohydrate [347]. Protein staining artifacts occurred to an equal or greater extent when this method was applied, and the sensitivity was lower. In addition, the method gave an appreciable light blue background (gels L and M).

Dye adsorbents

To reduce uptake of stain from the destaining solution by the gels, a cartridge [78] containing activated charcoal or mixed-bed ion-exchange resin (to absorb both the positively-charged pararosanilin and the negatively-charged Schiff-reaction products) was placed in the destaining solution. A mixture of Amberlite IR-120 and Amberlite IRA-400 was much more effective than Amberlite MB-3, which is itself a mixture of IR-120 and IRA-410. IRA-410 is a weaker anion-exchange resin than IR-400.

Model staining system

Gels of non-glycoproteins were incubated in sugar solutions (10-50 mg/ml in 5-7.5% acetic acid or in alcoholic fixative) and then PAS-stained. Whenever glycosides were present, gels became a uniform rose color while in Schiff's reagent. The rose color washed away on destain-
Figure 1. SDS gels of dextranucrase preparations

A–B   PAS background after destaining with (A) heavily-stained gel also in the destaining solvent, and (B) no other gels in destaining solvent.

C–F   (C) Heavily-stained gel with high background developed during several weeks of storage. (D)–(F) Same gel after storage in large volume of 10 mg/ml sodium metabisulfite for (D) 3 days, (E) 10 days, and (F) 25 days.

G–H   Otherwise identical gels stained by reductive dye-coupling (G) or PAS stain (H). Corresponding protein stain is gel N.

I–K   (I) Gel soaked in 50 mg/ml maltose, PAS-stained, and placed in tube of 7.5% acetic acid next to untreated gel (J) for 36 h. Both gels were then destained in 7.5% acetic acid. (K) Control: gel soaked in 50 mg/ml maltose and PAS-stained with the omission of the periodate oxidation step.

L–M   PAS stain (L) or periodic acid-Alcian Blue stain (M) on SDS gels of culture supernatant concentrate after a 5-min incubation with sucrose. Corresponding protein stain is gel N.

N–Q   Protease treatment. Gels were stained for protein after no protease treatment (N), or after 9-h incubation with 20 μg/ml pepsin (O), chymotrypsin (P), or trypsin (Q).

R–V   Insertion of protease treatment between incubation with sucrose and before PAS stain. Gels R and T show the protein stains corresponding to protease-treated, PAS-stained gels S and U, respectively. (V) Control: same as gel U, but sucrose incubation performed in presence of 1 mg/ml SDS instead of 10 mg/ml Tween 80, in order to inhibit dextranucrase and thereby prevent polysaccharide production.
ing in dilute acetic acid, except at protein positions, which remained colored. All glycosides caused proteins to stain, while free glucose did not. There was virtually no background (color remaining after the de-staining wash) with disaccharides or methyl α-glucoside. With the tri-saccharide raffinose, there was a slight background, and with higher oligosaccharides, such as stachyose (a tetrasaccharide) or α-cyclodextrin (a hexasaccharide), background was heavy and not reduced by extended washing in dilute acetic acid.

Gels I and J show, in this model system, how the diffusion of stained material from one gel (gel I) stained protein in another gel (gel J). Gel K shows that the original staining requires periodate oxidation.

To test the possibility that the protein staining was reversible, gels stained after incubation in maltose solutions were washed with 6 M guanidine hydrochloride or with a mixture of 3 M KCl and 10 mg/ml Triton X-100. Overnight washes with these solutions resulted in a considerable reduction in the intensity of stained protein bands. About the same degree of reduction in color was caused by metabisulfite. Longer washes gave no more reduction in color.

Blocking of amino and aldehyde groups

Attempts to prevent the protein staining reaction by post-electrophoretically blocking protein amino groups, either by reductive methylation [167] or by carbamylation [318], were unsuccessful; i.e., protein still stained in the model staining system. However, adding compounds to the destaining solution that form adducts with aldehydes, and are used histochemically to block aldehydes ([17], pp. 312-315 in Lillie and Fullmer [210]), was effective to some degree. The aldehyde blockers hydroxylamine, metabisulfite, and semicarbazide, but not dimedone, reduced or prevented staining in the model system. In the normal PAS staining procedure, however, they reduced the intensity of stained polysaccharide bands, despite claims in the histochemical literature that they are slowly displaced from aldehydes by Schiff’s reagent. They were
not completely effective in preventing artifactual staining of protein bands.

Semicarbazide bleached the PAS color. Over several days in 7.5% acetic acid (pH 2.6), it turned solutions of pararosaniline hydrochloride completely colorless. In this connection, it should be noted that histochemical aldehyde blockers are all reducing agents.

Protease treatment

Gels N–Q show the effect of protease treatment on the protein content of gels of crude dextransucrase. Comparison of protease-treated gels (gels O–Q) with a non-treated control (gel N) showed that trypsin (gel Q) and chymotrypsin (gel P) hydrolyzed the protein adequately, but that hydrolysis by pepsin (gel O) was negligible (wrong HCl concentration suspected). Chymotrypsin was easier to handle than trypsin because its crystals were larger. The trypsin preparation had the consistency of dust.

Equal amounts of one dextransucrase preparation were applied to gels R and S, and equal amounts of another preparation to gels T and U. Gels R and T were stained for protein, while gels S and U were incubated with sucrose, treated with protease, and then PAS-stained. No artifactually stained protein bands exist in gels S and U, despite the high initial protein content (gels R and T). A uniform pink background occurred in gel S, presumably caused by staining of the uniformly distributed protease. Gel V, a control, was the same as gel U, except that the sucrose incubation was done without reactivating the dextransucrase, so that no polysaccharide was produced. It showed that protease treatment caused no inherent background on subsequent PAS-staining.

Stain Sensitivity

Without exception, long incubations (12–18 h) in Schiff’s reagent, in methods based on that of Segrest and Jackson [308], gave much weaker final color than when short incubations (2 h) were used. This was true
even for the method of Matthieu and Quarles [225], where the long incubation in Schiff's reagent was coupled to "intensifying" metabisulfite washes.

In our hands, the procedure of Kapitany and Zebrowski [173] was as sensitive as any of the variant PAS-staining methods, except for the method of Konat et al. [195]. Because of the high-temperature ethanolic step used by Konat et al. [195], the chemistry of the stain may be somewhat different than for other PAS stains, in that the colored products may consist to a lesser degree of the usual [71, 254, 279] aminoalkyl-sulfonates and more of other compounds [27, 148, 272]. The method of Konat et al. [195] gave a high background if short incubations in Schiff's reagent were used.

The increased sensitivity reported by post-staining treatments in many variations on the method of Segrest and Jackson [308] (e.g., refs. 94 and 225) was not significantly different from the increase that occurred merely by washing with dilute acetic acid after removing the gels from Schiff's reagent. In particular, metabisulfite washes did not necessarily improve the sensitivity, which parallels the results of histochemical studies on model films [80], although an increase in stain intensity was sometimes observed when a brief metabisulfite treatment immediately followed incubation in Schiff's reagent. For commonly used conditions metabisulfite treatment (e.g., 30 min incubation in 0.2% sodium metabisulfite), any change in stain intensity was undetectable, but increasing either the metabisulfite concentration or the incubation time caused an obvious decrease, as did storage in metabisulfite solutions.
DISCUSSION

Nature of the Diffusing Stain

If one of several PAS-stained gels in the destaining solution contained a heavily stained polysaccharide band, all protein bands in all the gels in the destaining solution became stained during the destaining wash. Gels with stained polysaccharide bands that were too weak to cause protein staining during the destaining wash developed stained protein bands during storage, caused by diffusion from the PAS-stained areas.

Periodate-oxidized carbohydrate was required for protein to be stained. Three additional features of the staining were discovered in experiments in which gels were soaked in sugar solutions and then PAS-stained. First, the presence of glucose did not cause proteins to stain, while the presence of methyl glucoside or any other glycoside did. Second, placing a gel with proteins stained after incubation in a glycoside solution into destaining solvent next to an unoxidized, unstained gel caused the proteins in the unstained gel to become stained. Third, glycosides larger than maltose caused background color to develop that could not be removed by destaining. At constant weight concentration, larger glycosides produced darker background.

Based on these three findings, it seemed likely that the diffusing stain had low molecular weight, and had more than one dye-combined or free (or potentially free) aldehyde group per molecule, the latter because only glycosides resulted in staining. Because the staining also occurred when Alcian Blue reagent was substituted for Schiff’s reagent, any method including periodate oxidation probably will cause proteins to stain.

Staining may be caused by reaction of the proteins with small poly-aldehydes which are initially formed during the periodate oxidation of carbohydrate, and which then are incompletely derivatized by Schiff’s reagent. Periodate oxidation is known to cause fragmentation of polysaccharide chains through a free-radical process [305], and the Schiff
reaction is far from stoichiometric, both because the periodate oxidation
is usually incomplete [70, 273], and because the aldehydes generated do
not all react with Schiff's reagent [80, 273]. Compounds used histochem-
ically to block aldehydes (e.g., hydroxylamine and semicarbazide) pre-
vented protein staining in certain of the experiments.

Two types of solutions to the protein-staining artifact were stud-
ied. One was the prevention of stain diffusion, and the other was the
prevention of the reaction of diffused stain with protein.

Reduction of Stain Diffusion

Most of the methods described in this section prevented or slowed
protein staining during storage. Because they could be used only after
the initial destaining, they did not prevent the proteins from being
stained before or during the destaining process.

Crosslinking

Pararosaniline leucosulfite, the aldehyde-staining component of
Schiff's reagent, is potentially a crosslinking reagent, capable of re-
acting with six aldehyde equivalents, although steric and electronic
limitations may permit reaction with only one or two equivalents [273].
The PAS stain has been reported by Scott and Harbison [304] to be spe-
cific for polyaldehydes, as long as bisulfite washes are used after the
incubation in Schiff's reagent. These washes, which are included in most
PAS staining procedures, but whose function does not seem to be well
understood, were found by Scott and Harbison [304] to wash unsubstituted
and monosubstituted dye molecules out of tissue sections, leaving primar-
ily disubstituted dye molecules.

Consideration was given to the possibility that the PAS-staining
procedure of Kapitany and Zebrowski [173], by insufficient length of
incubation with periodic acid or Schiff's reagent, did not allow enough
crosslinking by the Schiff's reagent to occur. Therefore, both of these
incubations were lengthened, separately and in combination. Lengthening
the oxidation step did reduce diffusion of the stained carbohydrate, as judged by less colored matter released into the gel storage solvent. Lengthening the Schiff reaction step did not reduce the diffusion of the stained carbohydrate.

**Reductive dye coupling**

To circumvent electronic factors that might be reducing crosslinking by making pararosaniline leucosulfite less reactive after coupling with one or two aldehyde groups [279], a reductive dye-coupling method was developed. Reductive dye-coupling of mono-functional, fluorescent chromophores to periodate-oxidized carbohydrate has been described [110]. The multi-functional chromophore pararosaniline was chosen for its capacity to crosslink periodate-oxidized carbohydrate.

Although the sensitivity of the reductive dye-coupling procedure was not as high as with the standard PAS stain (gels G and H), and was much less than with fluorescent chromophores, it gave no background, even after gels had been stored for months. This method also had the advantage of using only stable reagents, in contrast to the notoriously unstable [20, 174] Schiff's reagent. However, protein bands did stain artifically by this method (gel G).

**Conventional crosslinking reagents**

PAS-stained gels were treated with several crosslinking reagents that are used to couple proteins to each other or to activated carbohydrate supports, in an attempt to slow the diffusion of the protein-staining material by crosslinking its amino groups. Treatments with divinylsulfone, 1,4-butanediol diglycidyl ether, quinone, and formaldehyde all slowed the diffusion. However, because these reactions could only be performed after destaining, some protein staining had already occurred. Only the staining that happens during storage could be prevented.

**Nonoptimal crosslinking conditions**

Reactions with divinylsulfone and bisoxirane (1,4-butanediol diglycidyl ether) were done at pH values
basic enough for reaction with carbohydrate hydroxyl groups. Since the reaction appears to occur with the amine functions of the dye, lower pH should work nearly as well, and decrease the length and number of washes needed.

Lowering the pH was successful for quinone treatment. In addition to being a more reactive reagent than either divinylsulfone or 1,4-butanediol diglycidyl ether, quinone had the advantage as a fixing agent that the resultant gel could be stored at a more acid pH (e.g., pH 2.6, the approximate pH of 7.5% acetic acid) than gels treated with divinylsulfone or formaldehyde. Reaction with divinylsulfone is reversible, and the reverse reaction is faster at low pH. The methylene crosslinks introduced by formaldehyde treatment are unstable below pH 3 [37]. Acidic storage of PAS-stained gels is desirable, because the stain fades due to reactions of the carbinol form of the dye [122, 206], whose proportion increases with pH.

Reductive dye coupling was not optimized, either. It is likely that more complete reaction and faster results could be obtained; e.g., by using DMSO as a solvent.

Reaction of Diffused Stain with Protein

Attempts were made to block the amino groups of proteins in the gels before PAS-staining. Procedures for amine modification by reductive methylation and by carbamylation failed to prevent protein staining, or to reduce it noticeably.

Treatment with alkali to hydrolyze Schiff base linkages of the chromophore to the protein, or reduction with cyanoborohydride to reduce the more reactive nonsubstituted and disubstituted dye molecules, reduced the intensity of stained protein bands no more than the intensity of the stained polysaccharide bands; i.e., the protein-staining artifact was not selectively reversed. Reduction or alkali treatment did decrease background temporarily. Oxidation with sodium periodate or with periodic acid reduce background more permanently. Extended washing with metabi-
sulfite was also effective at reducing background (gels C-F).

No method was found that specifically destained protein bands once they had become stained. This would be expected if the color were caused by the binding of PAS-stained or potentially stainable carbohydrate. Ways considered to avoid the problem were to decrease the sensitivity of the stain [123], to cut the gel into sections and incubate them separately with sucrose, and to apply less protein to the gel, so that protein bands would not be visible when stained.

A different approach was chosen instead. A protease treatment was inserted into the staining protocol prior to periodate oxidation, to eliminate the protein bands and thus the possibility of protein staining. This achieved the desired goals (gels R-V), but did produce a light, uniform pink background if the protease concentration was too high (gel S). As a method for the specific detection of glycansucrase activity, this procedure had the potential advantage of preventing the staining of glycoproteins, which would be removed by proteolysis.
SUMMARY AND GENERAL DISCUSSION

Stabilization

It was found in Part I that a broad variety of nonionic detergents, and mild ionic detergents such as sodium taurocholate, stabilized B-512F dextranucrase at or below their critical micelle concentrations. (Sodium cholate and sodium deoxycholate, mild ionic detergents that are more commonly used that sodium taurocholate, are insoluble at pH 5, because they are unionized.) Whether the enzyme is stabilized by binding detergent micelles or detergent monomers is not known, and neither are the mechanisms of inactivation the detergents protect against. It is not clear how much dextranucrase, which is considered to be a "soluble" enzyme, may actually resemble a peripheral membrane protein [98].

The nonionic polymers dextran, poly(ethylene glycol), and methyl cellulose also stabilized B-512F dextranucrase, as did yeast mannan, a phosphorylated polymer present in the bacterial growth medium. Ca\textsuperscript{2+} stabilized the enzyme only against the inactivation caused by bovine serum albumin and of Triton X-100. The inactivation in the presence of Triton X-100 is probably caused by the hydroperoxide impurity known to be present in preparations of this detergent [8, 234].

B-512F dextranucrase was activated by poly(ethylene glycol) and nonionic detergents. The degree of activation appeared to increase with enzyme purity, and may be inversely correlated with dextran content. For highly purified dextranucrase, the degree of activation may have been as high as 100%, but was more likely 30-40%, compared to 15-25% for crude dextranucrase from which little or no culture dextran had been removed.

Purification and Properties

B-512F dextranucrase was purified by two methods. One was a method where most purification took place during two gel filtration steps separated by a dextranase treatment. This method gave an enzyme preparation
with high specific activity (90-130 U/mg protein) and low carbohydrate content (less than protein by weight), and was simpler than the other method. It had the disadvantages of activity losses during concentration steps and of limitations in scale imposed by the relatively small sample volumes that can be applied to gel filtration columns.

The other purification method developed used a combination of ion-exchange and affinity chromatography. Compared to the methods of Robyt and Walseth [286], Kobayashi and Matsuda [188], and Monsan and Lopez [238], it gave a higher specific activity (90-170 U/mg protein) and a lower carbohydrate content (ranging from undetectable to 70% of protein by weight). As in the three published methods just cited, levansucrase and dextranase were absent from the purified enzyme. The procedure was not as simple as those of Robyt and Walseth or Monsan and Lopez, but because it was based on adsorption steps, was not limited as they are by the low capacity of laboratory gel filtration columns.

The high specific activity of the purified enzyme was caused to an unknown degree by the presence of Tween 80, which was not present in the other studies cited. Tween 80 was found to activate enzymes with high dextran content relatively little (15-25%). The specific activity immediately after the DEAE-cellulose chromatography step (130 U/mg protein when fresh culture supernatant was used) is probably comparable to the 122 U/mg protein reported by Monsan and Lopez [238].

Both methods developed here had high yields relative to most glucan-sucrase purification procedures in the literature. In addition to the usual reasons, this was desirable for a consideration stated in Furuta et al. [109]: "... the very low yields of GTases isolated is a common problem with reported purifications of these enzymes and has led to worry that, against the background of multiple forms of GTases, the isolated species may not be very representative of the total...."

The purified enzyme had the same $K_m$ for sucrose (12-16 mM) and the same pH activity profile (optimum at pH 5-5.5) as did the crude enzyme (culture supernatant). It also produced the same lightly branched dextran, the same acceptor products, and the same amount of glucose. It was
inhibited 10-15% by any concentration (1-10 mg/ml tested) of added dextran, and showed a 20-70% increase in its rate of de novo dextran synthesis that started at a de novo dextran concentration of about 0.1 mg/ml.

Studies have appeared [53, 254, 286] in which the PAS stain was applied to gels of purified glucansucrase preparations, causing most or all protein bands to stain. This has been taken as evidence that glucansucrases are glycoproteins. The PAS stain did not stain the enzyme on SDS gels, which, in concert with the undetectably low carbohydrate concentration of the most highly purified preparations, makes it unlikely that B-512F dextran sucrase is a glycoprotein. This agrees with the findings of Shimamura et al. [310].

The enzyme consisted mostly of a 177 kDa form when freshly prepared, but on aging was slowly converted into a 158 kDa form. This conversion took place both in the culture supernatant in the most purified preparations. An additional peptide at 117 kDa, having no glucansucrase activity, also formed in large amounts during storage, as well as small amounts of other inactive peptides. Similar changes for other glucansucrases have been attributed to proteolysis [123, 294].

The 177 kDa dextran sucrase was the largest protein in all preparations, assuming that several barely visible bands with slower migration on SDS gels were protein aggregates. The culture supernatant contained levansucrase at 116 and 92 kDa, but the purified enzyme had no levansucrase.

The enzyme was not stable after affinity chromatography on Sephadex G-200, even in the presence of Tween 80. In preparations of purified dextran sucrase, a precipitate slowly formed that initially could be dissolved by SDS, 2 M urea, salt, or sucrose, and partially by dextran. After many months of storage, the precipitate could no longer be dissolved by salt, sucrose, or dextran. In preparations of the purified enzyme, activity was lost from solution faster than protein was lost (i.e., faster than precipitate formation).
Metal Ion Kinetics

It was found that there are two kinetically important metal ion sites on B-512F dextranucrase, and probably no more than two. One of these sites is also the sucrose site, since the four divalent metal ions investigated (Ca$^{2+}$, Zn$^{2+}$, Ni$^{2+}$, and Co$^{2+}$) were all competitive with sucrose at high Ca$^{2+}$ concentration. High Ca$^{2+}$ concentration was needed to show this competitive inhibition because all four metal ions bound more strongly to the other metal ion site, where all but Ca$^{2+}$ caused a mixed-type inhibition. By contrast, Ca$^{2+}$ gave mixed-type activation at this site. Limited evidence suggests that the stabilizing effect of Ca$^{2+}$ is caused by binding at this site, which had a much higher affinity for Ca$^{2+}$ (apparent $K_{\text{diss}} < 10 \, \mu$M) than for the other three metal ions. While it was an activator, Ca$^{2+}$ was not essential for dextran synthesis, a finding originally made by Neely and Hallmark [249]. Dextran was necessary to prevent inactivation by Zn$^{2+}$.

Ca$^{2+}$ decreased the $K_m$ for sucrose, and Zn$^{2+}$ increased it, but both metal ions increased $V_{\text{max}}$ and decreased $K_{\text{IS}}$, the substrate inhibition constant for sucrose. Zn$^{2+}$ increased $V_{\text{max}}$ more than did Ca$^{2+}$. Zn$^{2+}$ did not activate the enzyme at high sucrose concentration because it increased substrate inhibition faster than did Ca$^{2+}$. Preliminary evidence suggests that metal ions are necessary for substrate inhibition of dextran formation to occur. Acceptors also appeared to increase the substrate inhibition of dextran formation.

Neither Ca$^{2+}$ nor Zn$^{2+}$ caused major changes in the enzyme products, as determined qualitatively from thin-layer chromatograms of dextranucrase reaction mixtures and of dextranase-hydrolyzed dextran samples (the latter checked only for Ca$^{2+}$). Sucrose concentrations above 150 mM were not used for these thin-layer chromatography experiments, and small changes in product distribution would not have been noticed. Therefore, metal ions could be causing kinetically significant changes in the products under some conditions, especially in the experiments on substrate inhibition.
The possibility of changes in product distribution bears on the issue of whether the kinetic assay used throughout this dissertation was correct. The assay measures the production of methanol-insoluble polysaccharide, and not the production of glucose, leucrose, or other methanol-soluble enzyme products. The difference between competitive kinetics, found for Ca$^{2+}$ activation by Neely and Hallmark [249] and for fructose inhibition by Chludzinski et al. [50], and the mixed-type kinetics found for both Ca$^{2+}$ and fructose here, may be in part due to the different assays used (although Chludzinski et al. [50] used methanol-insoluble polysaccharide formation). However, although the assay may not be the correct measure of rate for some purposes, the large size of the intercept changes seen in double-reciprocal plots, compared to the small size of changes in the amounts of methanol-soluble products (as determined from thin-layer chromatography), showed that the mixed-type kinetics are real, and not an artifact of the assay used.

Molecular Weight

Because the molecular weight of an enzyme determined by most methods depends on the enzyme's hydrodynamic and charge properties, they measure the protein's particle or "structural" size. Dextranucrase aggregates so readily that when these techniques fail to detect significant amounts of dextranucrase monomer, it cannot be reliably concluded that the monomer is not catalytically competent.

The molecular weight of an enzyme determined by radiation inactivation depends on an enzyme's catalytic properties, measuring a "functional" size. Radiation inactivation gave a size of 201 kDa for B-512F dextranucrase, which was close enough to the 158 kDa peptide size found by SDS gel electrophoresis for the irradiated preparation that it could be concluded that the functional unit is a single peptide; i.e., that a single dextranucrase peptide is competent to synthesize dextran.

More freshly prepared dextranucrase than was used for the irradiation experiment also contained another dextranucrase peptide of 177 kDa.
(size by SDS gel electrophoresis). No peptide displaying dextranucrase activity smaller than 158 kDa was found on SDS gels. It is possible, however, that a form of the enzyme smaller than 158 kDa exists that was not detected on SDS gels, because not all of the dextranucrase activity lost in the presence of SDS was recovered.

Protein-Staining Artifact of the PAS Stain

The PAS stain used to detect glucansucrase activity on SDS polyacrylamide gels was found to stain proteins that were not glycoproteins. This protein staining was not caused by commonly encountered protein-staining artifacts of the PAS stain [16, 121, 268, 348], but by the reaction of proteins with periodate-oxidized carbohydrate that diffused through the destaining and storage solvents. The problems that resulted were the development of new stained bands during destaining and storage, and the development of high background color during storage of the stained gels.

Two ways were found to reduce these problems. One was to react PAS-stained gels with a crosslinking reagent (formaldehyde, divinylsulfone, 1,4-butanediol diglycidyl ether, or quinone) to crosslink the stain products and to reduce the rate of their diffusion. The other way was to remove all the protein from the gels before staining by chymotryptic digestion.

Multiple Enzyme Forms

Most of the experimental sections of this dissertation contain evidence for multiple forms of B-512F dextranucrase. The section on stabilization shows that inactivation often stops well short of completion, as also found by Kaboli and Reilly [172]. This may be caused by variable stability of dextranucrase molecules with different amounts of associated dextran or in different aggregation states. In the section on molecular weight determination, the strong correlation of the partial
loss of activity caused by SDS treatment with partial loss from other causes also argues for dextranucrase species of differing stability. The mixed-type kinetics given by Zn\(^{2+}\) and Ca\(^{2+}\) could largely result from having a mixture of dextranucrase species with different kinetic parameters.

The discovery of two major molecular weight forms of dextranucrase, which are described in the sections on kinetics, on molecular weight determination, and on purification, offers a possible explanation for these phenomena. No differences in properties besides molecular weight has been found for the two enzyme forms, but this may be because little characterization of the two forms has been done. Grahame and Mayer [124] have reported that the two molecular weight forms of dextranucrase from \textit{S. sanguis} ATCC 10558 are kinetically indistinguishable, both in the presence and absence of dextran T10, and that there is no difference in the dextrans they make.

To characterize the two molecular-weight forms of B-512F dextranucrase separately, SDS gel electrophoresis could be used (e.g., according to techniques described in refs. 207 and 317). Alternatively, fresh and long-stored enzyme preparations may be acceptably close to 100\% of the 177 kDa enzyme form and 100\% of the 158 kDa enzyme form, respectively.

Some Unanswered Questions

1. What are the mechanisms of dextranucrase inactivation on dilution and in the presence of Zn\(^{2+}\) or EDTA (Part I)?
2. Does stabilization by detergents require detergent micelles (Part I)?
3. Why are dextranucrase inactivation and reactivation often incomplete (Part I)?
4. What causes the instability, and particularly the precipitation, of purified dextranucrase, and how can it be prevented (Part II)?
5. Is stabilization by Ca\(^{2+}\) mediated through the high-affinity Ca\(^{2+}\) site (Parts I, IV, and V, and Appendix C)?
6. What causes the 177 to 158 kDa size conversion, and why is the protease that might be its cause not removed during the purification (Parts II and VI)?

7. What is the nature of the tight, pH-dependent binding of the enzyme to DEAE-cellulose and DEAE-Trisacryl (Part II)?

8. Do the 177 and 158 kDa forms of the enzyme have different metal ion kinetics (Parts IV and V)?

9. Is the high affinity Ca^{2+} site the acceptor or branching site (Parts IV and V, and Appendix D)?

10. What causes substrate inhibition by sucrose, which appears to be absolutely metal-ion dependent (Appendix D)?

11. Are metal ions at low concentration important to the kinetics of streptococcal glucansucrases, and especially to substrate inhibition (Part V and Appendix D)?
CONCLUSIONS

1. Nonionic detergents, mild ionic detergents, poly(ethylene glycol), and high-molecular-weight dextran stabilize B-512F dextransucrase against thermal inactivation and against inactivation caused by dilution and manipulation. They do not prevent loss of activity caused by Ca\(^{2+}\) removal. The stabilizing ability of the polymers poly(ethylene glycol) and dextran increases with the molecular weight of the polymer. Stabilization by Tween 80 does not change the pH activity profile of the enzyme.

2. B-512F dextransucrase can be purified to contain no detectable carbohydrate and to have a specific activity of 170 U/mg protein.

3. B-512F dextransucrase consists of two forms of differing molecular weight. A 177 kDa form is biosynthesized initially, and is converted during aging to a 158 kDa form by an unknown but probably proteolytic mechanism.

4. The sucrose site of B-512F dextransucrase is also a divalent metal ion site, with a higher affinity for Zn\(^{2+}\), Ni\(^{2+}\), and Co\(^{2+}\) than for Ca\(^{2+}\).

5. A second metal ion site exists with a high affinity for Ca\(^{2+}\). Ca\(^{2+}\) at this site activates the enzyme, while Zn\(^{2+}\), Ni\(^{2+}\), and Co\(^{2+}\) at this site inhibit the enzyme. Ca\(^{2+}\) is a nonessential activator; i.e., it is not required for dextran synthesis.

6. Dextransucrase stabilization by Ca\(^{2+}\) may be mediated through the high-affinity Ca\(^{2+}\) site.

7. Substrate inhibition by sucrose of dextran synthesis may be caused by metal ions, and increased by presence of acceptors.

8. Dextran is not synthesized by the cooperative action of two or more peptides (subunits), but by single dextransucrase peptides.

9. The periodic acid-Schiff stain artifactually stained proteins when used to detect glycansucrase activity on polyacrylamide gels. This artifact could be prevented by removing all protein from gels proteolytically before staining, and could be considerably retarded by the reaction of crosslinking agents with the stained gels.
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APPENDIX A

CALCULATION OF FREE METAL ION CONCENTRATIONS
IN THE PRESENCE OF METAL ION-BINDING LIGANDS
For Part IV, free calcium concentrations were calculated with the use of the WATFIV (FORTRAN) program FREEC (FREE CALCIUM), which treats a combination of calcium-binding ligands as a single apparent ligand with a single apparent calcium-binding constant, after first computing a pH-dependent calcium-binding constant for each ligand. For example, an apparent stability constant can be defined at any pH as a function of total EDTA (EDTA + EDTA^- + EDTA^{2-} + EDTA^{3-} + EDTA^{4-}) for the binding of calcium to the EDTA^{3-} and EDTA^{4-} charge forms. For a full description of this method as applied to EDTA, see refs. 266 or 271.

For Part V and Appendix D, a more general approach was taken. A program to solve simultaneous ligand binding equations was written in Microsoft FORTRAN version 3.1 for the Zenith Z-100 microcomputer. The program, titled METALS, uses the method of iteration, which is described in any text on numerical methods. Unlike FREEC, METALS makes no simplifying assumptions, and can easily be modified to handle a larger number of equilibria.

Stability constants were taken when possible from Martell and Smith [222] (pp. 3-7 of Vol. 3 (1977) and pp. 75-76 of Vol. 5 (1982)), because it is a critical compendium. When not given in Martell and Smith, stability constants were taken from refs. 49 (p. 624) or 157 (pp. 38-48).
PROGRAM "FREEC"

The algorithm for this program assumes that inequality (1) holds for any two ligands, $L_1$ and $L_2$, having apparent stability constants $K_1$ and $K_2$ for their respective complexes, $ML_1$ and $ML_2$, with a single metal ion species, $M$ (in this case, calcium). The presence of the subscript "T" means total concentration, while its absence means free concentration.

\[
\frac{1}{K_1 - K_2} \frac{|ML_1|}{K_1 L_{1T}} << 1. \tag{1}
\]

If inequality (1) is true, then the exact cubic equation describing both equilibria (eqn. 2) has an approximate solution given by a quadratic equation (eqn. 3), where

\[
ML = ML_1 + ML_2
\]

and

\[
L_{1T} = L_{1T} \left[ 1 + \frac{K_2 L_{2T}}{K_1 L_{1T}} \right].
\]

\[
ML_1^2 - (M + L_{1T} + 1/K_1)ML_1 + \frac{(ML_1 - L_{1T})ML_1 K_2 L_{2T}}{K_1 L_{1T} + (K_2 - K_1)ML_1} + M L_{1T} = 0 \tag{2}
\]

\[
ML = \left[ \frac{M_T + L_{1T} + 1/K_1}{2} \right] - \sqrt{\left[ \frac{M_T + L_{1T} + 1/K_1}{2} \right]^2 - M_{1T}L_{1T}} \tag{3}
\]

The program that follows has a sample data set at the end.
C PROGRAM FREEC (FREE CALCIUM): VERSION OF 11-04-84.

C THIS PROGRAM COMPUTES FREE CALCIUM CONCENTRATION IN THE
C PRESENCE OF EDTA, GIVEN PH AND TOTAL CONCENTRATIONS OF CALCIUM,
C EDTA, AND SODIUM ACETATE.

C REFERENCE FOR EQUATIONS DEALING WITH EDTA IS
C H. PORTZEHL, P. C. CALDWELL, AND J. C. RUEGG.

C DATA LINES FOLLOW SENTRY. INPUT CONCENTRATIONS MUST BE IN
C UNITS OF MOLARITY. A NEW TOTAL CALCIUM CONCENTRATION IS READ
C IN WHENEVER AN EDTA CONCENTRATION LESS THAN ZERO IS READ.

C VARIABLE DEFINITIONS:

C BOUNDC = CONCENTRATION OF LIGAND-BOUND CALCIUM.
C EDTA = TOTAL EDTA CONCENTRATION.
C FREEC = FREE CALCIUM CONCENTRATION.
C H = HYDROGEN ION ACTIVITY (CONCENTRATION).
C K1-K5 AND KS1-KS3 = ANTILOGS OF PKA1-PKA5 AND LOGKS1-LOGKS3.
C KDISS = APPARENT DISSOCATION CONSTANT OF CHOICE.
C KEDTA3 = APPARENT STABILITY CONSTANT FOR CA-EDTA DUE TO
C CA-EDTA(1-).
C KEDTA4 = APPARENT STABILITY CONSTANT FOR CA-EDTA DUE TO
C CA-EDTA(2-).
C KOAC = APPARENT STABILITY CONSTANT FOR CA-ACETATE.
C KS1 = LOG OF TRUE STABILITY CONSTANT FOR CA-EDTA(2-).
C KS2 = LOG OF TRUE STABILITY CONSTANT FOR CA-EDTA(1-).
C KS3 = LOG OF TRUE STABILITY CONSTANT FOR CA-OAC (ACETATE).
C LIGAND = TOTAL APPARENT LIGAND.
C NAOC = TOTAL ACETATE CONCENTRATION.
C PH = PH.
C PKA1-PKA4 = PKA VALUES FOR EDTA.
C PKA5 = PKA VALUE FOR ACETIC ACID.
C TOTALC = TOTAL CALCIUM CONCENTRATION.
PKA1 = 10.17
PKA2 = 6.11
PKA3 = 2.68
PKA4 = 1.95
PKA5 = 4.56
LOGKS1 = 10.61
LOGKS2 = 3.51
LOGKS3 = 0.53

C
K1 = 10.**(PKA1)
K2 = 10.**(PKA2)
K3 = 10.**(PKA3)
K4 = 10.**(PKA4)
K5 = 10.**(PKA5)
KS1 = 10.**(LOGKS1)
KS2 = 10.**(LOGKS2)
KS3 = 10.**(LOGKS3)

C
LOOP
READ, TOTALC
AT END, STOP
PRINT 1000, 1.0E06*TOTALC
1000 FORMAT('0/' TOTAL CALCIUM= ',F7.2,' UM'/'OSODIUM ACETATE (MM),'
> 3X,'TOTAL EDTA (MM)',3X,'FREE CALCIUM (UM)',3X,'PH',5X,
> 'LOG KEDTA4',3X,'LOG KEDTA3',3X,'LOG KOAC'/)

C
LOOP
READ, EDTA, PH, NAOAC
AT END, STOP
IF (EDTA.LT.O.) QUIT
H = 10.**(-PH)

C
KOAC = KS3/(1 + H*K5)
KEDTA3 = KS2/(1 + 1/(K1*H) + H*K2 + (H**2)*K2*K3 +
> (H**3)*K2*K3*K4)
KEDTA4 = KS1/(1 + H*K1 + (H**2)*K1*K2 + (H**3)*K1*K2*K3 +
> (H**4)*K1*K2*K3*K4)

C
IF (EDTA.GT.0) THEN
   LIGAND = EDTA*(1 + KEDTA3/KEDTA4)
   LIGAND = LIGAND*(1 + KOAC*NAOAC/KEDTA4*LIGAND)
   KDISS = 1/KEDTA4
ELSE
   LIGAND = NAOAC
   KDISS = 1/KOAC
END IF

TERM = (TOTALC + LIGAND + KDISS)/2.0
BOUNDC = TERM - DSQRT(TERM**2. - TOTALC*LIGAND)
FREEC = TOTALC - BOUNDC
C
    PRINT 2000, 1.0E03*NAOC, 1.0E03*EDTA, 1.0E06*FREEC, PH, >
      DLOG10(KEDTA4), DLOG10(KEDTA3), DLOG10(KOAC)
2000 FORMAT(5X,F9.4,11X,F9.4,8X,F11.6,8X,F4.2,1X,3(3X,F9.5))
ENDLOOP
C
ENDLOOP
STOP
END
ENTRY
132.0E-06
0.0E-03  5.20  25.0E-03
1.0E-03  5.20  25.0E-03
1.4286E-03  5.20  25.0E-03
2.5E-03  5.20  25.0E-03
5.0E-03  5.20  25.0E-03
10.0E-03  5.20  25.0E-03
-1.0  0.0  0.0
132.0E-06
0.0E-03  5.20  25.0E-03
1.0E-03  5.20  25.0E-03
1.4286E-03  5.20  25.0E-03
2.5E-03  5.20  25.0E-03
5.0E-03  5.20  25.0E-03
10.0E-03  5.20  25.0E-03
//
PROGRAM "METALS"

This program solves simultaneous chemical equilibrium equations of the type \( ML_n = K \times M \times L^n \), where \( M \) is free metal (or hydrogen) ion concentration. \( ML_n \) is the concentration of its complex with \( n \) equivalents of ligand \( L \). \( L \) is the concentration of free ligand, and \( K \) is the appropriate stability constant. Following the program are sample input and output data files.

Program METALS

SDEBUG

PROGRAM METALS

******************************************************************************

* THIS PROGRAM DETERMINES THE CONCENTRATIONS OF FREE AND
* COMPLEXED METAL IONS IN ACETATE BUFFER.
* A SYSTEM OF SIMULTANEOUS EQUILIBRIUM EQUATIONS IS SOLVED
* BY THE METHOD OF ITERATION.
* INPUT CONCENTRATIONS ARE IN MILLIMOLAR:
* INPUT STABILITY CONSTANTS ARE FOR CALCULATIONS WITH MOLARITY.

******************************************************************************

IMPLICIT REAL*8 (A-Z)
INTEGER I, ITER

****** CREATE I/O DISK FILES.
OPEN(5, FILE="IONDATA")
OPEN(6, FILE="IONCONC", STATUS="NEW")

****** READ PH AND STABILITY CONSTANTS.
****** PARAMETERS FOR DETERMINING ACETATE CONCENTRATION.
****** CONVERGENCE CRITERION.
****** MAXIMUM NUMBER OF ITERATIONS ALLOWED.
READ(5,8100) PH, LOGKA1, LOGKC1,
>  LOGKZ1, LOGKZ2, LOGKZ3, LOGKZ4,
>  LOGKN1, LOGKN2, LOGKN3

READ(5,8200) ACETATE, COEFC, COEFZ, COEFN
READ(5,8300) TEST0

READ(5,8400) ITER

H  = 10.**(-PH)
KA1 = 10.**LOGKA1
KC1 = 10.**LOGKC1
KZ1 = 10.**LOGKZ1
KZ2 = 10.**LOGKZ2
KZ3 = 10.**LOGKZ3
KZ4 = 10.**LOGKZ4
KN1 = 10.**LOGKN1
KN2 = 10.**LOGKN2
KN3 = 10.**LOGKN3

***** PRINT INPUT CONSTANTS AND COLUMN HEADINGS.

WRITE(6,9050) PH, H, LOGKA1, KA1, LOGKC1, KC1, LOGKZ1, KZ1,
>  LOGKZ2, KZ2, LOGKZ3, KZ3, LOGKZ4, KZ4,
>  LOGKN1, KN1, LOGKN2, KN2, LOGKN3, KN3
WRITE(6,9100) ACETATE, COEFC, COEFZ, COEFN, TEST0
WRITE(6,9150)
WRITE(6,9200) ITER
WRITE(6,9250)

***** READ TOTAL METAL ION CONCENTRATIONS.

200 READ(5,8500,END=2000) CO, NO, ZO

***** CONVERT MILLIMOLAR CONCENTRATIONS TO MOLAR CONCENTRATIONS.

CO = 0.001*CO
NO = 0.001*NO
ZO = 0.001*ZO

***** ASSIGN INITIAL FREE METAL ION CONCENTRATIONS.

C = CO
N = NO
Z = ZO

***** INITIALIZE CONCENTRATIONS OF ACETATE COMPLEXES.
AO = 0.001*ACETATE + COEF*C0 + COEFZ*ZO + COEFN*NO
A = AO

HA1 = 0.
CA1 = 0.
NA1 = 0.
NA2 = 0.
NA3 = 0.
ZA1 = 0.
ZA2 = 0.
ZA3 = 0.
ZA4 = 0.

***** RECALCULATE CONCENTRATIONS ITERATIVELY.

DO 1500 I = 1, ITER

HA1 = KA1*H*A
A = A0-HA1-CA1-ZA1-2*ZA2-3*ZA3-4*ZA4-NA1-2*NA2-3*NA3

IF (CO.EQ.0.) THEN
TEST1=0.
GOTO 400
ELSE
OLD = C
CA1 = KC1*C*A
A = A0-HA1-CA1-ZA1-2*ZA2-3*ZA3-4*ZA4-NA1-2*NA2-3*NA3
C = CO - CA1
TEST1 = DABS(1.-C/OLD)
ENDIF

400 IF (ZO.EQ.0.) THEN
TEST2=0.
GOTO 800
ELSE
OLD = Z
ZA1 = KZ1*Z*A
A = A0-HA1-CA1-ZA1-2*ZA2-3*ZA3-4*ZA4-NA1-2*NA2-3*NA3
Z = ZO - ZA1 - ZA2 - ZA3 - ZA4
ZA2 = KZ2*Z*A*A
A = A0-HA1-CA1-ZA1-2*ZA2-3*ZA3-4*ZA4-NA1-2*NA2-3*NA3
Z = ZO - ZA1 - ZA2 - ZA3 - ZA4
ZA3 = KZ3*Z*A*A*A
A = A0-HA1-CA1-ZA1-2*ZA2-3*ZA3-4*ZA4-NA1-2*NA2-3*NA3
Z = ZO - ZA1 - ZA2 - ZA3 - ZA4
ZA4 = KZ4*Z*A*A*A*A
A = A0-HA1-CA1-ZA1-2*ZA2-3*ZA3-4*ZA4-NA1-2*NA2-3*NA3
Z = ZO - ZA1 - ZA2 - ZA3 - ZA4
TEST2 = DABS(1.-Z/OLD)
ENDIF

800 IF (NO.EQ.0) THEN
  TEST3=0.
  GOTO 1200
ELSE
  OLD = N
  NA1 = KN1*N*A
  A = AO-HA1-CA1-ZA1-2*ZA2-3*ZA3-4*ZA4-NA1-2*NA2-3*NA3
  N = NO - NA1 - NA2 - NA3
  NA2 = KN2*N*A*A
  A = AO-HA1-CA1-ZA1-2*ZA2-3*ZA3-4*ZA4-NA1-2*NA2-3*NA3
  N = NO - NA1 - NA2 - NA3
  NA3 = KN3*N*A*A*A
  A = AO-HA1-CA1-ZA1-2*ZA2-3*ZA3-4*ZA4-NA1-2*NA2-3*NA3
  N = NO - NA1 - NA2 - NA3
  TEST3 = DABS(1.-N/OLD)
ENDIF

1200 TEST = DMAX1(TEST1,TEST2,TEST3)
IF (TEST.LE.TEST0) GO TO 1600

1500 CONTINUE
1600 CONTINUE

***** PRINT RESULTS.
WRITE(6,9300) 1000*A0,1000*A,1000*C0,1000*C,
> 1000*ZO,1000*Z,1000*NO,1000*N
IF (CO.EQ.0.) THEN
  WRITE(6,9350)
ELSE
  WRITE(6,9400) 100*CA1/C
ENDIF
IF (ZO.EQ.0.) THEN
  WRITE(6,9450)
ELSE
  WRITE(6,9500) 100*ZA1/Z,100*ZA2/Z,100*ZA3/Z,100*ZA4/Z
ENDIF
WRITE(6,9550) I,TEST
IF (I.EQ.ITER+1) WRITE(6,9600)
WRITE(6,9650) 100*HA1/A
IF (NO.EQ.0) THEN
ELSE
  WRITE(6,9700) 100*NA1/N,100*NA2/N,100*NA3/N
ENDIF

WRITE(6,9800)

GOTO 200

2000 STOP

8100 FORMAT(///////////7F10.6/3F10.6)
8200 FORMAT(4F10.6)
8300 FORMAT(D10.5)
8400 FORMAT(I4)
8500 FORMAT(3F10.6)

9050 FORMAT(’P00’/’PH = ’,4X,F7.3,10X,’[H+] = ’,F8.6/
  ’LOG KAl = ’,F6.3,10X,’KAl = ’,D9.4/
  ’LOG KC1 = ’,F6.3,10X,’KC1 = ’,F9.6/
  ’LOG KZ1 = ’,F6.3,10X,’KZ1 = ’,F9.6/
  ’LOG KZ2 = ’,F6.3,10X,’KZ2 = ’,F9.6/
  ’LOG KZ3 = ’,F6.3,10X,’KZ3 = ’,F9.6/
  ’LOG KZ4 = ’,F6.3,10X,’KZ4 = ’,F9.6/
  ’LOG KN1 = ’,F6.3,10X,’KN1 = ’,F9.6/
  ’LOG KN2 = ’,F6.3,10X,’KN2 = ’,F9.6/
  ’LOG KN3 = ’,F6.3,10X,’KN3 = ’,F9.6)/

9100 FORMAT(/F10.2,’ MM SODIUM ACETATE’/
  F10.2,’ ADDITIONAL MOLES ACETATE PER MOLE CALCIUM’/
  F10.2,’ ADDITIONAL MOLES ACETATE PER MOLE ZINC’/
  F10.2,’ ADDITIONAL MOLES ACETATE PER MOLE NICKEL’/
  D10.3,’ = CONVERGENCE CRITERION (FRACTIONAL CHANGE’)/)

9150 FORMAT(’%MAX/M = AMOUNT OF COMPLEX MAX AS % OF FREE M’/
  ’[M = H (H+), C (CA2+), N (NI2+), Z (ZN2+); ‘,
  ’A = ACETATE ANION; X = 1, 2, 3, 4])

9200 FORMAT(’MAXIMUM NUMBER OF ITERATIONS = ’,I4)

9250 FORMAT(’/TOTAL’,4X,’FREE’,5X,’TOTAL’,4X,’FREE’,1X,
  2(4X,’TOTAL’,3X,’FREE’)/
  ’ACETATE’,2X,’ACETATE’,2X ’(CA2’,5X),
  2(’ZN2’,4X),2(’NI2’,4X),
  4(’(MM)’,5X),4(’(MM)’,4X),’%CA1/A’,2X,’%CA1/N’,2X,’%CA1/N’,
  2X,’%Z2A/N’,10X,’ITERATION’,3X,’TEST MAX.’/
  4(’ ’,2X),9(’ ’,2X),9(’ ’,2X),’ ’,2X,’ ’/)

9300 FORMAT(2(1X,F6.2,2X),2(F7.3,2X),3(F6.2,2X),F6.2))

9350 FORMAT(2X,’———’)

9400 FORMAT(2X,F6.2))

9450 FORMAT(2X,’———’,2(2X,’———’),2X,’———’)

9500 FORMAT(2X,F6.2,2(2X,F6.2),2X,F6.2))

9550 FORMAT(6X,I4,3X,D9.3)
Sample input file

***** FIRST 9 LINES ARE RESERVED FOR COMMENTS. DATA LINES ARE:
*****1. PH, LOG KA1, LOG KC1, LOG KZ1, LOG KZ2, LOG KZ3, LOG KZ4
*****2. LOG KN1, LOG KN2, LOG KN3
*****3. MM SODIUM ACETATE (A), MM A/MM C, MM A/MM Z, MM A/MM N
*****4. CONVERGENCE CRITERION (TESTO)
*****5. MAXIMUM NUMBER OF ITERATIONS (ITER).
***** NEXTFollows LIST OF TOTAL METAL ION CONCENTRATIONS, IN ORDER
*****   CALCIUM, NICKEL, ZINC.
*****
  5.0  4.76  0.53  1.1  1.9  1.60  1.36
  0.74  1.15  0.40
  47.89  0.00  1.17  1.83
  1.0D-10
  1000
  7.17  30.0  15.00
  7.17  22.5  15.00
  7.17  15.0  15.00
  7.17   7.5  15.00
  7.17   0.0  15.00
  7.17   0.0  15.00

Sample input file

***** FIRST 9 LINES ARE RESERVED FOR COMMENTS. DATA LINES ARE:
*****1. PH, LOG KA1, LOG KC1, LOG KZ1, LOG KZ2, LOG KZ3, LOG KZ4
*****2. LOG KN1, LOG KN2, LOG KN3
*****3. MM SODIUM ACETATE (A), MM A/MM C, MM A/MM Z, MM A/MM N
*****4. CONVERGENCE CRITERION (TESTO)
*****5. MAXIMUM NUMBER OF ITERATIONS (ITER).
***** NEXTFollows LIST OF TOTAL METAL ION CONCENTRATIONS, IN ORDER
*****   CALCIUM, NICKEL, ZINC.
*****
  5.0  4.76  0.53  1.1  1.9  1.60  1.36
  0.74  1.15  0.40
  47.89  0.00  1.17  1.83
  1.0D-10
  1000
  7.17  30.0  15.00
  7.17  22.5  15.00
  7.17  15.0  15.00
  7.17   7.5  15.00
  7.17   0.0  15.00
  7.17   0.0  15.00
Sample output file

.P00

PH = 5.000  [H+] = .000010
LOG KA1 = 4.760  KA1 = .5754D+05
LOG KC1 = .530  KC1 = 3.388442
LOG KZ1 = 1.100  KZ1 = 12.589254
LOG KZ2 = 1.900  KZ2 = 79.432823
LOG KZ3 = 1.600  KZ3 = 39.810717
LOG KZ4 = 1.360  KZ4 = 22.908677
LOG KN1 = .740  KN1 = 5.495409
LOG KN2 = 1.150  KN2 = 14.125375
LOG KN3 = .400  KN3 = 2.511886

47.89 MM SODIUM ACETATE
.00 ADDITIONAL MOLES ACETATE PER MOLE CALCIUM
1.17 ADDITIONAL MOLES ACETATE PER MOLE ZINC
1.83 ADDITIONAL MOLES ACETATE PER MOLE NICKEL
.100D-09 = CONVERGENCE CRITERION (FRACTIONAL CHANGE)

%MAX/M = AMOUNT OF COMPLEX MAX AS % OF FREE M
[M = H (H+), C (CA2+), N (NI2+), Z (ZN2+); A = ACETATE ANION; X = 1,
MAXIMUM NUMBER OF ITERATIONS = 1000

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<th>FREE ACETATE (MM)</th>
<th>TOTAL CA2+ (MM)</th>
<th>FREE CA2+ (MM)</th>
<th>TOTAL ZN2+ (MM)</th>
<th>FREE ZN2+ (MM)</th>
<th>TOTAL NI2+ (MM)</th>
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<td>%ZA3/Z</td>
<td>%ZA4/Z</td>
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<td>38</td>
<td>.578D-10</td>
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<td>57.54</td>
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</tbody>
</table>
APPENDIX B

FITTING AND TESTING OF KINETIC MODELS
INTRODUCTION

When a kinetic mechanism is unknown, the chief value of statistical analysis is its ability to discriminate among rival models; i.e., to test models, rather than to fit them. While a choice of models can often be made by ruler-and-straightedge graphical methods, this is not always possible even for simple models. A classic example is where inhibition that appears to be uncompetitive in double-reciprocal plots can be shown by numerical methods to be mixed-type. My own experience is that statistical analysis always shows that the real situation is more complex than it appears by simple graphical methods.

Good reviews of the use of numerical methods in enzyme kinetics include two by Mannervik [218, 219] and several in a volume of symposium proceedings edited by Endrenyi [89]. The chapter by Endrenyi on experimental design [90] can be enlightening. Other well-known reviews are those by Cleland [57] and by Garfinkel et al. [111]. Statistics as a subject in itself, but in relation to enzyme kinetics, is handled intelligibly in appendices in both of Cornish-Bowden's introductory books on enzyme kinetics [59, 60]. The general topic of regression, including model selection, is covered in a surprisingly comprehensible text by Draper and Smith [75]. A good short review of all the topics above is given by Sagnella [296].

For the work in this dissertation, the statistical package SAS [297] was used almost exclusively. This package supports nonlinear regression, and resulted in programs that I found easier to modify. References to some other programs for analyzing enzyme kinetics are cited in refs. 194 and 303.
GENERAL METHODS

Weighting of Kinetic Data

Currently, the most popular method for weighting enzyme kinetic data makes use of the approximation that the variance of the initial rate \( v_i \) is a simple power function of the rate itself (eqn. 1) \[219\], rather than of the independent variables, which are the substrate and effector concentrations. K and \( \alpha \) are constants for a given experiment, and \( u_v \) (the expected value of \( v_i \)) is in practice replaced by its estimator, \( \hat{v}_i \).

\[
\text{Var}( u_v ) = K \times \hat{v}_i^\alpha \quad (1)
\]

The weights for values of \( 1/v_i \) can then be approximated as being proportional to \( v_i^{(4-\alpha)} \). As found by Askelof et al. \[9\], more general variance functions, such as eqn. 2, did not prove superior.

\[
\text{Var}( u_v ) = K_0 + K_1 \times v_i^{(4-\alpha)} \quad (2)
\]

For the work in this dissertation, the weighting parameter \( \alpha \) was initially set at 2.0 for each experiment, and was then corrected to a better value by the residual analysis described by Mannervik et al. \[220\]. The data were then re-fit using the new \( \alpha \). It was never necessary to correct \( \alpha \) more than once.

Other approaches to coping with the problem of weighting have been described by Cornish-Bowden \[60, 61\] (nonparametric, or distribution-free, analysis), and by Cornish-Bowden and Endrenyi \[62\] (bi-weight regression).

Analysis of Residuals

In the absence of bias, which is usually caused by the choice of an incorrect model, and with proper weighting, plots of residuals against
any variable used in a regression, dependent or independent, should appear as a band of constant width, symmetrically distributed about zero. This is only true, however, when the residuals have first been normalized in some way. One way [58] is to studentize them (divide them by their standard errors). SAS computes studentized residuals for linear regression, but not for nonlinear regression. For nonlinear regression, therefore, each residual was instead divided by an estimate of its standard error based on eqn. 1; specifically, by the square root of the predicted value of $v_i^a$ [166]. The variable name assigned to these normalized residuals was NORMRES.

Normalized residuals were plotted against the regressors and and against their squares, reciprocals, or logarithms, depending on which transformation spread the points most evenly along the abscissa. This helped to avoid the obscuring effect of clustering, which made good values of $v_i$ look too large or too small.

Use of Nonlinear Regression

Many of the models used in this study could not be linearized by transforming the data, necessitating the use of nonlinear regression. Nonlinear regression is inefficient relative to linear regression, and gives less useful test statistics (see Draper and Smith [75], p. 282). It has been argued [111, 168] that nonlinear regression on untransformed data (e.g., $v_i$ and [substrate]) nevertheless has certain advantages over linear regression on transformed data (e.g., $1/v_i$ and $1$/[substrate]). These advantages are mostly related to assumptions made about the error structure of the variables, which is transformed whenever the variables are. As an example, if errors in $v_i$ are normally distributed, then errors in $1/v_i$ will not be; yet linear regression methods, both weighted and unweighted, require normal distribution of errors for parameter estimates to be unbiased and for the commonly used statistical tests to be valid. While there is some dispute about whether the distributions of $v_i$ and $1/v_i$ are strictly normal, non-normality rarely causes problems in
practical enzyme kinetics.

There is one definite, if minor, practical advantage of nonlinear regression: the standard errors (experimental precisions) of the kinetic constants are estimated directly. With linear regression, the standard errors of the constants must be extracted from standard errors determined for apparent constants that are products of two or more kinetic constants and/or reciprocals of kinetic constants.

Model Selection

Enzyme kinetic models that can be justified on theoretical grounds often contain more terms than the data can fit, because some of the terms have very small numerical values. Where this appeared to be a problem, and where linear regression was appropriate, an initial selection of minor terms to include in models was made with the aid of PROC STEPWISE, a model-building routine in SAS. Mallow's $C_p$ statistic (defined and briefly discussed in the SAS User's Guide [297]) was plotted against $p$ to determine when adding more terms to the model could no longer be justified on a statistical basis. Where linear regression was not appropriate, model simulation using the graphics capabilities of the microcomputer software package LOTUS 1-2-3 served the same purpose.
Examples of the principal programs written for enzyme kinetic analysis are appended, and are given with most of the options, gimcracks, and flapdoodles that proved useful with even the least tractable data encountered in the course of this work.

Description of Programs

The first program listed in the following pages is NLNMODEL (NON-LINEAR MODELS), which performs nonlinear regression on a data set taken from a disk file (A.U4503.EDTA3), using an internally assigned value of the weighting parameter α (ALPHA). The residuals and predicted velocities are written back to disk in the form of an SAS data set (NLNDAT.NLN), which is used to refine the value of α.

Following NLNMODEL is a section of a program that was substituted for the corresponding section of NLNMODEL when linear regression was used. The remainder of the program was then rewritten so that residual plots used STUDENT (the studentized residuals) instead of NORMRES.

Next is listed ALPHAEST (ALPHA ESTIMATOR) which uses the SAS data set NLNDAT.NLN created by NLNMODEL to do weighted nonlinear regression according to eqn. 1. In this procedure, described by Mannervik et al. [220], the squared residuals are used as estimates of the variance of \( \nu_i \). The data were then analyzed a second time with NLNMODEL, using the revised value of α.

Program NLNMODEL

```
//KINETICS JOB U4503,'MILLER'
//S1 EXEC SAS
//VALUES DD UNIT=DISK,DISP=SHR,DSN=A.U4503.EDTA3
//RESDATA DD DSN=A.U4503.NLNDAT,DISP=(NEW,CATLG),
//   SPACE=(TRK,(5,1),RLSE),UNIT=DISK
//SAS.SYSIN DD *
```
* PROGRAM NLNMODEL (NONLINEAR MODELS): VERSION OF 10-27-84;  

* FITS ENZYME KINETIC DATA BY NONLINEAR REGRESSION,  
  USING SECANT METHOD (DUD);  

* REFERENCE: "SAS USER'S GUIDE: STATISTICS, 1982 EDITION."  
  SAS INSTITUTE INC., CARY, NORTH CAROLINA (1982);  

* READ IN EXPERIMENTAL VALUES FROM DATA SET ON DISK:  
  S = SUBSTRATE, I = INHIBITOR, A = ACTIVATOR, VI = ARRAY OF VELOCITIES.  
  MISSING VALUES ARE ALLOWED;  

DATA B;  
ARRAY VI VI1-VI3;  
INFILE VALUES MISSOVER;  
INPUT S I VI1-VI3 A1-A3;  
ID=99; ALPHA=1.42; KIA=74300.;  
DROP VI1-VI3 A1-A3; A=A1;  

* CALCULATE NON-NORMALIZED WEIGHTS;  
DO OVER VI:  
  V=VI;  
  W=V**(-ALPHA);  
  OUTPUT; END;  
DATA A; SET B; IF V=. THEN DELETE;  

* NORMALIZE THE WEIGHTS;  
PROC MEANS NOPRINT; VAR ID W;  
OUTPUT OUT=B MEAN=ID WMEAN;  
DATA C; MERGE A B; BY ID; DROP ID;  
  W=W/WMEAN;  

* PERFORM REGRESSION;  
PROC NLIN METHOD=DUD; _WEIGHT_=W;  

* DATASET EDTA3;  
* NONESSENTIAL ACTIVATION + COMPETITIVE INHIBITION;  
* EQUATION MODIFIED FROM SEGEL, P. 840;  

PARMS KM = 66  
  KA1 = 4.7  
  KA2 = 0.54  
  KA3 = 4.0  
  KA4 = 2.2  
  VMAX = 560;
\[ \text{KM\_COEF} = \frac{(1 + A \times (1 + A/K\_IA)/K\_IA)}{(1 + A/K\_A2)} \]
\[ \text{S\_COEF} = \frac{(1 + A/K\_A3)}{(1 + A/K\_A4)} \]

MODEL \( V = \frac{V_{\text{MAX}}S}{(\text{KM\_COEF} \times K\_M + \text{S\_COEF} \times S)} \)

OUTPUT OUT=A PREDICTED=VHAT RESIDUAL=RES;

* ABORT EXECUTION IF CONVERGENCE HAS NOT OCCURRED;

DATA B; SET A;
IF VHAT= . THEN ABORT RETURN;

* DEFINE VALUES TO BE PLOTED. SCALE = ARBITRARY SCALING FACTOR:

\[ W_2 = (\text{VHAT}^{(-\text{ALPHA})})/W_{\text{MEAN}} \]
\[ \text{NORMRES} = \frac{\text{RES} \times (W_2^{0.5})}{\text{SCALE}} \]

\[ \text{SCALE} = 1000; \]
\[ Y = \frac{\text{SCALE}}{V}; \]
\[ \text{YHAT} = \frac{\text{SCALE}}{\text{VHAT}}; \]
\[ \text{SINV} = \frac{\text{SCALE}}{S}; \]
\[ \text{SINVSQ} = \text{SINV} \times \text{SINV}; \]
\[ \text{LOGA} = \log_{10}(A); \]
\[ \text{LOGVHAT} = \log_{10}(\text{VHAT}); \]

DROP ALPHA WMEAN SCALE;
PROC PRINT;

* CREATE PLOTS:

PROC PLOT;
LABEL SINV=1/SUBSTRATE
Y=1/RATE;
PLOT Y*SINV YHAT*SINV='*' OVERLAY HZERO VZERO HREF=0 VREF=0 HPOS=64;
TITLE DOUBLE-RECIPROCAL PLOT;

PROC PLOT;
LABEL VHAT=PREDICTED RATE
LOGVHAT=LOG OF PREDICTED RATE
S=SUCROSE
SINV=1/SUCROSE
SINVSQ=1/SUCROSE SQUARED
I=TOTAL EDTA
A=FREE CALCIUM
LOGA=LOG FREE CALCIUM
NORMRES=RESIDUAL/STD. DEV. OF PREDICTED RATE;
PLOT NORMRES*VHAT/VREF=0 HPOS=64;
PLOT NORMRES*LOGVHAT/VREF=0 HPOS=64;
PLOT NORMRES*S/VREF=0 HPOS=64;
PLOT NORMRES*SINV/VREF=0 HPOS=64;
PLOT NORMRES*SINVSQ/VREF=0 HPOS=64;
PLOT NORMRES*I/VREF=0 HPOS=64;
PLOT NORMRES*A/VREF=0 HPOS=64;
PLOT NORMRES*LOGA/VREF=0 HPOS=64;
TITLE RESIDUAL PLOTS:

* CREATE SAS DATA SET TO USE IN REFINING VALUE OF WEIGHTING PARAMETER 'ALPHA';

DATA B; SET A; KEEP VHAT RES; IF RES NE .;
DATA RESDATA.NLN; SET B;

Modified section of NLNMODEL

DO OVER VI;
  V=VI; Y=1/V; X1=1/5; X2=X1*I; X3=X1*X2; X4=I;
  W=Y**(ALPHA-4);
  OUTPUT; END;
DATA A; SET B; IF V NE .;

* CALCULATE NORMALIZED WEIGHTS;

PROC MEANS NOPRINT; VAR ID W;
OUTPUT OUT=B MEAN=ID WMEAN;
DATA C; MERGE A B: BY ID:
W=W/WMEAN;
DROP ID WMEAN;

* PERFORM REGRESSION;

PROC REG; MODEL Y=X1 X2 X3 X4/CORRB SEGB; WEIGHT W;
OUTPUT OUT=A PREDICTED=YHAT RESIDUAL=RES STUDENT=STUDENT;
Program ALPHAEST

//KINETICS JOB U4503, 'MILLER'
//S1 EXEC SAS
//RESDATA DD UNIT=DISK,DISP=OLD,DSN=A.U4503.EDTARES
//SAS.SYSIN DD *

* PROGRAM ALPHAEST (ALPHA ESTIMATOR): VERSION OF 10-31-84:

* PROGRAM TESTS CHOICE OF ALPHA IN MODEL-FITTING PROGRAM BY NONLINEARLY REGRESSING LOCAL MEANS OF SQUARED RESIDUALS ON LOCAL MEDIAN OR MEANS OF V(THAT RAISED TO ALPHA POWER), GIVING NEW ESTIMATE OF ALPHA. IF NEW ESTIMATE DIFFERS SIGNIFICANTLY (BY 0.2 OR MORE) FROM INITIAL ESTIMATE, RUN MODEL-FITTING PROGRAM AGAIN WITH NEW ESTIMATE:

* REFERENCE: B. MANNERVIK, I. JAKOBSEN, AND M. WARHOLM. BIOCHIM. BIOPHYS. ACTA 567, 43-48 (1979);

* INPUTS ARE LIST OF PREDICTED VELOCITIES (V(THAT) AND CORRESPONDING RESIDUALS (RES). MISSING VALUES ARE NOT ALLOWED;

DATA A; SET RESDATA.EDTA4;
   IF RES=. THEN ABORT RETURN;
   PROC SORT; BY VHAT;

* DIVISOR OF 'N' SHOULD BE SET SO THAT THE NUMBER OF GROUPS ('GROUP') CONTAINS AT LEAST FIVE OBSERVATIONS:

DATA B; SET A;
   N + 1;
   GROUP = CEIL(N/5);
   RESSQ=RES*RES;
   PROC PRINT;

PROC SUMMARY; CLASS GROUP; VAR VHAT RESSQ;
   OUTPUT OUT=A MEAN=VMEAN RSQMEAN;

* MEDIAN REPLACES MEAN IF THE TWO LINES ABOVE ARE REPLACED BY:

* PROC UNIVARIATE NOPRINT; * VAR VHAT RESSQ; * BY GROUP;
* OUTPUT OUT=A MEAN=X RSQMEAN MEDIAN=VMEAN:

DATA B; SET A; IF GROUP NE.;
   W=1/RSQMEAN;
   LRSQMEAN=LOG(RSQMEAN);
   LOGVMEAN=LOG(VMEAN);
   PROC PRINT;
PROC NLIN METHOD=MARQUARDT PLOT; _WEIGHT_=W;

PARMS K= 0.1 0.03 0.01 0.003 0.001 0.0003
       ALPHA= 1.0 1.4 1.8 2.2 2.6;

X=VMEAN**ALPHA;

MODEL RSQMEAN=K*X;
     DER.K=X;
     DER.ALPHA=K*X*LOGVMEAN;

OUTPUT OUT=A PREDICTED=PREDICT RESIDUAL=RESIDUAL;

DATA B; SET A; LOGPRED=LOG(PREDICT);
   IF PREDICT=. THEN ABORT RETURN;
   PROC PRINT;

PROC PLOT;
   PLOT LRSQMEAN*LOGVMEAN LOGPRED*LOGVMEAN='*' /OVERLAY HPOS=64;
   //
APPENDIX C

STABILIZATION OF DEXTRANSUCRASE BY DEXTRAN AND CALCIUM
This appendix summarizes the effects of Ca\textsuperscript{2+}, Zn\textsuperscript{2+}, EDTA, and dextran T10 on dextransucrase stability.

It was shown in Part I that EDTA inactivates both crude dextransucrase, which contains dextran, and purified enzyme, which does not. Crude enzyme is not denatured by Zn\textsuperscript{2+} (Part V), but purified enzyme is denatured (Part I). Inactivation is reduced but not prevented by the presence of the stabilizing detergent Tween 80.

Table I shows that purified enzyme was not inactivated by Zn\textsuperscript{2+} when dextran was present, and was inhibited the same amount by Zn\textsuperscript{2+} in the presence and absence of dextran. Dextran T10 was therefore included in kinetic experiments on purified enzyme using Zn\textsuperscript{2+}, although the inclusion of a saturating concentration of one of the enzyme products in the assays limited the scope of experiments on kinetic mechanism. At 10 mg/ml, dextran T10 stabilized purified enzyme as well as did native B-512F dextran.

Preliminary experiments on purified dextransucrase (not shown) gave the following results:

1. EDTA does not always increase the rate of inactivation.
2. Dextran T10 reduces the rate of inactivation, both in the presence and absence of EDTA.
3. Inactivation by 5-10 mM Zn\textsuperscript{2+} is two or more times as rapid at 0.04 mM Ca\textsuperscript{2+} as at 1 mM Ca\textsuperscript{2+}.
4. Part of the activity lost during prolonged incubation at low Ca\textsuperscript{2+} concentration (<0.1 mM) returns over a period of hours following the addition of 5 mM Ca\textsuperscript{2+}. This occurs both in the presence and absence of Zn\textsuperscript{2+}, but only in the absence of dextran.

From these results, it can be tentatively concluded that stabilization by Ca\textsuperscript{2+} and inactivation by Zn\textsuperscript{2+} are controlled by occupancy of the high-affinity Ca\textsuperscript{2+} site. It may also be that dextran can fully replace Ca\textsuperscript{2+} in its stabilizing function. Ca\textsuperscript{2+} definitely cannot fully replace dextran as a stabilizer.
Table I. Inactivation of purified dextranucrase by $\text{Zn}^{2+}$ in the presence and absence of dextran T10$^a$

<table>
<thead>
<tr>
<th>Incubation Concentrations</th>
<th>Dextran T10 (mg/ml)</th>
<th>Zn$^{2+}$ (mM)</th>
<th>Dextranucrase Activity (Arbitrary Units)</th>
<th>Incubation</th>
<th>Activity Lost (°)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Before 6-h Incubation</td>
<td>After 6-h Incubation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0</td>
<td>100</td>
<td>102</td>
<td>-2</td>
</tr>
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<td>18</td>
<td>0</td>
<td>105</td>
<td>105</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>9</td>
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<td>21</td>
<td>48</td>
</tr>
<tr>
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<td>18</td>
<td>9</td>
<td>42</td>
<td>40</td>
<td>5</td>
</tr>
</tbody>
</table>

$^a$ Dextranucrase purified by DEAE-Trisacryl M chromatography was assayed at the beginning and end of a 6 h incubation at 25° by adding an equal volume of 300 mM $^{14}$C-sucrose and measuring the rate of formation of methanol-insoluble polysaccharide. Incubations had 1 mM Ca$^{2+}$ and 0.2 mg/ml Tween 80.
APPENDIX D

EFFECT OF ZINC, CALCIUM, AND ACCEPTORS ON
THE SUBSTRATE INHIBITION OF DEXTRANSUCRASE
INTRODUCTION

In Parts IV and V, it was determined that the metal ions Ca$^{2+}$, Zn$^{2+}$, Ni$^{2+}$, and Co$^{2+}$ compete with sucrose for the substrate site. There is at least one other kinetically important metal ion site in dextran synthesis: the high-affinity Ca$^{2+}$ site. If this could be shown to be the acceptor site, and the binding of metal ions and acceptors could be shown to be competitive at this site, many of the important differences among glucansucrases might be explained by different metal ion affinities for the two metal ion sites. For example, the low percentage of branching in B-512F dextran might be the result of its high affinity for Ca$^{2+}$ at the acceptor (branching) site. Streptococcal glucansucrases, which have no demonstrable Ca$^{2+}$ dependence, might be highly branched because Ca$^{2+}$ rarely blocked the acceptor site, in contrast to the case with B-512F dextran-sucrase. The higher specific activities found for B-512F dextran-sucrase relative to streptococcal dextran-sucrases might be due to an increase in catalytic rate and in the affinity for sucrose caused by the binding of Ca$^{2+}$ to the acceptor site, which could happen if Ca$^{2+}$ made the sucrose site sterically more accessible by blocking the binding of dextran to the acceptor site.

Acceptors have been shown to decrease substrate (sucrose) inhibition [31, 226]. This would result if the mechanism of substrate inhibition were the competition of sucrose and acceptors for the acceptor site. If metal ions also decreased substrate inhibition, it would be evidence that sucrose, acceptors, and metal ions all compete for the acceptor site. A preliminary experiment on the effects of the metal ions Zn$^{2+}$ and Ca$^{2+}$ on substrate inhibition is described in this appendix.
RESULTS

Initial rates of methanol-insoluble polysaccharide formation were determined under the standard conditions described in Part V (pH 5.0, no Tween 80). Single determinations of velocity were made at the five sucrose concentrations shown in Fig. 1, the extremes being 20 mM and 500 mM. Sucrose concentrations higher than 500 mM were not used, to minimize the effects of changes in water activity which may have biased other studies [31, 86] in which sucrose concentrations as high as 1.6 M were used.

Initial velocities were fit to eqn. 1, in which $S$ is sucrose concentration and $K_{is}$ is the substrate (sucrose) inhibition constant. Fig. 1 shows two sets of sample data fit to eqn. 1.

\[
\frac{1}{v} = \frac{1}{V_{\text{max}}} + \frac{K_m}{V_{\text{max}} S} + \frac{S}{V_{\text{max}} K_{is}} \quad (1)
\]

Negative values and high errors were calculated for $K_{is}$ for reactions containing EDTA, because there was not enough information to determine a statistically meaningful $K_{is}$. Therefore, where EDTA was present, the data were refit to eqn. 1 with the last term set equal to zero. This was equivalent to setting $K_{is}$ equal to infinity, which was the value assigned to $K_{is}$ for these experiments in Fig. 3. The data for one set of conditions where EDTA was present are shown in Fig. 1B. The solid line shows the first fit, which included substrate inhibition and gave a negative $K_{is}$, and the dotted line shows the second fit, which did not include substrate inhibition.

Because the experiment was designed only for determining concentrations to be used in later experiments, precision was low compared to the experiments shown in Parts IV and V. High coefficients of variation (100 x standard error/mean) occurred for some sets of conditions, as shown in Fig. 2. (As a rule of thumb [219], enzyme kinetic parameters
Figure 1. Double-reciprocal plots of sample data

Data for the conditions "C" and "E", described in the legend to Fig. 2, are shown in (A) and (B), respectively. Solid lines show the fits to eqn. 1, while the dotted line in (B) shows the fit to eqn. 1 with $K_{is}$ set to a value of infinity.
Figure 2. Errors in the parameters determined from eqn. 1

Coefficients of variation for $V_{\text{max}}$, $K_m$, and $K_i$ (substrate inhibition constant) are shown for each set of concentrations.

Concentrations:

"C," 1 mM Ca$^{2+}$;
"C, F\textsubscript{2}," 1 mM Ca$^{2+}$, 200 mM fructose;
"C, M\textsubscript{2}," 1 mM Ca$^{2+}$, 100 mM maltose;
"E," 0.02 mM Ca$^{2+}$, 10 mM EDTA;
"E, F\textsubscript{2}," 0.02 mM Ca$^{2+}$, 10 mM EDTA, 200 mM fructose;
"E, M\textsubscript{2}," 0.02 mM Ca$^{2+}$, 10 mM EDTA, 100 mM maltose;
"F\textsubscript{1}," 0.02 mM Ca$^{2+}$, 100 mM fructose;
"F\textsubscript{2}," 0.02 mM Ca$^{2+}$, 200 mM fructose;
"M\textsubscript{1}," 0.02 mM Ca$^{2+}$, 50 mM maltose;
"M\textsubscript{2}," 0.02 mM Ca$^{2+}$, 100 mM maltose;
"Z," 0.02 mM Ca$^{2+}$, 1 mM Zn$^{2+}$;
"Z, F\textsubscript{2}," 0.02 mM Ca$^{2+}$, 1 mM Zn$^{2+}$, 200 mM fructose;
"Z, M\textsubscript{2}," 0.02 mM Ca$^{2+}$, 1 mM Zn$^{2+}$, 100 mM maltose.
having coefficients of variation higher than 50% can be treated as zero.)

These high coefficients of variation all resulted from low velocities, or more precisely, from little $^{14}$C incorporation.

Fig. 3 shows the effect of the acceptors fructose and maltose at constant metal ion concentrations. The effects of the acceptors, summarized in Table I, appeared to be the same for the three different metal ion conditions, except for their effects on $K_m$ and $K_{is}$ in the presence of $Zn^{2+}$, where errors were too high to draw conclusions.

Fig. 4 shows the effects of changing the metal ion concentrations at constant acceptor concentrations. The general trends are summarized in Table I. Two qualifications should be made. The first is that $Ca^{2+}$ and $Zn^{2+}$ increased $V_{max}$ more in the absence of acceptors than in their presence. In the presence of acceptors, the effect of $Zn^{2+}$ and $Ca^{2+}$ on $V_{max}$ differed little if any, while in the absence of acceptors, $Zn^{2+}$ increased $V_{max}$ more than did $Ca^{2+}$. The second exception is that $Zn^{2+}$ did not decrease $K_{is}$ as much in the presence of acceptors as it did in their absence. More specifically, in the presence of acceptors, $Zn^{2+}$ decreased $K_{is}$ less than did $Ca^{2+}$, while the reverse was true in the absence of acceptors.
Figure 3. Effect of acceptors on $V_{\text{max}}$, $K_m$, and $K_i$ at constant metal ion concentrations

The labels on the abscissa are defined in the legend to Fig. 2. The numbers given above the bars are coefficients of variation, not bar heights.
$V_{\text{max}}$ (cpm/min)

$K_m$ (mM)

$K_{is}$ (M)

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>F1</th>
<th>F2</th>
<th>M1</th>
<th>M2</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>5</td>
<td>21</td>
<td>35</td>
<td>67</td>
<td>104</td>
</tr>
<tr>
<td>C, F2</td>
<td>68</td>
<td>13</td>
<td>19</td>
<td>125</td>
<td>164</td>
</tr>
</tbody>
</table>
Figure 4. Effect of Ca\(^{2+}\) and Zn\(^{2+}\) on \(V_{\text{max}}\), \(K_m\), and \(K_{is}\) at constant acceptor concentrations

The labels on the abscissa are defined in the legend to Fig. 2. The numbers given above the bars are coefficients of variation, not bar heights.
Table I. Effect of modifiers on rate of dextran synthesis by crude\textsuperscript{a} dextransucrase

<table>
<thead>
<tr>
<th>Modifier</th>
<th>$V_{\text{max}}$</th>
<th>$K_m$</th>
<th>$K_{is}$</th>
<th>Change in parameter in presence of modifier\textsuperscript{b}</th>
<th>Literature reports</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA</td>
<td>-</td>
<td>+</td>
<td>++</td>
<td>This experiment reports</td>
<td>$0_{d,e}$ $+d,e$ N.D.</td>
</tr>
<tr>
<td>Zn$^{2+}$</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>Literature reports</td>
<td>N.D. N.D. N.D.</td>
</tr>
<tr>
<td>Ca$^{2+}$</td>
<td>+</td>
<td>-</td>
<td>+d,e</td>
<td>Literature reports</td>
<td>N.D. N.D. N.D.</td>
</tr>
<tr>
<td>Fructose</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>Literature reports</td>
<td>$0_{c,f}$ $+c,f$ N.D.</td>
</tr>
<tr>
<td>Maltose</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>Literature reports</td>
<td>$+d,g$ $+d,g$ $+d,g$</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Dialyzed concentrate of culture supernatant.

\textsuperscript{b} Symbols: -, decrease; +, increase; ++, large increase; 0, no change; N.D., not determined.

\textsuperscript{c} Based on rates of polysaccharide formation.

\textsuperscript{d} Based on rates of reducing value increase.

\textsuperscript{e} Ref. 249.

\textsuperscript{f} Ref. 50.

\textsuperscript{g} Refs. 31 and 226.
DISCUSSION

Acceptors Effects

Acceptors like maltose, which activate dextranucrase when velocity measurements are made by reducing value, also decrease substrate inhibition (i.e., increase $K_{is}$ in eqn. 1) [31, 226]. In the experiment described in this appendix, where velocities were measured by polysaccharide formation, maltose had the opposite effect, inhibiting the enzyme both by decreasing $V_{max}$ and by increasing $K_m$. Inhibition of dextran synthesis in the presence of activating acceptors is well-documented [282], and is easily explained by the higher proportion of total enzyme products that are methanol-soluble (i.e., that are acceptor products) when synthesis occurs in the presence of an acceptor.

What has not been reported is that maltose increases substrate inhibition (decreases $K_{is}$) when velocity measurements are made by polysaccharide formation (Fig. 3C, Table I). This means not only that the fraction of total enzyme products that are acceptor products increases in the presence of maltose, but that this fraction becomes larger as the sucrose concentration increases.

Fructose is one of the few acceptors that inhibits dextranucrase even when velocities are measured by reducing value [11]. It has been reported to be a competitive inhibitor of dextran formation [50]. From the more limited data here (Fig. 3), fructose appeared to be a mixed-type inhibitor of dextran synthesis, since it decreased $V_{max}$ (Fig. 3A) and increased $K_m$ (Fig. 3B). This is similar to Ca$^{2+}$, which was reported to be a competitive activator [249], but which was found in Part IV to be a mixed-type activator.

In the experiment described here (Fig. 3), fructose showed no qualitative differences from maltose, with the possible exception being the smaller changes in $V_{max}$ that occurred with fructose compared to maltose with changing metal ion concentrations.
Metal Ion Effects

The four metal-ion conditions compared were (1) essentially no metal ions (EDTA present), (2) low Ca\(^{2+}\) concentration (0.02 mM), (3) moderate Ca\(^{2+}\) concentration (1 mM), and (4) low Ca\(^{2+}\) concentration (0.02 M) combined with moderate Zn\(^{2+}\) concentration (1 mM). Low Ca\(^{2+}\) concentration in the absence of acceptor was not examined; however, an earlier preliminary experiment showed no differences between 0.02 and 1 mM Ca\(^{2+}\) in the absence of added acceptors.

The most important conclusion drawn from Fig. 4 is that substrate inhibition of dextran formation does not occur in the absence of metal ions. Zn\(^{2+}\) and Ca\(^{2+}\) both cause substrate inhibition. In the presence of fructose or maltose, less substrate inhibition occurred with Zn\(^{2+}\) than with Ca\(^{2+}\), but in the absence of fructose or maltose, more substrate inhibition occurred with Zn\(^{2+}\) than with Ca\(^{2+}\).

The other Zn\(^{2+}\) and Ca\(^{2+}\) effects, summarized in Table I, mostly duplicate findings in Parts IV and V. Only two will be mentioned. The first is the validation of the finding in Part V that Zn\(^{2+}\) increases $V_{\text{max}}$ at low Ca\(^{2+}\) concentration (Fig. 4A). The second is that Ca\(^{2+}\) appears to be unique in its ability to reduce $K_m$ (Fig. 4B).

Comparison of Metal Ions with Acceptors

Zn\(^{2+}\) and Ca\(^{2+}\) increased the inhibition of dextran formation by sucrose, as did acceptors. Unlike acceptors, thin-layer chromatograms of dextran-sucrase reaction products at 10-100 mM sucrose (not shown) showed no obvious changes in the relative proportions of dextran, acceptor products, and free glucose caused by the presence of moderate to high concentrations (1-10 mM) of Zn\(^{2+}\) or Ca\(^{2+}\). Also unlike acceptors, Zn\(^{2+}\) and Ca\(^{2+}\) increased the $V_{\text{max}}$ of dextran formation. The effects of Zn\(^{2+}\) and Ca\(^{2+}\) on the kinetic parameters are less clear in the presence of acceptors than in their absence.
Mechanism of Metal Ion Action

The low experimental precision of the results described here limits the conclusions, and few inferences will be drawn. The implications of the increase in substrate inhibition caused by fructose and maltose have not been considered in detail, particularly as they might relate to the effects of Zn\(^{2+}\) and Ca\(^{2+}\).

The question of whether metal ions compete with acceptors for the acceptor site remains unanswered. The increase in substrate inhibition observed in the presence of metal ions can be interpreted in at least two ways. If metal ions bind to the acceptor site, and thereby prevent the binding of acceptors, the metal ion-caused increase in substrate inhibition might be due to an increase in sucrose hydrolysis at high sucrose concentration. Thin-layer chromatography experiments have not been performed to exclude this possibility.

Metal ions could cause substrate inhibition in a much different manner, by increasing the affinity of the acceptor site for sucrose and/or acceptors. The binding of sucrose or acceptors would increase substrate inhibition (of dextran formation) (Fig. 3). In this case, there would be no mutually exclusive site for metal ions and acceptors.
"I'd just like to know what in hell is happening, that's all! I'd like to know what in hell is happening! Do you know what in hell is happening?"