The structure of native porcine pancreatic [alpha]-amylase and the purification and properties of the dextranucrases of Streptococcus mutans OMZ 176

Alice Jean Corrigan

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The structure of native porcine pancreatic α-amylase and the purification and properties of the dextran sucrases of *Streptococcus mutans* OMZ 176

by

Alice Jean Corrigan

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

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Iowa State University
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1977
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<tr>
<td>A</td>
<td>absorbance</td>
</tr>
<tr>
<td>Ǻ</td>
<td>angstrom</td>
</tr>
<tr>
<td>bis</td>
<td>(N,N'\text{-methylenе-bis-acrylamide})</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CNT</td>
<td>3-carboxylato-4-nitrothiophenolate</td>
</tr>
<tr>
<td>cpm</td>
<td>counts per minute</td>
</tr>
<tr>
<td>DFP</td>
<td>diisopropylfluorophosphate</td>
</tr>
<tr>
<td>D</td>
<td>Dalton</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
</tr>
<tr>
<td>D.P.</td>
<td>degree of polymerization</td>
</tr>
<tr>
<td>DTNB</td>
<td>(5,5'\text{-dithiobis-(2-nitrobenzoic acid)})</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethyleneglycol-bis(амinоethyl)tetraacetic acid</td>
</tr>
<tr>
<td>GLC</td>
<td>gas-liquid chromatography</td>
</tr>
<tr>
<td>ma</td>
<td>milliampere</td>
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<tr>
<td>PMSF</td>
<td>phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>POPOP</td>
<td>1,4-bis(2-(5-phenyloxazolyl)) benzene</td>
</tr>
<tr>
<td>PPO</td>
<td>2,5-diphenyloxazole</td>
</tr>
<tr>
<td>psi</td>
<td>pounds per square inch</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>T(_{17.7})</td>
<td>dextran, average molecular weight 17,770 D</td>
</tr>
<tr>
<td>T(_{72.6})</td>
<td>dextran, average molecular weight 72,600 D</td>
</tr>
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</table>
TEMED  \( N,N,N',N'\text{-tetramethylethlenediamine} \)

TFA  trifluoroacetic acid

TMS  trimethylsilyl

tris  2-amino-2-(hydroxymethyl)-1,3-propanediol

PPA  porcine pancreatic \( \alpha\)-amylase

PPP  porcine pancreatic protease
DEDICATION

to

Mike
PART I. STRUCTURE OF PORCINE PANCREATIC α-AMYLASE
I. INTRODUCTION

Amylases (1) hydrolyze amylose, amylopectin, glycogen, and their derived dextrins. Amylases may be divided into three subdivisions: The glucoamylases which release glucose residues from the nonreducing end via an exomechanism; β-amylases which release β-maltose residues from the nonreducing end via an exomechanism until an α(1→6) branch linkage is reached; and α-amylases which release low molecular weight oligosaccharides via an endomechanism. It is this third type of amylase which will be discussed here.

Porcine pancreatic α-amylase (α-1,4-glucan-4-glucohydrolase, E.C.3.2.1.1.) has been studied for many years. Roberts in 1881 investigated its amylolytic (2) strength and defined a unit of activity as that maximal concentration of starch on a percent basis that could be cleaved in a unit of time to the achromic point, that is, the stage where iodine will no longer react with starch to give a blue color. In 1925, Sherman et al. (3) first identified this enzyme as a protein. This was based on the stabilizing effect of amino acids. He noted the enzyme is more stable at the proper pH and at low temperatures. Amylase was a confusing enzyme at that time. It seemed to possess two activities: The amylolytic activity which cleaved starch molecules and the saccharogenic activity which released an increased amount
of reducing sugars. These two activities seemed to copurify in the same ratio.

Porcine pancreatic α-amylase (PPA) is a protein with a molecular weight of 50,000-54,000 daltons (4-6) as determined by several methods. It has been shown to be a single homogeneous molecule by boundary electrophoresis, flat gel polyacrylamide electrophoresis, gel filtration, and ultracentrifugation (7). The two activities Sherman et al. (3) investigated have been elucidated by Robyt and French (8) and can be summed up by the term multiple attack. An initial cleavage reduces the long amylose molecule into two shorter chains (amyloclastic) and, before one of these is released, multiple cleavage results in the release of maltose or maltotriose (saccharogenic) activity (9). The binding site is envisioned to span five glucose units with the active site included (10).

The following will be a survey of the published material on the structure of this amylase including some of the known properties, calcium effect, sulfhydryl reactivity, protease susceptibility, amino and carboxy terminals, isozymic forms, subunits, and sequence. The dissertation work spanned a time from September 1971 to October 1974. Several of the references were published after this final date and are included to complete the picture.

Beaupoil-Abadie et al. (11) have shown that PPA is a
glycoprotein. The carbohydrate values were obtained by
gas chromatographic analysis of the TMS derivatives of the
hydrolyzed and reduced sugars. Two PPA isozymes were ob­
served, I and II (4, 5, 7). Each mole of the enzymes con­
tains 0.75 mole of fucose, 0.45 mole of galactose, and 0.52
mole of mannose. Isozyme I contained 1.2 moles of glucos­
amine and isozyme II contained 0.8 mole. Traces of sialic
acid were also found (11). The pH optimum is 6.9 (12).

Lifshitz and Levitzki (13) have found one chloride bind­
ing site per molecule. Chloride increases the $K_{cat}$ of the re­
action by 30-fold while the affinity toward the substrate
(Km) remains unchanged. This means that chloride exerts
its effect on the initial catalytic step and not on the
multirepetitive steps (13°). The ε-amino group of one lysine
has been identified (13) as the chloride binding site. The pK
has been determined to be 9.1, somewhat lower than the usual
pK for ε-amino groups of 10.3. The chloride binding site can
be blocked with 2,4-dinitrofluorobenzene without affecting
substrate binding. Chloride binding induces a small con­
formational change, too small to be detected by circular
dichroism, fluorescence, or specific probes. This change
has been determined (13) by proton exchange methods. Bind­
ing causes a suppression in the exchange of 26 protons and
is accompanied by an increase of 240-fold in the calcium
binding constant. Albumin, sodium taurodeoxycholate,
Triton X-100, polyethylene glycol, and DMSO activate the
enzyme by some sort of surface phenomenon (15).

Krysteva and Elödi (16) have made several physical measurements on the enzyme such as optical rotary dispersion, denatured difference spectra, and perturbation difference spectra and have found the structure possesses about 15% helical content. Eighty percent of the tryptophans (12 residues) and 30% of the tyrosines (5 residues) are accessible to 70% ethylene glycol while 100% of the tryptophans (15 residues) and again 30% of the tyrosines are available to 92% D₂O. These findings indicate a fairly compact molecule. Amino acid analysis (4) indicates a high amount of tyrosine (18/470) and tryptophan (15/470) (10.8%) (6) which accounts for its high absorbance at 280 nm. There are also high amounts of threonine (23/470) and serine (32/470) (9.5%) and aspartic (61-66/470) and glutamic acid (38/470)(24%). These last two groups are known to favor hydrogen bonding and are also indicative of a compact structure. Another indicator is the favorable ratio (1.18) of polar to nonpolar residues (6). Most investigators agree that two sulfhydryls exist but they disagree as to whether or not they are necessary for enzymatic activity. Schramm (17) says they are required for high enzymatic activity. The sulfhydryls will react rapidly in the presence of both EDTA and SDS. 7 M urea and SDS allow only a slow reaction. Derivatization with DTNB (5,5'-dithiobis[2-nitrobenzoic
acid]) or N-ethylmaleimide causes 80% inhibition in enzymatic activity. I\(_2\) is the only reagent that can rapidly attack the sulfhydryls in the absence of EDTA. Amylase consists of a tight, compact structure, which in effect masks the sulfhydryls from rapid reaction with many sulfhydryl reagents. This compact structure is attributed to the action of the calcium ion. Once it is removed, unfolding can take place and it is this unfolding which is the rate determining step in sulfhydryl reactivity. Chloride ion also has an effect. The reaction requires four hours in the presence of the chloride ion and only 15 min in its absence (14). The effect of the chloride ion apparently lies in its ability to increase the affinity for Ca\(^{++}\) (14). Both act together to keep the structure compact. In the absence of Ca\(^{++}\) the first sulfhydryl reacts with a burst in about three seconds while the second reacts more slowly, displaying a reactivity half-life of six min (14).

Teledgi and Straub (18a) added equimolar amounts of DTNB in the presence of EDTA to PPA and observed that two moles of CNT (3-carboxylato-4-nitrothiophenolate) were released per mole of DTNB. Normally one mole of DTNB reacts with one mole of sulfhydryl releasing one CNT.

\[
\text{Protein} - \text{S}^- + \varnothing - \text{S} - \text{S} - \varnothing \rightarrow \text{Protein} - \text{S} - \text{S} - \varnothing + \varnothing - \text{S}^- \\
(\text{protein sulfhydryl}) + (\text{DTNB}) \rightarrow (\text{Protein} - \text{S} - \text{CNT}) + (\text{CNT})
\]

(mixed disulfide) (18b)
Thus Teledgi and Straub's observation can then be explained by assuming the two sulfhydryls are located close together. Once the first reactive sulfhydryl becomes derivatized (CNT-S-) then the second sulfhydryl becomes exposed and subsequently displaces the CNT group from the first, resulting in two moles of CNT released from one DTNB plus the formation of a new disulfide bond. They propose that the sulfhydryl groups are not essential for activity but only for the stability of the enzyme.

In contrast to Schramm (17) and Telegdi and Straub (18a) Pommier et al. (19) did not observe this first initial burst of the activity. He states that both react with DTNB with the same rate constant in the presence of EDTA and/or urea. No activity was observed after derivatization. From his studies he concluded that one of the sulfhydryls was not present at the active center since this derivatization caused only a 25% loss in activity. Derivatization is blocked by maltotriose indicating that the sulfhydryls are part of or close to the binding site. The sulfhydryl groups are necessary for enzyme activity such that blocking them with CNT causes local modification or steric hindrance of the active site. The enzyme itself loses only 50% of its activity after 3 hr of incubation in EDTA which may be due to protease contamination. This may represent their difference of 100% inhibition and others which only obtained
80% inhibition. A further publication from the same laboratory by Granger et al. (20) observed no loss in activity after derivatizing the sulfhydryls with mercuric ions, iodoacetamidonapthol, or DTNB in the absence of EDTA. (CNT)₂-amylase is still partially active (25-35%). This enzyme preparation contained no contaminating proteases which were apparently present in Pommier et al. (19) enzyme preparation. The gradual loss in activity does not parallel blockage of the sulfhydryl groups and substrate binding is not affected by CNT binding.

Eisele et al. (21a) and Porter and Preston (21b) have shown that protease activity is manifested in the presence of SDS at 37°C. This activity is responsible for the false assumption that pullulanase from *Aerobacter aerogenes* consists of subunits with molecular weights of 111,000 and 34,700 or 87,400 and 55,800. Actually the enzyme consists of a single chain weighing about 140,000 daltons. The apparent subunits arise from preferential splitting at either of two labile peptide bonds (21a).

Granger et al. (22) have shown that one-half of the PPA in the presence of EDTA can be split with trypsin in 15 min. After 30 min most of the enzyme is in two forms (A and B) which have molecular weights of 35,000 and 12,500 by SDS gel electrophoresis. No further decomposition is observed on prolonged trypsin treatment. This enzymatically inactive
preparation when passed through a Bio-Gel P-100 column gives two peaks, \( P_1 \) and \( P_2 \). \( P_1 \) contains A and B bound together by disulfide bridges and is retarded by the column similar to the way the active amylase is retarded. \( P_1 \) contains the two free sulfhydryls and \( P_2 \) is the link peptide between A and B (22).

Several investigators have shown that PPA contains at least one gram atom of \( \text{Ca}^{++} \) firmly bound per mole. \( \text{Ca}^{++} \) is required for activity (14, 23a). This ion provides stability and aids in the enzyme's crystallization (23b). 0.25 gram atom of \( \text{Zn}^{++} \) per mole was found (23a). Crystallizations from urea and organic solvents require \( \text{Ca}^{++} \) for stability (23b). Telegdi and Straub (18a) and Stein and Fischer (24) state that \( \text{Ca}^{++} \) protects the enzyme from denaturation and proteolytic degradation. Repeated crystallizations do not free the preparations of contaminants which may well be present in their zymogen forms. DFP can inactivate these only when the zymogen has been activated. Use of \( \text{Ca}^{++} \) and DFP may not guarantee the absence of proteolytic activity, however (7, 25-27).

Most investigators agree the carboxy terminal is a leucine residue (6, 22). When the literature concerning the amino terminal is examined, several different amino acids have been reported: lysine (13); phenylalanine, alanine, and glycine (26); and glycine (27). Reports that the amino
terminal was derivatized have been made (6, 22); a N-acetyl derivative (28, 29) and pyroglutamic acid (30) have been suggested. The most recent evidence seems to support the blocked amino end with the free amino ends probably arising from proteolytic action. Fábián (30) submitted his manuscript for publication July 22, 1970, and Cozzone and Marchis-Mouren (29) submitted theirs July 10, 1970. The latter two had a paper in print suggesting that the blockage may be due to an acetyl group but they only stated the fact that it is blocked and not how it is blocked. Fábián hydrolyzed the enzyme with trypsin, chymotrypsin, and then nagarse and isolated a spot which barely reacted with ninhydrin. After several purification steps, he compared the mobilities of N-acetyl glutamic acid and pyroglutamic acid standards with his unknown and identified it as pyroglutamic acid. He stated, however, that his method of purification did not exclude acetyl lysine, acetyl arginine, or acetyl histidine but since he could not find acetyl groups by gas chromatography, he proposed pyroglutamic as the amino terminal. Cozzone and Marchis-Mouren (29) propose an N-acetyl group based upon a NMR acetyl singlet due to the methyl group. The published spectrum does show an acetyl group but the evidence may not necessarily demonstrate that the position of the acetyl group is on the amino terminal. Beaupoil-Abadie et al. (11) have found that glu-
cosamine is present at 1.2 residues (for amylase I) and 0.8 residue (for amylase II) per molecule. Their method of isolation could not distinguish whether glucosamine was present as a free amino sugar or derivatized with an N-acetyl group. It is very likely that the glucosamine is acetylated and that it is this acetyl group that the NMR is picking up. They did not determine the position of the glucosamine in the protein molecule. The amino terminal CNBr peptide contained 26 amino acids. This may contain glucosamine which may be N-acetylated. If glucosamine is not present, then the acetyl group may be attached to the amino terminal.

Multiple enzymatic forms have been observed by several investigators (4, 5, 7, 25-27, 31, 32) on DEAE-cellulose, DEAE-Sephadex, and polyacrylamide gel electrophoresis. Most investigators have identified two isozymes; the faster moving one on DEAE-cellulose is labeled I. Isozyme I elutes in its original position and isozyme II elutes in its original position when subjected to rechromatography on DEAE-cellulose. Isozyme I migrates in gel electrophoresis as a single band separated from that of isozyme II. Isozyme separation depends on the pH of the eluting buffer (27). I and II could not be separated below pH 8.5. Their thermal and pH stabilities were identical and the two forms could not be interconverted by trypsin at 25° or 37°.
Szabó and Straub (31) found two to four active fractions that were dependent on the presence or absence of urea and/or mercaptoethanol. Each was equally as active as any of the others but some were interconvertible. Two of these forms seem to be artifacts of the isolation procedure. Further investigations by Juhász and Szabó (33) found only two isozymes in the presence of mercaptoethanol and these possessed the same pH optimum and Km.

Banks et al. (5) found four fractions on DEAE-cellulose which were identical in their hydrolysis patterns, optimum pH, multiple attack pattern, and amino acid content. These extra peaks are thought to be artifacts due to isolation procedures in the commercial preparation.

Cozzone et al. (6) did amino acid analysis on the two amylases and found five more aspartic acid residues in I than in II. Both possessed a common carboxyl terminal of -Ile-Val-Tyr-Leu-COOH and a blocked amino terminal. Fifty-five amide groups were found. The only difference between amylase I and II seemed to be in the glucosamine content and in the aspartic acid content. Cozzone et al. (34) cleaved the two isozymes with CNBr and found no difference in the purified peptides. They could not find where the extra five aspartic acid or asparagine residues were located, or whether they were randomly scattered or clustered at some point. If these aspartates were located at the amino
terminal of one of the CNBr peptides and if onto the amino
terminal of these five asparates was attached a proline
residue, then this cluster may have been lost during the
isolation procedures. It has been shown that Asx-Pro is
labile to organic acids (34). These peptides can be ordered
to correspond to that of the native peptide based upon
pulse labeling in pancreatic slices with 3H-valine or 14C-
protein hydrolysate. As the chain grows from the amino
terminal, the specific activity increases. They found no
overlapping specific activities and were able to order them
unambiguously. Fortunately the two terminal peptides agreed
in their terminal amino acid with that found in the native
enzyme. Nine peptides were purified which accounted for
the eight methionines (35).

Straub et al. (36) found 10 carboxymethylated cysteine
peptides after tryptic hydrolysis of the completely reduced
and carboxymethylated amylase. They thus also found one
peptide chain for the enzyme. 14C-labeling of the free
sulphydryls by carboxymethylation, disulfide reduction, and
carboxymethylation of the newly released sulphydryls results
in two to three radioactive spots which varied in intensity.
This indicates microheterogeneity of the isozymes due to
disulfide-sulphydryl exchange. The disulfide bonds in the
two amylases are formed from different cysteine residues,
causing the isozymes to contain different sulphydryl groups
Treatment in concentrated urea or salting out with ammonium sulfate causes this rearrangement. Treatment with 3 mM EDTA and 20 mM DTT for 2 hr at 0° causes the original disulfide sulfhydryls to reform again into the original position. All four are near enough for interchange to be possible. This is a modification of the explanation of isozymes as proposed by Robyt et al. (25) and Lee (38).

McGeachin and Brown (26) added 1-fluoro-2,4-dinitrobenzene and found three amino terminal amino acids, unlike Cozzone et al. (6,34). This would indicate a minimum of three peptide chains with amino terminals of phenylalanine, alanine, and glycine. It is believed that these arose from limited proteolytic hydrolysis. Even these partially hydrolyzed molecules are held together in a fairly compact structure since only 12 out of the 17 possible lysine residues reacted and none of the histidines or tyrosines reacted. A single peak was found in the ultracentrifuge and by free electrophoresis (26). The authors observed that DFP was required at all times since it can inhibit only activated protease molecules. The zymogens that perhaps were entrapped within the amylase crystals could not yet have reacted with DFP. Either the DFP concentration was not high enough to prevent partial proteolysis or limited hydrolysis of DFP by water reduced the DFP concentration below a critical point, resulting in proteolysis that pro-
duced the free amino terminals. No carboxy terminal data were presented.

Straub et al. (39) found isozyme II more abundant than isozyme I. Treatment with mercaptoethanol in the presence of 8 M urea followed by carboxymethylation or treatment with alkaline sulfite and Cu^{++} in the presence of 8 M urea produced two fractions after DEAE-cellulose chromatography. These were pooled with a 30-40% yield, dialyzed, and treated with performic acid. Peptide A had a molecular weight of 32,000 daltons and B of 21,000 daltons. These subunits, however, could have been derived from the 30 min, 37° heat treatment that the amylase was subjected to during isolation.

Scharpe et al. (7) found three fractions on 7.5% acrylamide with isoelectric points of 6.0, 5.7, and 5.4. All had similar activities. Proteolytic action may be responsible for this result.

Although the previous reports of finding subunits seem to be explained away by proteolysis, the basic idea of subunits seemed plausible. Similar subunits could be held together by disulfide bonds in different ways and could give rise to nearly identical isozymes that differed only in apparent charge. It would simplify the understanding of the allosteric activation by chloride if the enzyme consisted of subunits. Another interesting fact about this enzyme is that it seems to contain two binding sites (32),
which was determined by equilibrium dialysis. Two moles of maltotriose have been shown to bind to one mole of enzyme. This determination was based on an earlier observation that a precipitin-like reaction (40) occurs between glycogen and amylase. Levitzki et al. (40) showed that under the proper conditions 80% of the amylase could be precipitated by glycogen in the presence of Ca$^{++}$. No precipitation was observed in the Ca$^{++}$-free enzyme. Ca$^{++}$, Sr$^{++}$, or Ba$^{++}$ added back to the system restores the phenomenon. As in the usual precipitin reaction, an excess of either amylase or glycogen decreases the quantity of the insoluble complex.

Robyt et al. (25) were able to explain all of the known facts with a proposed structure. The amylase was reduced with dithiothreitol and passed over a reducing DEAE-cellulose column. Two subunits emerged with molecular weights of 25,000 daltons, each having two sulfhydryls, and two disulfide bonds. Treatment with DTT in the presence of EDTA followed by derivatization with iodoacetamide resulted in only one peak on DEAE-cellulose. Performic acid oxidation (38) followed by SDS gel electrophoresis revealed one band at about 12,000 daltons. Amylase was proposed to exist as four identical chains of 12,500 daltons each, held together by disulfide linkages in such a way that a more labile disulfide could easily be cleaved to yield two 25,000 dalton
chains. This structure would easily explain two or three similar isozymes differing only in the disulfide bond arrangement which would reveal different parts of the peptides to result in different apparent charges. Two subunits working together also explains the allosteric effect of chloride and the existence of two binding sites. These data, however, were in conflict with Cozzone et al. (6) where they find only one chain.

The earliest evidence for enzyme or hormone precursors (zymogens) was for the posterior pituitary nonapeptide hormone, vasopressin, as reported by Steiner et al. (41). Other hormones have since been shown to have precursors such as parathyroid hormone, gastrin, glucagon, sheep pituitary peptide B-lipotropin, and insulin. Some of these need to be activated by two actions such as in proinsulin which shows a trypsin and a chymotrypsin-like cleavage (42). There is evidence for a "pre" form for proinsulin (42). Other enzymes that possess a "pre" form are trypsin, chymotrypsin, carboxypeptidase, pepsin, and all the proteolytic enzymes involved in blood clotting (43). All of these "pre" forms are inactive or of low activity. A slightly different example is that of ribonuclease S which can be cleaved with subtilisin into two peptides which are held together to maintain an active complex. The bonds that are involved in the structure have been identified (44).
The following are some of the questions that I set out to answer in part A of my dissertation on the structure of porcine pancreatic α-amylase: Could PPA exist in a "pre" form? After proteolytic cleavage, does it still hold together like ribonuclease S? Were the subunits due to proteolysis? Was Cozzone's et al. (6,34) data due to poor reactants which were not strong enough to reveal the subunits? My first question was, does freshly prepared PPA, free of proteolytic activity, contain subunits?
II. MATERIALS AND METHODS

A. Materials

The following is a list of materials obtained from Calbiochem, San Diego, CA.

Egg albumin (2 x's crys.)
Aspergillus oryzae α-amylase (3 x's crys.)
Aquacide II-A
Dithiothreitol A grade (DTT)
5,5'-Dithiobis-(2-nitrobenzoic acid) A grade (DTNB)

The following is a list of materials obtained from Eastman Organic, Rochester, NY.

Riboflavin
Acrylamide
N,N,N',N'-Tetramethylethlenediamine (TEMED)
α-toluenesulfonyl fluoride (PMSF)

The following is a list of materials obtained from Sigma Chemical Co., St. Louis, MO.

Cytochrome c
α-chymotrypsin (3 x's crys.)
Bovine serum albumin (BSA)
Pepsin (2 x's crys.)
Glycogen (Shellfish)
N,N'-Methylene-bis-acrylamide (bis)
Iodoacetamide
Iodoacetic acid
4-vinylpyridine
Diisopropylfluorophosphate (DFP)
Sodium lauryl sulfate (SDS)
Dansyl chloride
DEAE-cellulose
β-glycerophosphate

The following is a list of materials obtained from Worthington Biochemicals, Freehold, NJ.
Porcine pancreatic α-amylase (3 x's crys.) (PPA)
Trypsin-TPCK
Lysozyme

The following is a list of materials obtained from Merck, Rahway, NJ.
Casein
Soluble starch Merck (special for diastatic powder determination)

The following is a list of materials obtained from Mallinckrodt Chemical Works, St. Louis, MO.
Ammonium persulfate
2-amino-2-(hydroxymethyl)-1,3-propanediol (tris)

The following is a list of materials obtained from J. T. Baker Chemical Co., Phillipsburg, NJ.
Maltose
Bromophenol blue

The following is a list of materials and from where they were obtained.

Yorkshire pancreas from the Meats Laboratory, Iowa State University, Ames, IA.
Leucine aminopeptidase (pig kidney), P-L Biochemicals Inc., Milwaukee, WI.
Amylose obtained in our laboratory, Iowa State University, Ames, IA.
Ethylenediaminetetraacetic acid (EDTA), Fischer Scientific Co., Fairlawn, NJ.
Fluram (fluorescamine), Roche Diagnostics, Nutley, NJ.
Coomassie Brilliant Blue R-250, Mann Research Laboratories, Orangeberg, NY.

All other chemicals were reagent grade unless otherwise stated.

B. Methods

1. Analytical methods
   a. Reducing value was measured by the alkaline ferri cyanide method as adapted for use on the Technicon AutoAnalyzer (45).
   b. Blue value was determined by absorbance at 620 nm of a 0.2 ml sample and 5.0 ml water mixed with 20 µl of an
iodine solution containing 0.1 M KI and 0.1 M I₂ and allowed to stabilize for 10 min. Blue value is defined as

\((A_t/A_o) \times 100\), where \(A_o\) and \(A_t\) are the absorbancies of the iodine complex of the digest at zero time and at \(t\) min of hydrolysis (8).

c. Protein concentration was determined in the crude preparations by the method of Folin-Ciocalteu. Bovine serum albumin was used as a standard and the absorbance was determined at 750 nm (46). Protein concentration in the purified preparation was determined by absorbance at 280 nm, using the extinction coefficient \(E_{1%}^{1cm} = 22.9\) (27) on a Beckman model DU spectrophotometer with a Gilford Instruments Digital Readout absorbance meter attachment or a Beckman Acta CII Spectrophotometer.

d. Protein was detected by Coomassie Brilliant Blue R-250. The reagent was prepared by dissolving 1.25 g Coomassie Brilliant Blue R-250 in 454 ml of 50\% methanol and 46\% glacial acetic acid. The insoluble materials were removed by filtration through Whatman No. 1 filter paper. Staining of the gels was at room temperature overnight followed by diffusion destaining with several changes of a 7.5\% acetic acid and 5\% methanol solution (47).

e. Starch iodide stain for amylase activity in polyacrylamide gels consisted of soaking the gels in a 1\% starch solution at room temperature for 10-30 min, washing the
gels with cold water, and adding an iodine solution consisting of 0.1 M KI and 0.1 M I$_2$ in 50% methanol. Amylase activity is seen as a clearing against a blue background.

f. Amino acid detection on thin layer chromatographic plates, in the range of nanomoles, can be accomplished through the use of Fluram: 15 ml of fluorescamine dissolved in 100 ml acetone is sprayed onto the silica gel plate. After one min the spots are visible under ultraviolet light (48).

g. Concentration of protein solutions can be accomplished under mild conditions by the slow removal of water through a dialysis bag placed onto Aquacide II-A.

2. Amylase preparation

a. Preparation by the method of Marchis-Mouren and Paséro (28). The pancreas is freed of fat and connective tissue and homogenized in 10$^{-4}$ M DFP. This is then centrifuged for 2 hrs at 16,300 x G and then for another 1$^{1/2}$ hrs at 40,300 x G. After lyophilization, the powder is dissolved in 20 mM β-glycerophosphate, 10 mM NaCl, 3 mM CaCl$_2$, and 10$^{-4}$ M DFP standard pH 7 buffer. This is then precipitated with 2 vol cold acetone and centrifuged. The precipitate is dissolved and reprecipitated by 40% sat. ammonium sulfate dissolved in the same buffer, dialyzed overnight against buffer, and lyophilized.
b. Preparation by the method of Schramm and Loyter (49). Again the pancreas was freed of fat and connective tissue and homogenized in $2 \times 10^{-4}$ M PMSF. It was centrifuged after passage through a cheese cloth and lyophilized as above. The resulting powder is then dissolved in 40% ethanol, 12 mM β-glycerophosphate, 14 mM NaCl, 3 mM CaCl$_2$, and $2 \times 10^{-4}$ M PMSF (pH 6.9) buffer. After stirring and centrifuging to remove the precipitate, the amylase activity was determined as described by Schramm and Loyter to be that quantity (1 unit) which will release 1 mg equivalent of maltose hydrate in 3 min at 30°. The amylase is then precipitated with glycogen at the ratio of 1 mg of glycogen to 500 of Schramm's amylase units. This precipitate is then removed by centrifugation and washed with 40% ethanol. The precipitated complex is then suspended in the buffer at a concentration of about 20 mg protein per ml and allowed to stand 1-2 hrs. Under these conditions, the amylase digests the glycogen in the complex to low molecular weight dextrins. The pH is then adjusted to 8.5 by addition of ammonium hydroxide and the solution is centrifuged at 20° for 5 min at 6,000 x G to remove the undigested glycogen-amylase-complex precipitate from the supernatant enzyme solution. This precipitate is then washed repeatedly with 20 mM β-glycerophosphate buffer (pH 8.5) to remove remaining amylase activity. These washes are combined and concentrated
10-fold by Aquacide II. This concentrated wash is then combined with the supernatant enzyme solution and the pH is adjusted to 7.0 with acetic acid and cooled to 0° for 30 min. Under these conditions PPA precipitates and is removed by centrifuging at 2° for 20 min at 9,000 x G. The precipitate is dissolved at 20° in 20 mM Tris-HCl pH 8 and 2 x 10⁻⁴ M PMSF to a protein concentration of about 20 mg/ml and charcoal is added to remove the dextrins. The enzyme is then dialyzed against pH 6.9, 20 mM β-glycerophosphate, 3 mM CaCl₂, 7 mM NaCl, and 2 x 10⁻⁴ M PMSF. The enzyme is then precipitated by adding 90 ml of acetone followed by 210 ml of a 1:1 mixture of ethanol: ether per 100 ml amylase solution. These are added over a 20 min period with stirring at -5°. Acetic acid (1 to 2 drops) is also added to aid precipitation. This is centrifuged and repeated starting at the precipitation from the pH 8.0 step above. The enzyme is stored at 4° at about 20-30 mg/ml in the standard pH 7 buffer.

3. Amylase assay
   a. Continuous assay method (45): 1 ml of an appropriate enzyme concentration is added with rapid mixing to 9 ml of a 1% starch solution in 20 mM β-glycerophosphate and 10 mM NaCl buffered at pH 7.0 and maintained at 37°. The sample line from the AutoAnalyzer is immediately placed into the solution and allowed to sample continuously. The
μg of maltose produced is determined from a standard maltose curve and plotted as a function of reaction time. The initial slope of this plot gives the initial velocity (μg/min). The International Unit is computed from the initial velocity. One International Unit is equal to 1 μmole of apparent maltose released from soluble starch per min of reaction time.

b. Agar plate method: 1.5 g of agar is dissolved by heating in 50 ml of pH 7 10 mM β-glycerolphosphate and 10 ml of 6% starch is added. This solution is autoclaved for 25 min at 121° along with the petri dishes to be used. Ten ml of the agar is added to the bottom of the dish and allowed to solidify. The enzyme is allowed to diffuse into 6 mm Whatman 3 MM filter disc by capillary action. The disc is placed onto the agar gel and incubated overnight at 37° and then removed from the gel. The gel is then stained with 1% KI, 1% I₂ solution. Zones of clearing are visible against a blue background. A linear relationship exists between the diameter of these zones and the concentration expressed in units of 10-fold dilution. Figure 1 is an example of the kinds of results obtained by this assay. The diameter of the zone of clearing shows a linear relationship when plotted against the logarithm of the enzyme concentration. The proteinase agar plate assay discussed below shows a similar relationship.
Figure 1. Amylase assay by the agar plate method
Diameter (cm)

Concentration of PPA (log µg/ml)

r = 0.998
4. Proteinase assay

Casein is dissolved (10 mg/ml) in 10 mM NaOH and added to a 2.5% agar solution to a resulting concentration of 0.5 mg/ml. The agar is dissolved as above. After autoclaving for 20 min at 121°, 10 ml hot agar solution is added to the lower plate and allowed to solidify. The test solution is incubated as above. After removal of the filter paper disc, the plate is stained with Coomassie blue. The developed plate is destained with several changes of 7.5% acetic acid and 5% methanol as described in section 1d in Methods.

5. Detection of carbohydrates on paper chromatograms

A silver nitrate dip procedure was used (50). The chromatogram is first dipped into a silver nitrate solution (2.5 ml saturated AgNO\textsubscript{3} in 1 l acetone) and allowed to dry. This is then dipped into the developer (10 ml 40% (w/v) NaOH in 1 l methanol). After the spots have developed, this is then dipped into the fixer (240 g Na\textsubscript{2}S\textsubscript{2}O\textsubscript{3}·5 H\textsubscript{2}O, 25 g Na\textsubscript{2}S\textsubscript{2}O\textsubscript{5}, and 10 g Na\textsubscript{2}SO\textsubscript{3} in 1 l H\textsubscript{2}O) until the background is white with dark brown spots. The fixer is then washed out in a water bath for 30 min.

6. Ion exchange chromatography

Dry DEAE-cellulose was washed with 15 vol (w/v) of 0.5 N HCl (51), allowed to settle 30 min, filtered, and washed with water until the pH was 4.0. The exchanger was
resuspended in 15 vol NaOH, allowed to settle 30 min, filtered, and washed with water until the pH was neutral. Buffer was added and the fines decanted off about 10 times. This was degassed by gentle vacuum from a water aspirator and poured into a column via a funnel reservoir. Enzyme was eluted at room temperature and 2 ml fractions were collected on a Research Specialties Co. fraction collector.

7. Recrystallization of acrylamide

Acrylamide (70 g) is dissolved in 1 l chloroform at 50° and filtered while still hot with suction and cooled to -10°. The crystals are filtered off with suction and washed briefly with cold chloroform (52). The remaining chloroform is then removed by suction from a vacuum pump overnight. The crystals are stored in a dark bottle.

8. End group analysis by dansylation

Worthington crystalline PPA (2 mg) is added to 0.5 ml of 30% H₂O₂ and 4.5 ml of 88-90% formic acid. The reaction is complete in 15 min at 50°. Water (20 ml) is added to the reaction mixture which then is lyophilized; 1 mg of lyophilized material is added to a small tube and dissolved with heating in 200 μl of 1% SDS. To this cooled solution 200 μl of N-ethylmorpholine is added with 300 μl of 25 mg of dansyl chloride per ml of dimethyl formamide. After 24 hr at room temperature, 3 ml of acetone is added to precipitate
the protein. This is then centrifuged, washed with 80% acetone, and centrifuged again. Hydrolysis is performed with 6 N HCl for 4-16 hr. This is then concentrated and extracted with 0.5 ml of H2O-saturated ethylacetate (53). Dansylated amino acids are separated on Brinkman M:N Polygram Polyamide G thin layer plates by benzene:acetic acid (9:1) or water:formic acid (100:37) (53). Dansylated amino acids may also be separated on silica gel plates by benzene:pyridine:water (40:10:1) (54), diethyl ether:methanol:acetic acid (100:5:1) (55), or n-butanol saturated with 0.2 N NaOH (54). The spots are visible under ultraviolet light.

9. End group analysis by leucine aminopeptidase

Worthington PPA (5 mg) is added to 0.1 ml of 0.1 M tris and 2.5 mM MgCl2 buffered at pH 8.5. Leucine aminopeptidase (600 mU) is added and the reaction is allowed to proceed at 37° for three days. Aliquots are removed with time and added to citric acid to stop the reaction (56). The released amino acids are analyzed on silica gel with n-propanol:water (70:30), n-butanol:glacial acetic acid:water (4:1:1), or phenol:water (75:25) (57). The freed amino acids may also be analyzed after dansylation. Fluram is used to detect the free amino acids.
10. Sulfhydryl and disulfide modification

a. Dithiothreitol at a concentration of 10 mM in the presence of 10 mM EDTA cleaves disulfide bonds to sulfhydryls (58).

b. Vinyl pyridine gives a stable derivative of disulfide bonds, when used in 100-fold excess over sulfhydryl (59).

c. DTNB (Ellman's reagent) also derivatizes sulfhydryls, but this reagent is reversible. Reaction occurs after buffering the protein (2 ml, 0.5-10.0 x 10^{-5} M sulfhydryl) in 0.1 ml of buffer (1 M tris, 1 M phosphate pH 8.1) and adding 0.5 ml of 2 mM DTNB (10 mM phosphate pH 8.1). The reaction is usually complete within 30 min at 37°C (18b).

d. Iodoacetic acid or iodoacetamide (100 mM) will form a derivative with sulfhydryls after treatment with DTT and in the presence of DTT (25).

11. Protease inhibition by DFP and PMSF

During the first PPA preparation DFP was used at the concentration of 10^{-4} M to inhibit any protease that may be present. It is possible that it was not sufficient to destroy the protease. An experiment was performed to compare the efficiency of DFP to PMSF (60, 61) as protease inhibitors. A stock solution of trypsin was prepared at a concentration of 1 mg/ml (2 x 10^{-5} M) in β-glycerophosphate. This was then
treated with DFP at $10^{-4}$ M or $10^{-3}$ M or PMSF in 5% 2-propanol at $5 \times 10^{-4}$ M or $5 \times 10^{-5}$ M. These incubation mixtures were then tested for the residual protease activity on casein plates after 24 hr reaction time.

12. Gel electrophoresis on polyacrylamide gels

The electrophoresis procedure is that of Davis (62) and modified by the Canalco formulation sheet of 1965.

a. Separating gel:

Reagent A; $48 \text{ ml } 1 \text{ N } \text{HCl} + 36.3 \text{ g tris}$

$+ 0.23 \text{ ml TEMED} + \text{ dilution to } 100 \text{ ml with distilled water.}$

Reagent B; $28.0 \text{ g recrystallized acrylamide}$

$+ 0.735 \text{ g bis} + \text{ made up to } 100 \text{ ml}$

with distilled water.

Reagent C; $0.140 \text{ g ammonium persulfate}$

made up to 100 ml with distilled water fresh before use.

One part reagent A + 1 part reagent B + 2 parts reagent C. The solutions are deaerated separately, mixed in the indicated proportions, and 2 ml added to a 105 x 6 mm column, which is overlaid with 4 mm of water. Polymerization is complete within 20-30 min.

b. Stacking gel:

Reagent D; $48 \text{ ml } 1 \text{ N HCl} + 5.98 \text{ g tris} + 0.46 \text{ ml TEMED} + \text{ dilution to } 100 \text{ ml with distilled
water.

Reagent E; 10.0 g recrystallized acrylamide
+ 2.5 g bis + made up to 100 ml with
distilled water.

Reagent F; 4 mg riboflavin made up to 100 ml
with distilled water.

One part reagent D + 2 parts reagent E + 1 part reagent
F + 4 parts distilled water. The reagent is deaerated and
0.2 ml of the stacking gel is added onto the top of the
separating gel after removal of the water layer. Water (4
mm) is added to the top of this layer. The gel is formed
by photopolymerization within 30-45 min.

c. Sample layer: The top water layer is again removed
and the sample of 50-200 \( \mu \text{g} \) in 0.15 ml, to which 2 drops of
glycerol and 1 drop of 0.5% bromophenol blue in 60% glycerol
have been added, is layered on top.

d. Electrophoresis buffer: The buffer consists of
3.0 g tris and 14.4 g glycine in 1 l distilled water.

e. Electrophoresis: Buffer is carefully overlaid on
the sample layer and the tubes placed in the apparatus with
buffer in the upper and lower chamber. The proteins are
then separated for 2 hr at 5 mA/tube and 210-280 V. The
proteins are then identified by staining with Coomassie
blue (see section 1d under Methods) or the active enzyme
bands are found by the starch-iodide stain (see section 1e under Methods).

13. SDS gel electrophoresis for molecular weight determination

Electrophoresis is that as described by Weber and Osborn (63) with slight modification. Preincubation of the proteins is in 4 M urea, 1% SDS, 1% mercaptoethanol, and 0.2% EDTA at pH 8.0. This is maintained at 37° for 1-2 hr.

Reagent G; 7.8 g NaH$_2$PO$_4$·H$_2$O + 50 g Na$_2$HPO$_4$·12 H$_2$O + 2.0 g SDS + made up to 100 ml with distilled water.

Reagent H; 20 g recrystallized acrylamide + 0.4 g bis + made up to 100 ml with reagent G.

Reagent I; 15 mg/ml ammonium persulfate in distilled water made up fresh before use.

13.5 ml of reagent G + 15 ml of reagent H + 1.5 ml reagent I + 10 µl TEMED. The reagents are deaerated separately, mixed in the indicated proportions and 2 ml added to a 105 x 6 mm column, which is overlaid with 4 mm of water. Polymerization is complete within 30 min.

The gels are then preelectrophoresed in a 1:1 dilution of the reagent G for 1 hr at 8 ma/tube. The water above
the gels is removed and the sample is added after the addition of 1 drop of glycerol, 5 \( \mu l \) mercaptoethanol, and 3 \( \mu l \) of 0.05% bromophenol blue. Buffer is then carefully layered onto the protein solution. Electrophoresis is complete in 4.5 hr at 8 mA/tube. Proteins of known molecular weights were used as standards to construct a standard curve by plotting the log of the molecular weight against the relative mobility as compared to that of bromophenol blue. Location of this dye front after staining with Coomassie blue is facilitated by marking the dye front, after removal of the gel from the glass tube, with a short piece of wire.

14. Protease degradation of PPA

a. First experiment: PPA (1 mg) is dissolved in 0.6 ml of 20 mM \( \beta \)-glycerophosphate, 3 mM NaCl, 7 mM CaCl\(_2\) buffer pH 6.9 (standard PPA buffer). To this, 10 \( \mu l \) aliquots of stock trypsin or \( \alpha \)-chymotrypsin solution (1 mg/ml) is added at 0, 10, and 20 hr. To a third, 5 \( \mu l \) of the 40% ethanol insoluble protease (PPP) is added at the same time periods. The fourth incubation mixture is a control and receives buffer at the three addition periods. Aliquots (80 \( \mu l \)) are removed with time and added to 50 \( \mu l \) of 10 M urea, 20 mM DTT, and 20 mM EDTA. This is allowed to react at 4\( ^\circ \) for 22-24 hr and made 100 mM in iodoacetic acid. Reaction is complete in 5 hr at room temperature, after which the derivatized product
is denatured by the addition of 15 μl of 10% SDS and frozen. The course of the reaction is followed by electrophoresis of the derivatized protein on SDS gels to determine the molecular weight of the products.

b. Second experiment: The experiment is similar to that listed above except additions occurred at 0 and 15 hr and trypsin was not used. Aliquots were removed with time and treated with DTT as above or added to buffer. At 51 hr an additional aliquot was added to urea and EDTA but not DTT. These were also analyzed by SDS gels and the remaining activity was determined by the continuous assay method.

c. Third experiment: This experiment consisted of one control and one test using α-chymotrypsin. Additions were made at 0, 10, 20, and 30 hr. Aliquots were removed and incubated in:

10 mM EDTA, 25 mM tris, 4 M urea (pH 8.1), and allowed to react 24 hr at 4°C before 100 mM iodoacetamide was added. After 24 hr the solution was made 1% in SDS and frozen;

or

10 mM EDTA, 25 mM tris, 4 M urea pH 8.1 and 1% SDS and frozen;

or

standard PPA buffer and 10 mM DDT and frozen;
or

standard PPA buffer and frozen.

The products of reaction were analyzed by SDS gel electrophoresis, gel electrophoresis, product specificity pattern (blue values vs. % apparent maltose) and the continuous assay method.

15. Product specificity pattern

Amylose is dissolved (8) at a concentration of 1 mg/ml in 15 mM β-glycerophosphate, 7.5 mM NaCl, and 3 mM CaCl₂, buffer at pH 6.9. To this, 2.5 mU of PPA is added. Fractions (2 ml) are removed with time and boiled for 15 min to stop the reaction. After cooling, Amberlite MB-3 was added to desalt the reaction mixture. After removal of the resin, the aliquot is concentrated on the test tube evaporator and dissolved in 100 µl of water (46). This is spotted on a 17.8 x 17.8 cm Whatman 3 MM paper and subjected to 3 ascents at 65° (64), modified from French et al. (65). The experiment was performed on the untreated control and on the α-chymotrypsin treated PPA 40 hr sample obtained in the third experiment under section 14 in the Methods section.

16. Blue value vs. reducing value

PPA is allowed to react with 1% amylose, and aliquots are removed with time. The reducing value and the blue value
(see sections la and lb under Methods) for each time point are determined. The % apparent maltose was determined by the following formula:

\[
\text{% apparent maltose (% theoretical maltose)} = \frac{\mu g/ml \text{ apparent maltose} \times 100}{1000 \mu g \text{ starch/ml}} + \frac{342 \mu g/\mu \text{mole maltose}}{324 \mu g/\mu \text{mole anhydro-maltose}}
\]

The blue value is then plotted against the % apparent maltose for the untreated control and for the α-chymotrypsin treated PPA 40 hr sample obtained in the third experiment under the heading of protease degradation of PPA in the Methods section.

17. Thin-layer gel filtration chromatography

Thin layer gel filtration is performed with Sephadex G-100 superfine and Bio-Gel P-100 superfine which is swollen in water as described in the apparatus book from Pharmacia (66). A slurry is prepared and layered on a 20 x 40 cm plate with the gel spreader that is provided, with a thickness of 0.6 mm. The gel is then equilibrated with buffer (20 mM phosphate and 500 mM NaCl, pH 7.25) overnight. The gel is connected to the buffer reservoirs via a Whatman 3 MM filter paper wick. The plate is elevated to a 10° angle for equilibration and for separation of samples. Protein samples (5 μl) are applied to the horizontal gel with
a Hamilton syringe. Separation is complete after 6.5 hr at
an angle of 10°. Protein spots are absorbed with the liquid
by carefully rolling a precut (20 x 40 cm) Whatman 3 MM
filter paper onto the gel surface. Care is taken to avoid
air bubbles from forming under the paper that might interfere
with the transfer process. After about 3 min most of the
liquid had been absorbed into the paper. The paper is re­
moved and dried in an oven at 100° for about 10 min. Pro­
teins are detected by staining the paper with Coomassie
blue R-250 (0.25% in methanol:acetic acid 90:10) and de­
staining the background with methanol:glacial acetic acid:
water (50:10:50) (66). This rinse may be reused several
times by passage through a funnel containing charcoal. A
plot of the log of the molecular weight vs. the relative
mobility to BSA is linear. A standard curve was prepared
using proteins of different molecular weight (12,400 D to
87,000 D). Amylase activity may be detected by presoaking
the paper in a solution of 1% starch. The amylase reacts
with the starch in the paper which is incubated on the gel
at 37° for 30 min. The paper is removed and sprayed with
an iodine solution containing 0.1 M KI and 0.1 M I₂. The
amylase-containing spots are seen as white to orange spots
against a blue background.
18. The $r$ function

This function gives an estimate of the goodness of fit of least squares-predicted points and the actual experimental points. It is called the correlation coefficient and is a measure of the linear correlation of two variables, $x$ and $y$. The sign of the function may be positive or negative and is indicative of the sign of the slope of the fitted line. The magnitude of the function varies from 0 to 1. A value of 0 indicates the two variables are uncorrelated and a value of 1 indicates the correlation is exact. This value is calculated as follows:

$$r = \frac{m \sigma_x}{\sigma_y}$$

where

$$(\sigma_x)^2 = \frac{\sum_{i=1}^{N} X_i^2}{N} - \bar{X}^2,$$

$$(\sigma_y)^2 = \frac{\sum_{i=1}^{N} Y_i^2}{N} - \bar{Y}^2,$$

and

$$m = \frac{\sum_{i=1}^{N} \frac{X_i Y_i - \bar{X} \bar{Y}}{\sigma_x^2}}{\sum_{i=1}^{N} \frac{1}{\sigma_x^2}}.$$
III. RESULTS AND DISCUSSION

Several preliminary studies were performed to compare the PPA which I isolated by the method of Marchis-Mouren and Paséro (28) to that reported in the literature. This PPA had a molecular weight which averaged about 53,000 D and a minor band of about 39,000 D. The literature value gives a range from 50,000 D to 54,000 D (4-6).

Isozymes I and II were partially separated on DEAE-cellulose with 20 mM β-glycerophosphate at pH 8.3. This buffer was used because it did not interfere with the alkaline ferricyanide reducing sugar assay. The buffering powers are poor at pH 8.5 and I was unable to maintain the pH of the column at the optimal pH 8.5 (27) for the best separation. The buffer used in the literature was phosphate (4) which interferes with the assay procedure. I chose not to use their assay procedure because it was not as accurate as the alkaline ferricyanide procedure (67). Figure 2 shows the elution profile of the purified PPA on DEAE-cellulose and indicates the presence of a proteolytic impurity. Selected fractions were then assayed for amylase activity and/or concentrated by Aquacide II before gel electrophoresis. Table 1 indicates the isozymic band mobility and the activity of the analyzed fractions. Fractions before #43 show no bands on electrophoresis. These proteolytic enzymes (trypsin and
Figure 2. DEAE-cellulose elution profile of PPA purified by the method of Marchis-Mouren and Pasero (28)

- O- $A_{280}$ protein determination
- ••• diameter of the zone of clearing in the casein agar plate, proteinase determination
Table 1. Analysis of the DEAE-cellulose fractions of PPA prepared by the method of Marchis-Mouren and Paséro (28)

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>Band mobility&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Amylase activity&lt;sup&gt;b&lt;/sup&gt; U/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>35</td>
<td>-</td>
<td>1.82</td>
</tr>
<tr>
<td>40</td>
<td>-</td>
<td>N.D.</td>
</tr>
<tr>
<td>43</td>
<td>.16&lt;sup&gt;c&lt;/sup&gt;</td>
<td>289</td>
</tr>
<tr>
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</tr>
<tr>
<td>48</td>
<td>N.D.</td>
<td>822</td>
</tr>
<tr>
<td>50</td>
<td>.19</td>
<td>N.D.</td>
</tr>
<tr>
<td>52</td>
<td>.19</td>
<td>N.D.</td>
</tr>
<tr>
<td>56</td>
<td>.19, .22&lt;sup&gt;c&lt;/sup&gt;</td>
<td>N.D.</td>
</tr>
<tr>
<td>62</td>
<td>.19&lt;sup&gt;c&lt;/sup&gt;, .22</td>
<td>N.D.</td>
</tr>
<tr>
<td>65</td>
<td>.17&lt;sup&gt;c&lt;/sup&gt;, .22</td>
<td>1660</td>
</tr>
<tr>
<td>72</td>
<td>.23</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

<sup>a</sup>Relative mobility of the protein band with respect to that of the tracking dye, bromophenol blue. N.D. not determined.

<sup>b</sup>International units.

<sup>c</sup>Relatively faint band.
chymotrypsin) and do not stain well with Coomassie blue. Fractions #43-52 contain isozyme I. Fractions #56-65 show a mixture of isozymes I and II. Isozyme I is decreasing and isozyme II is increasing. Fraction #72 consists exclusively of isozyme II. The large amount of protease present in this preparation precludes its use for further structural studies.

Dansylation of the freshly purchased commercial PPA prepared by the method of Caldwell et al. (68) failed to yield any dansylated α-amino acids. This agrees with the more reliable reports in the literature (6, 22, 28-30). Treatment with leucine aminopeptidase also released no amino terminal that could be detected with Fluram on silica gel plates. This is in agreement with the dansylation data. I found no references to anyone else using this technique to investigate the amino terminal linkage. My data seem to agree with those who propose a derivatized amino terminal (6, 22, 28-30).

After treatment of PPA (preparation 2a in Methods) with DTT and iodoacetamide or iodoacetic acid, two peaks were separated on DEAE-cellulose in a location that was distinct from the isozyme I and II elution positions. This is in agreement with observations by Robyt et al. (25). Molecular weight determination on these pooled and concentrated peaks indicated partial breakdown of the original intact
structure. Two major bands were observed with molecular weights of about 53,000 D and 38,000 D. Two very faint bands were observed with molecular weights of 25,000 D and 13,000 D. These lower two bands were observed by Lee (38) to be relatively more intense than I was able to find. The explanation for the presence of the two higher molecular weight bands lies in the fact that the gels contain a rather high concentration of persulfate which was used to catalyze polymerization. This then removes the carboxymethyl groups from the derivatized sulfhydryls and allows the disulfide bonds to reform. Without this interference by persulfate, only the 25,000 D band would be visible and possibly also the 13,000 D due to complete reduction of the highly resistant disulfide bands holding these 12,500 D subunits together (38). Other investigators could find only one band at 53,000 D after reduction with mercaptoethanol, a weak reducing reagent (4). Treatment with vinylpyridine or DTNB after reduction showed the same result as that obtained using iodoacetic acid or iodoacetamide. These two were tried in an attempt to halt the deleterious effect of persulfate. Protease activity may be responsible for the lower molecular weight bands. Proteinase analysis of both the crystalline Worthington PPA and that which I prepared (preparation 2a in Methods) revealed the presence of contaminating proteolytic activity.
A way to remove this unwanted activity would be to increase the inhibitor concentration or find a better inhibitor. At this point, I decided to compare the relative strength of DFP and PMSF. Figure 3 shows a casein plate test of the remaining proteolytic activity after preincubation for 24 hr with the two inhibitors at different concentrations. The diameter of clearing in the untreated trypsin sample is an expression of the tryptic activity. This zone has a diameter of 1.8 cm. DFP treatment at a concentration of $10^{-4}$ or $10^{-3}$ does inhibit the trypsin ($2 \times 10^{-5}$ M) somewhat as can be seen by a reduction in the zone of clearing down to 1.7 cm but this is only 0.1 cm less than the untreated trypsin. PMSF, however, at concentrations lower than those used with DFP was capable of inhibiting trypsin to a far greater extent. PMSF at a concentration of $5 \times 10^{-5}$ M reduces the protease activity to the point that it is only able to produce a 1.2 cm diameter zone of clearing. With PMSF at a concentration of $5 \times 10^{-4}$ M the diameter is reduced to only 0.9 cm. A comparison between this latter value and that of buffer alone indicates that very little protease activity remains. A drop of one-half of the original diameter size does not indicate one-half of the enzyme remains since the relationship is not linear with the concentration but it is linear with the logarithm of the concentration. This can be seen in Figure 1. Here a drop of one-half indicates a
Figure 3. Casein agar plate proteinase assay comparing the inhibitory properties of DFP and PMSF after 24 hr of incubation

A buffer control
B trypsin (2 x 10^{-5} M)
C trypsin (2 x 10^{-5} M) and DFP (10^{-3} M)
D trypsin (2 x 10^{-5} M) and DFP (10^{-4} M)
E trypsin (2 x 10^{-5} M) and PMSF (5 x 10^{-5} M)
F trypsin (2 x 10^{-5} M) and PMSF (5 x 10^{-4} M)
drop of about 100 times. The diameter of the filter paper
disc is about 6 mm so buffer may cause a slight clearing
up to 6 mm. After 12 days of incubation (not shown), no
protease activity remains with this concentration of in-
hibitor. This experiment indicates that PMSF is far super-
ior to DFP in its protease inhibitory powers. Even so some
activity does remain which may be sufficient to cleave par-
ticularly labile peptide bonds in PPA if they exist. This
would make it impossible to determine the number of peptide
chains originally present in PPA. A method of purification
must therefore be used which will not carry with it any
contaminating proteolytic activity.

After comparison of various preparation procedures the
method of Schramm and Loyter (49) appeared to be the best
method for purifying PPA since the preparation does not con-
tain any contaminating proteolytic activity. PPA was pur-
ified (see section 2b under Methods) by their method using
$10^{-4}$ M PMSF as an added precaution. This purification is
based on the affinity of PPA to bind to glycogen and not
simply on protein purification techniques. The crucial
purification step involves the precipitin-like reaction be-
tween PPA and glycogen in 40% ethanol. This complex pre-
apitates out, leaving all contaminating protease in solu-
tion. Casein agar plates showed no proteolytic activity
was present in this preparation. This preparation was then
used for all the following structural studies.

Treatment with DTT followed by iodoacetic acid yielded no bands below 52,000 D. Dilution of the enzyme with 3 volumes of PMSF-free standard PPA buffer for 3 days at room temperature followed by reduction and carboxymethylation (see section 14a in Methods) produced no bands below 52,000 D. The Worthington preparation showed an extra band at 42,000 D. The results indicate that the purified, protease-free enzyme consists of one peptide chain of about 53,000 D. Treatment with DTT and derivatization of the sulfhydryls did not reveal the presence of subunits held together by disulfide bonds. The only way to prove that the apparent subunits did arise from proteolytic action was to add protease to an aliquot and run a control. If degradation products occur in the presence of CaCl$_2$, which is supposed to protect PPA from proteolysis (18a, 24), then the subunits did arise from contaminating proteolytic activities in the preparations which also contained Ca$^{++}$.

The most natural choice for the initial studies is a preparation from the pancreas itself. During the purification of the PPA, there was one step that was particularly rich in protease. After homogenization, lyophilization, and dissolution of the PPA in 40% ethanol, a precipitate was removed. This precipitate was used as a proteolytic agent and is referred to below as porcine pancreatic proteinase (PPP).
This was dissolved in 1 l of standard PPA buffer. PPA (preparation 2b) was treated with PPP, trypsin, and α-chymotrypsin as explained in the Methods section (14a). No change was observed with time in the control or in the trypsin (1.6 x 10^{-6} M) treated amylase, as can be seen in Figure 4a and 4b. These SDS gels were run after reduction and carboxymethylation. Both the PPP treatment and the α-chymotrypsin treatment (Figure 5a and 5b) resulted in a reduction of the intensity of the 53,000 D band and the appearance of lower molecular weight bands in the region of 39,000 D, 26,000 D and 15,000 D. The formation of lower molecular weight forms increased with time. A band appeared above the 53,000 D band. PPA always shows this dimeric band at about 110,000 D. This band seems fairly resistant to the denaturing conditions present in the SDS gels. The PPP treated PPA in Figure 4a showed a greater destruction than that treated with α-chymotrypsin (Figure 4b). It is not known whether this is due to a greater concentration of α-chymotrypsin present or due to a combination of protease activities. It is interesting to note that these experiments were performed in the presence of added Ca^{++}. Stein and Fischer (24) have stated that PPA is resistant to protease action in the presence of Ca^{++}. It appears that although it may be resistant to the action of trypsin, in the presence of Ca^{++}, it is not completely resistant to α-chymotrypsin. Granger et al. (22)
Figure 4. SDS gel electrophoresis of the first experiment

a. Control PPA at 5, 10, 15, 20, and 25 hr

b. PPA treated with trypsin (1.6 x 10^{-6} M) at 5, 10, 15, 20, and 25 hr. Trypsin was added at 0, 10, and 20 hr.

All samples were denatured with 5 M urea, 10 mM EDTA, and 10 mM DTT for 24 hr followed by sulphydryl derivatization with iodoacetic acid before electrophoresis.
Figure 5. SDS gel electrophoresis of the first experiment

a. PPA treated with PPP

b. PPA treated with α-chymotrypsin (1.6 x 10^{-6} M)

Protease was added at 0, 10, and 20 hr. All samples were denatured with 5 M urea, 10 mM EDTA, and 10 mM DTT for 24 hr followed by sulfhydryl derivatization with iodoacetic acid before electrophoresis.
state that trypsin cleaved PPA into two peptides that were inactive, but this was in the absence of Ca\(^{++}\) and in the presence of EDTA. I have found no references in the literature where anyone else has studied proteolytic degradation in the presence of Ca\(^{++}\). The finding of amino terminals that could be released as free amino acids might be accounted for by limited proteolysis. Presumably adequate Ca\(^{++}\) was present. In these cases, no study was performed to prove this supposition. Granger et al. (22) found inactive peptides. The PPA in the other investigators' work (13, 26, 27) showed no decrease in specific activity. My results show that PPA is susceptible to limited \(\alpha\)-chymotrypsin hydrolysis. Is it possible then that \(\alpha\)-chymotrypsin can act on PPA and still allow full activity?

The second experiment described in the Methods section (14b) explores the question of \(\alpha\)-chymotrypsin and PPP attack further. The experiment was performed in the presence and absence of added PMSF. In the absence of added PMSF, the solution contained about \(10^{-7}\) M PMSF due to that already present in the undiluted PPA. In the presence of added PMSF, the solution contained \(10^{-4}\) M PMSF. As can be seen in Figures 6 and 7, even this concentration was not sufficient to inhibit the \(\alpha\)-chymotrypsin or PPP. This concentration only slowed down their action. Samples were removed with time and aliquots were reduced and carboxy-
Figure 6. SDS gel electrophoresis of the second experiment

a. Control experiment in the absence of added PMSF. The final concentration of PMSF was $10^{-2}$ M

b. Control experiment in the presence of added PMSF. The final concentration of PMSF was $10^{-4}$ M

The first 4 gels in each figure have been treated with DTT and iodoacetamide. The last gel, labeled 5ln did not receive this. All gels were denatured in 5 M urea and 10 mM EDTA.
Figure 7. SDS gel electrophoresis of the second experiment

a. PPA treated with α-chymotrypsin in the absence of added PMSF. The final concentration of PMSF was $10^{-7}$ M

b. PPA treated with α-chymotrypsin in the presence of added PMSF. The final concentration of PMSF was $10^{-4}$ M

The first 4 gels in each figure have been treated with DTT and iodoacetamide. The last gel labeled 5ln did not receive this. All gels were denatured in 5 M urea and 10 mM EDTA
methylated. After 51 hr, one aliquot was not reduced but it was still treated with urea and SDS. This allowed any peptides that were held together by disulfide bonds to remain intact during the SDS gel electrophoresis.

Figure 6 shows the SDS gels of the control experiment in the absence of any added proteolytic activity. Figure 6a contains the control in the absence of added PMSF and Figure 6b contains the control in the presence of $10^{-4}$ M PMSF. No degradation is observed within the 51 hr. Figure 7 shows nicely the progressive degradation of the native PPA by $\alpha$-chymotrypsin action. Figure 7a shows the action in the absence of added PMSF. At 15 hr the 53,000 band is already decreasing and at 26 hr it is very faint, and after 51 hr it has completely disappeared. The 38,000 D band begins to appear after 15 hr, becomes very prominent at 26 hr and then it also begins to disappear at 51 hr. The lower bands slowly appear and disappear also. This progression is delayed in the presence of PMSF (Figure 6b) such that the 53,000 D band never disappears but the lower bands slowly become visible. Those gels labeled 51m are the 51 hr samples which were not reduced and carboxymethylated. Comparison to those labeled 51 shows disulfide bonds still hold together much of the degraded PPA.

Figure 8 shows the results of the 40% protease treatment. Figure 8a shows this in the absence of PMSF. The
Figure 8. SDS gel electrophoresis of the second experiment

a. PPA treated with PPP in the absence of added PMSF. The final concentration of PMSF was $10^{-5}$ M

b. PPA treated with PPP in the presence of added PMSF. The final concentration of PMSF was $10^{-4}$ M

The first 4 gels in each figure have been treated with DTT and iodoactamide. The last gel labeled 51n did not receive this. All gels were denatured in 5 M urea and 10 mM EDTA
gel seems to indicate the complete destruction of the enzyme in 40 hr. No bands are visible in the 40 hr or the 51 hr gels. The gel that was not treated with DTT (5ln) indicates much of the 53,000 D PPA is still intact. Some of the 38,000 D band is also present. Figure 8b shows similar results in the presence of added PMSF but at a slower rate.

Figure 9 shows an example of how the molecular weights were determined from an SDS gel. The example was taken from the α-chymotrypsin treated enzyme in the absence of added PMSF and DTT treatment. The mobilities of the bands of the standards of known molecular weights were calculated with respect to Bromophenol blue. The mobilities are then calculated for the unknowns and located on the linear plot. From this curve the peptide molecular weights are determined.

A comparison of the gels in Figures 7 and 8 to those in Figure 6 seems to indicate a significant destruction of the enzyme, at least in the integrity of the original peptide. There seems to be some unifying property, at least as observed by the disulfide maintenance of at least part of the 53,000 D structure. One might expect a significant decrease in the enzymatic activity. Table 2 compares the percentage of activity remaining in each of the samples at each time point as it compares to the control. About 70% of the amylase activity remains in the α-chymotrypsin treated enzyme. Over 85% of the possible activity still remains in
Figure 9. Molecular weight determination by SDS gel electrophoresis

The gel used was nC-51n in Figure 7a

Standards:  BSA  66,000
            A. oryzae α-amylase  51,000
            α-chymotrypsin  24,000
            cytochrome c  12,300

Gel bands:  A  52,400
            B  39,300
            C  27,100
            D  13,400
Table 2. Analysis of the relative activity remaining after the various treatments in the second experiment

<table>
<thead>
<tr>
<th>Added protease</th>
<th>Added PMSF</th>
<th>Fig. symbol</th>
<th>15 hr</th>
<th>26 hr</th>
<th>40 hr</th>
<th>51 hr</th>
</tr>
</thead>
<tbody>
<tr>
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<td>no</td>
<td>nB</td>
<td>100</td>
<td>100</td>
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<td>B</td>
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<td>%</td>
<td>110</td>
<td>96</td>
<td>81</td>
<td>88</td>
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</tbody>
</table>

<sup>a</sup><i>a</i>-chymotrypsin treated.

<sup>b</sup><i>PPP</i> treated (40% protease treated).

the PPP enzyme after 51 hr. This is the preparation that shows no bands in the SDS gels after reduction and carboxymethylation. Although extensive proteolysis has obviously occurred, the amount of remaining activity is remarkable. The intact disulfide bonds seem to provide stability for the partially degraded PPA based on the amount of remaining activity. This also indicates the active site seems to remain intact. The question arises whether the conformation remains fairly well-intact, or unfolds to form a random coil, or alters to a conformation somewhere in between these two extremes. If it does unfold,
hydrogen bonds may be broken and new hydrophobic or hydrophilic residues may be exposed. Changes in the geometric structure or the charge will be revealed by altered mobility of the protein bands in gel electrophoresis. Gel electrophoresis may then be used as a test for altered conformational structure.

The third experiment described in the Methods section (14c) compares the action of α-chymotrypsin to a control. The control showed no degradation (not shown). Samples removed between 0-40 hr showed large increases in the degradation of the PPA with time. Those samples that were not reduced indicated that the peptides were held together by disulfide bonds, i.e., there was a much greater preponderance of the 50,000 D band than any of the lower molecular weight bands. These are not shown because they were very similar to those experiments shown in Figures 6 and 7. Figures 10 and 11a show the gel electrophoresis patterns for the different α-chymotrypsin hydrolysis times. No differences can be seen between the control and the treated sample for each time or throughout the course of the experiment. Treatment of the samples with DTT (Figure 11b) without the denaturing condition (i.e., without urea and EDTA) show similar results. The structure is apparently so compact that DTT is unable to enter and break up the disulfide bonds that hold it together. Ca^{++} should still be present and thus aid
Figure 10. Gel electrophoresis pattern of the control and the α-chymotrypsin treated PPA in the third experiment on samples removed from 0-25 hr. B is the control PPA and C is the α-chymotrypsin treated PPA
Figure 11. Gel electrophoresis of the control and the α-chymotrypsin treated PPA in the third experiment

a. Samples from 30-40 hr

b. Samples 0, 20, and 40 hr after 10 mM DTT treatment in the absence of urea and EDTA. B is the control PPA and C is the α-chymotrypsin treated PPA in the third experiment
in the maintenance of the structure. Gel electrophoresis cannot distinguish between the intact and partially degraded enzyme indicating the conformation is maintained enough that the enzyme is still active. Only SDS gels are capable of distinguishing the difference after DTT treatment in denaturing medium. Table 3 gives the relative remaining activity of the treated PPA compared to the control and also gives the PPA activity after each treatment with DTT. Nearly all of the activity still remains in all of the samples.

The 40 hr α-chymotrypsin treated sample still maintains the same product specificity pattern as compared with untreated enzyme (control). Figure 12 illustrates this activity pattern for the 40 hr control. Figure 13 illustrates the pattern obtained from the α-chymotrypsin 40 hr treated sample. No differences can be detected between them in their specificity pattern. This is but another test for the integrity of the active site.

Figure 14 shows the blue value vs. % apparent maltose curves for these two samples. No significant differences are seen between these two samples by this criterion. This also indicates the integrity of the active site.

It appears that the tertiary structure of PPA is so stable that even though it undergoes limited proteolytic hydrolysis, it loses very little enzymatic activity. This suggests that it is capable of high enzymatic activity even in the
Figure 12. Product specificity pattern of the control PPA 40 hr sample from the third experiment
Figure 13. Product specificity pattern of the α-chymotrypsin treated PPA from the 40 hr sample from the third experiment
Figure 14. Blue value vs. % apparent maltose curves of the 0 control and α-chymotrypsin treated PPA of the third experiment.
Table 3. Analysis of the relative activity remaining after the various treatments in the third experiment in the absence and the presence of DTT

<table>
<thead>
<tr>
<th>Sample</th>
<th>0 hr</th>
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<th>10 hr</th>
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<th>20 hr</th>
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<td>82</td>
<td>84</td>
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</tr>
</tbody>
</table>

|a| α-chymotrypsin treated.  
|b| Reduced with DTT 10 mM prior to analysis in 1.0 mM DTT.

highly proteolytic environment of the intestine. The enzyme remains intact and cannot be distinguished by gel electrophoresis, product specificity pattern, or in its products as determined by the blue value vs. % apparent maltose curves. The primary structure of PPA then consists of a single peptide of about 53,000 D, which is held together in an extremely compact tertiary structure by disulfide bonds and Ca++ chelation. This compactness may account for its reported anomalous behavior on gel chromatography which is discussed next.

Gel chromatography has inherent disadvantages (69). The beads are sensitive and subject to crushing at a high
pressure head. If the pressure head is not low then the column becomes compact, eventually shrinks, and becomes resistant to flow after a couple of samples. The use of a small pressure head (7 cm for a 100 cm column) allows the column to be reused several times, but slows the flow rate resulting indiffusion of the sample and broad peaks. Since only one sample can be run at a time, it takes about a week to standardize the column so molecular weight studies can be performed on it. By this time the column has compacted enough to be off by a few percent. The simplest method for determining molecular weights by gel chromatography is then by thin layer gel chromatography where many samples can be analyzed simultaneously without the problems of pressure and gel changes mentioned above. This method was then used to determine the molecular weight of the active species of PPA. Both normal (25) and anomalous (70) behavior of PPA on gel chromatography has been reported. A possible explanation for these differences might be in the methods of treating the gel before chromatography. In the report of the normal behavior, the gel was first pretreated with 50 mM HCl, washed, and then treated with 40 mM NaOH, and washed to pH 7 and the PPA was reported to emerge at the position expected for a molecular weight of 53,000 D. The anomalous behavior of PPA has been explained (70) as being due to the interaction of PPA with the Sephadex which has a carbohydrate
structure similar to the natural substrate of PPA, viz. starch and glycogen. Passage over Bio-Gel, a noncarbohydrate support, then should eliminate this as the primary source of the anomalous behavior. PPA and proteins of known molecular weight were tested on treated and untreated Sephadex G-100 and on Bio-Gel P-100. Figure 15a shows the analysis on untreated and treated Sephadex. Both *Aspergillus oryzae* α-amylase and PPA do not fall on the line. The apparent molecular weight for *A. oryzae* α-amylase is 31,000 D. This is lower than the actual value of 51,000 D. Prior treatment of the Sephadex does not seem to make any difference. The anomalous behavior is still present for both amylases. This is not in agreement with the previous report (25). Figure 12b shows the analysis on Bio-Gel. Here the anomalous behavior observed with *A. oryzae* α-amylase nearly disappears such that it now lies just slightly off the standard curve at an apparent molecular weight of 44,000 D. PPA, however, still lies off the standard curve with an apparent molecular weight of 22,000 D. This is the active PPA location as determined by absorption into starch treated filter paper as described in the Methods section. The location of the activity of each of the amylases corresponded to the protein spot as marked on the graph in Figure 12b. Carbohydrate interaction seems to account for most of the anomalous behavior observed with *A. oryzae* α-amylase such that changing from
Figure 15. Thin layer gel chromatography for molecular weight analysis of the native PPA

a. Sephadex G-100 superfine
   \( \bullet \bullet \bullet \) treated Sephadex (50 mM HCl, washed, 50 mM NaOH, washed)
   \(-\) untreated Sephadex

b. Bio-Gel P-100

\( \times \) purified PPA experimental, \( \equiv \) PPA expected 53,000 \( \equiv \) A. oryzae \( \alpha \)-amylase experimental, \( \bowtie \) expected 51,000.
Standard values: BSA 66,000; ovalbumin, 86,000 and 43,000; peroxidase 40,000; \( \alpha \)-chymotrypsin 21,000; cytochrome c 12,300 and 24,600
MOBILITY COMPARED TO BSA

A

MOLAR WEIGHT x10^4

(○ r = 0.982)
(● r = 0.972)

B

(△ r = 0.980)
Sephadex to Bio-Gel nearly eliminated the problem. This, however, is not the major factor in the anomalous behavior demonstrated by PPA. The use of Bio-Gel did not alter the anomalous migration. Thus, the anomalous migration of PPA on gels is not due to the interaction of PPA with the support, but due to some inherent property of the PPA structure. The PPA observed at the expected position on these gels may have been due to the dimer seen on SDS gels.

Since PPA is known to have a compact structure, it may be the structure itself that causes this anomalous gel behavior. Gel filtration separates on the basis of Stoke's radius. If the Stoke's radius is small, as it might be in an abnormally compact molecule, the elution might be such that the molecular weight would appear smaller than the actual value.
IV. CONCLUSIONS

1. Twice crystallized Worthington porcine pancreatic α-amylase contains a protease and is not suitable for structural studies.

2. Porcine pancreatic α-amylase purified by the method of Marchis-Mouren and Paséro (28) also contains protease and is not suitable for structure studies.

3. Phenylmethylsulfonylfluoride (PMSF) is a far superior protease inhibitor than is diisopropylfluorophosphate (DFP).

4. A proteinase free amylase preparation was obtained by the method of Schramm and Loyter (49), utilizing the formation of a complex between amylase and glycogen and the addition of PMSF.

5. Porcine pancreatic α-amylase consists initially of one peptide chain of 53,000 D which may be partially cleaved by α-chymotrypsin and possibly other proteases to give a molecule that is still enzymatically active and whose structure and conformation around the active site is not altered significantly.

6. α-Chymotrypsin cleaves relatively specific bonds in porcine pancreatic α-amylase to give a series of amylase molecules containing two, three, and four peptides of combinations of 38,000 D, 25,000 D, and 12,500 D giving a total
structure of 53,000 D, the peptides being held together by disulfide bonds.

7. The compact structure of PPA may be partially responsible for the observed molecular weight of 21,000 D using Sephadex or Bio-Gel gel chromatography.
PART II. PURIFICATION AND PROPERTIES OF THE
DEXTRANSUCRASE FROM STREPTOCOCCUS MUTANS OMZ 176
Dental disease has been common for many years. Benjamin Franklin, one of our founding fathers, had a set of wooden teeth replacing the ones that dental caries had destroyed. Dental caries is an infective disease that breaks down tooth enamel and dentine (71). Very few people know anyone who has not needed some sort of dental work done. Every morning one's consciousness of its potential is realized by simply passing the tongue over the teeth. The pebbly texture of the deposited plaque signals the presence of cariogenic organisms. Since no one can effectively fight the unknown, knowledge must be obtained concerning every aspect of dental disease. The following is a brief survey of the knowledge concerning dental caries. Guggenheim published a good review of the view of dental disease during the late 1800's and early 1900's (72).

Starting from the margin of the gum called the gingiva, a film, the acquired pellicle, begins to form several hours after the tooth has been cleaned (71). Saliva and serum proteins are deposited and continue to spread over the surface of the tooth. During the first stage of plaque formation, bacterial aggregates begin to adhere and multiply over the tooth. In the absence of food, this layer is termed fasting plaque and consists mostly of bacteria and protein.
Since the pH is higher than that of the saliva, this plaque is not cariogenic. When food is added, polysaccharides form and the plaque becomes thicker. Soon the polysaccharides cause a diffusion barrier (71) and entrap the bacteria and oligosaccharides next to the tooth surface. The pH drops and eventually the tooth becomes hypomineralized, resulting in lesions (71). This plaque is cariogenic. The process is fairly specific in that it entraps only cariogenic bacteria.

In 1924, Clarke (73) was the first to identify *Streptococcus mutans* as a cariogenic bacterial species. He observed that after 24 hr a liquid culture that was originally pH 7.0 will have dropped to pH 4.2. He identified them as being able to ferment glucose, lactose, raffinose, "mannitite", inulin, and "salicin" to lactic acid. *Streptococcus sanguis* also colonizes the tooth surface (71). When Clarke (73) identified *Streptococcus mutans*, he had no idea of the number of strains and complexity that would become associated with this species. In 1970, Bratthall (74) listed 70 strains of *S. mutans* which he divided into five groups based on their cross reaction with specific antisera. These groups were a, b, c, d, and Lancefield group E and carried the a, b, c, d, and e antigen (74, 75). Coykendall (76) found a genetic basis for these groups. The DNA from several strains were identified by their G-C percentage, melting temperature, and percent reassociation with other strains to form stable
hybrids. From these experiments four groups of *S. mutans* were identified. *S. sanguis* was shown to be unrelated to *S. mutans* (77). New strains were also studied. The genetic groupings correlated identically (77, 78) with Bratthall's serological groups (74). Hamada and Mizuno (79) studied and correlated the membrane proteins. Table 4 lists the Bratthall (74) groups for those strains that will be discussed here. The glucosyltransferase enzymes, a 50% sat. ammonium sulfate precipitate of the culture medium, are antigenically related (80). Groups c and e are antigenically related and are distinct from groups a, d, and g. All are distinct from group b. The different strains of *S. mutans* are grouped on the basis of serological (74, 81), cell wall (82-83), genetic (76-78, 83), enzymatic (79, 83), and ultra-structural (83) differences.

*S. mutans* can also be grouped by their cell wall reducing sugars (83). Serotypes c and d contain glucose, galactose, and rhamnose. Serotype b contains galactose and rhamnose. Serotypes a and e contain glucose and rhamnose. All these strains also contain glucosamine and muramic acid but do not contain galactose. Type b contains glycerol and phosphorus suggesting the presence of teichoic acids. These results were obtained by two Englishmen, Hardie and Bowen (82) who state that they have been unable to find any type a or type b infecting English mouths.
Table 4. Bratthall's serological grouping of some strains discussed in the introduction

<table>
<thead>
<tr>
<th>S. mutans strain</th>
<th>Bratthall's(^a) serological group</th>
</tr>
</thead>
<tbody>
<tr>
<td>HS-6</td>
<td>b</td>
</tr>
<tr>
<td>E-49</td>
<td>a</td>
</tr>
<tr>
<td>OMZ 61</td>
<td>a</td>
</tr>
<tr>
<td>AHT</td>
<td>a</td>
</tr>
<tr>
<td>BHT</td>
<td>b</td>
</tr>
<tr>
<td>FA-1</td>
<td>b</td>
</tr>
<tr>
<td>GS-5</td>
<td>c</td>
</tr>
<tr>
<td>C-67</td>
<td>c or e(^d)</td>
</tr>
<tr>
<td>JC-1</td>
<td>c</td>
</tr>
<tr>
<td>JC-2</td>
<td>c</td>
</tr>
<tr>
<td>IB</td>
<td>c</td>
</tr>
<tr>
<td>Ingbritt</td>
<td>c</td>
</tr>
<tr>
<td>OMZ 176</td>
<td>d</td>
</tr>
<tr>
<td>CHT</td>
<td>e(^c)</td>
</tr>
<tr>
<td>6715</td>
<td>g(^b)</td>
</tr>
<tr>
<td>Berergren</td>
<td>ND(^f)</td>
</tr>
<tr>
<td>BF-1</td>
<td>ND</td>
</tr>
<tr>
<td>SL-1</td>
<td>ND</td>
</tr>
<tr>
<td>OMZ 51</td>
<td>ND</td>
</tr>
<tr>
<td>10(^4)400</td>
<td>ND</td>
</tr>
<tr>
<td>21 Typ</td>
<td>ND</td>
</tr>
</tbody>
</table>

\(^a\)Bratthall (74).
\(^b\)Hamada and Mizuno (79).
\(^c\)Bratthall (81).
\(^d\)Hardie and Bowen (82).
\(^e\)Coykendall (78).
\(^f\)Not determined.
Another important feature of \textit{S. mutans} is their ability to produce bacteriocidal substances during the stationary phase that are lethal to other streptococcal species. These bacteriocins are not inhibitory to groups a, b, c, or d (84). The bacteriocin that was isolated from strain GS-5, a serotype c organism, is protein in nature and has a molecular weight above 20,000 D. It is sensitive to trypsin, pronase, and phospholipase c but not to catalase. It is stable to heating at 100° and to pH 2-7. This factor plays a role in regulating the bacterial ecology of the oral cavity by allowing \textit{S. mutans} to select against other streptococcal species, and for other \textit{S. mutans} strains (84, 85). Dextrans from \textit{S. mutans} BHT and GS-5 did not prevent the synthesis or release of active bacteriocins. Saliva had no effect. Sensitive strains are \textit{S. mutans} FA-1, \textit{S. salivarius} 13419, \textit{S. salivarius} 10556 and \textit{S. bovis} 9809. This protein can diffuse through dextran and levan and exert its effect against a wide variety of organisms such as the pathogens \textit{S. pyogenes} and \textit{Staphylococcus aureus}, in effect protecting the mucosa (85).

Streptococci belong to the family \textit{Lactobacillaceae} which also includes other genera, \textit{Lactobacillus} and \textit{Leuconostoc}. This family is characterized by its ability to produce glucosyltransferases or glucansucrases that synthesize extracellular dextrans from sucrose. Many also
synthesize intracellular glycogen-like polysaccharides (71). Sucrose is not the only substrate for the extracellular enzymes. Lactulosucrose (β-D-Galp-(1→4)-β-D-Fruf-(2→1)-α-D-Glcp) (73, 86) and glucosyl fluoride (86-88) have also been reported. The activity is somewhat less with these substrates compared to sucrose. Shaw (89) claims to have observed insoluble polysaccharide formation in rats from glucose and from amylose. He states that sucrose has no unique caries producing effect. This seems to be at odds with everyone else’s observations.

The glucan from strain OMZ 176 is a spheroid with the dimensions of $284.7 \pm 56.2 \, \text{Å} \times 52.0 \pm 12.7 \, \text{Å}$. The levan from S. salivarius strain ATCC 13419 is a spheroid with dimensions of $282.1 \pm 82.5 \, \text{Å} \times 46.4 \pm 13.6 \, \text{Å}$. The dextran from S. sanguis strain 804 is spheroid with dimensions of $248 \pm 86.9 \, \text{Å} \times 41.3 \pm 16.6 \, \text{Å}$ (90). All of these form products of about the same size when viewed via negative staining or platinum shadowing by the electron microscope. The molecules tend to clump together but may be dispersed by NaCl. These elaborated dextrans are important because they allow selective colonization of S. mutans and S. sanguis on teeth. S. salivarius does not produce insoluble glucans and it is mainly found in the saliva and on the tongue. S. sanguis produces about as much insoluble glucan as S. mutans and 10 times as much soluble glucan (91). This glucan is sticky and will adhere
to smooth surfaces, e.g. glass or teeth.

*S. mutans* has been studied more than *S. sanguis*. Henceforth when no species is given but a strain number is given, it will be assumed that the species is *S. mutans* unless otherwise stated. The adherence to smooth surfaces has been studied on tooth surfaces *in vivo*, on extracted teeth, on wires, and on glass surfaces. In all cases, organisms are able to adhere when grown in a sucrose medium if they are able to produce insoluble glucans. Isolated and washed, live or heat treated, cells grown in a glucose medium can adhere to glass surfaces that have previously been coated with dextran. This dextran was formed during incubation of the glucosyltransferase and sucrose (92). Enzyme from strain GS-5 formed a dextran layer on the glass surface that was able to bind GS-5, HS-6, FA-1, and OMZ 176 to varying degrees. *S. salivarius* GS-15 and *S. sanguis* 10556 cells did not bind (92).

Antiserum against the soluble extracellular glucosyltransferases inhibit the cells from adhering to smooth surfaces. This is presumably accomplished with those cells that contain the α-δ antigen via inhibition of the cell-associated polysaccharide synthesis. SL-1 does not contain the α-δ antigenic determinant and there is no cross reactivity with the IgA antiserum (75). In the presence of antibodies or the absence of sucrose, colonization of the sur-
face of the tooth still takes place, but to a lesser degree. Initially colonization takes place at the location of fissures but this requires higher cell inocula (93). Tests with strain 6715 show that the absence of sucrose does not lead to elimination of the organism, but a gradual decrease in the colonies does occur. Firm attachment only occurs when de novo glucan synthesis is allowed to occur with the weakly associated cells. Preformed glucans added to the system are not sufficient for firm attachment (93).

To further study this adhesion property, Sidebotham et al. (94) compared the soluble glucans of the cariogenic strains Ingbritt and OMZ 51 to that produced by the non-cariogenic strain ATCC 10558 and found no differences. Mutants were then prepared to study the adherence phenomenon. The noncariogenic mutant C67-25 (95) was derived from the cariogenic parent strain C67-1. The only difference between the two was the inability of the mutant to form a sticky insoluble extracellular polysaccharide in sucrose solution (broth). The same amount of acid was produced in each culture but the mutant was less viable in acidic solutions. The extracellular polysaccharide seemed to protect the parent strain against the acid (95). The two cultures were identical in their fermentation spectrum and rates of growth, extracellular polysaccharide production, and intracellular glycogen production. This mutant prepared by
ethylmethane sulfonate did not revert back during subcul-turing. The mutant was unable to bind insoluble polysaccharide and would not agglutinate upon the addition of dextran (96).

Strain SNG-1 (97), a mutant of strain GS-5, was prepared. This mutant, like its parent, adhered to glass when grown in the presence of sucrose. The glucose-grown mutant, however, did not adhere to smooth glass when sucrose was added under nongrowing conditions (97). It can be shown that the mutant has reduced levels of cell-associated glucosyltransferase activity. The total extracellular activity is about a tenth and the cell-associated about two percent of the parent strain. The mutant enzyme is less stable to heat denaturation. Kuramitsu (97) believed that the extracellular enzyme and the cell bound enzyme may simply be different forms of the same enzyme and that both accounted for adherence.

Just as the mutant of strain GS-5 was identified by its smooth colony appearance (97) so was the mutant strain 6715-13 identified by its loss in fibrillar morphology (98). The latter mutation was obtained by treatment with nitrosoguanidine. The lack of adhesive characteristics was related to its decreased fibrillar glucan synthesis and its increased extracellular, water soluble glucan synthesis. Another mutant of the same strain was prepared (87). This mutant
was just the opposite. It had 3-6 times the glucan synthesizing ability and was able to adhere at twice the rate of the parent strain. This mutant was obtained by ultraviolet irradiation. The mutant was identical to the parent, strain 6715, except strain S19 produced more enzyme.

McCabe and Smith (99) agreed with Kuramitsu (97). They described a process whereby soluble dextransucrase from strain Kl-R, when exposed to sucrose, initiates the synthesis of an insoluble dextran and causes the enzyme to become insoluble. This was termed reversibly bound enzyme since it could be eluted from the cells by clinical dextran. If exposed to further dextran synthesis, the result was termed irreversibly bound enzyme since it could not be solubilized. With time, this enzyme was eventually inactivated. When heat killed, sucrose grown cells, were suspended with soluble dextransucrase and later separated and assayed for enzyme activity, 70% of the original activity could be found in the cellular fractions after 2 hr. Clinical dextran prolonged the active life of the enzyme in the presence of sucrose (99).

Mukasa and Slade (100) determined that the adsorption of dextransucrase from strain HS-6, serotype a, onto the cells was complete in 1 min at either 5°C or 37°C. This result indicates no energy is involved in the adsorption process. Mukasa and Slade (100), however, in contrast
to McCabe and Smith (99) could not release the enzymes from heat killed cells by pH, salts, sugars, polymers, or antibodies. Heat killed cells possessed the ability to adhere when polysaccharide was synthesized (101). Mukasa and Slade (101) described a two part process for adherence. First was the synthesis of a water insoluble dextran-levan polymer by the cell bound enzymes. Second was the binding site participation on the cellular surface. The polymer synthesized by soluble enzyme did not cause adherence when added to the cells (101). These enzymes have been purified 1,100 fold by use of agarose and hydroxylapatite. Their size is between 400,000 and 2,000,000 D and they contain 5-30% polysaccharide. Adherence properties were purified along with and paralleled the insoluble polysaccharide synthesizing capability (102).

It seems that the ability to adhere to smooth surfaces is related to the proportion of α(1→3) bonds that the enzyme(s) can produce. Since the tooth surface is like hydroxylapatite, the binding of the enzymes during purification is also related to the polysaccharides that are formed. The total amount of polysaccharide adsorbed is related to the molecular size, and the strength of the adsorption is related to the proportion of α(1→3) linkages (103). In addition to their adhesive characteristics that lead to plaque formation, these bacteria also possess a co-
hesive property which is a glucan-mediated agglutination. Mutants of strain 6715-13 that had lost their ability to adhere to smooth surfaces still retained the ability to agglutinate. The extracellular enzyme produced decreased amounts of alkali soluble polysaccharides and increased amounts of water soluble ones. This demonstrated that the two capabilities, adhesion and agglutination, were distinct and dissociable and that adhesion was related to the water insoluble polysaccharide (10^4). Dextran-induced agglutination is independent of cell bound dextransucrase. The enzyme could be inhibited by toluene, Hg^{++}, Cu^{++}, and heat without altering agglutination. Trypsin and EDTA blocked agglutination but not enzyme activity. Heat and EDTA treated strain 6715-49 required Ca^{++} to activate the cell surface dextran receptor or to form intercellular Ca^{++} bridges. It was not the cell bound dextransucrase that bound dextran (105). More than one cell must bind to the same dextran molecule, thus only high molecular weight dextran will cause agglutination. The process has a wide pH range with the optimum around pH 8.5. It has been shown that as little as 6 ng of dextran (added to 6 x 10^8 cells in \( \frac{1}{2} \) ml) with a molecular weight of 2 x 10^6 D (about 3 molecules per cell) can cause agglutination. This could be inhibited by a high concentration of glucose, levan, or low molecular weight dextran (2.0 x 10^4 D) (106). Gibbons and Fitzgerald (106)
worked with the strains GS-5 and 6715 and suggested that it may be the cell bound enzyme that is the receptor site. They found no agglutination with organisms that have been heated for 10 min at 100°.

Kelstrup and Funder-Nielsen (107) have shown that 5-50 ng of Leuconostoc dextran \((2 \times 10^6 \text{ D})\) per ml can cause agglutination of 15 different strains. They stated that the dextran receptor site was not identical to the enzyme reactive site on the cell, in agreement with Freedman and Tanzer (104). Periodate oxidation with or without \(\text{NaBH}_4\) treatment prevented agglutination as did cyclohexylisocyanate, 4 M urea, 10 mM EDTA, or 0.1% SDS. \(\text{Ca}^{++}\) or \(\text{Mg}^{++}\) reversed the effect of EDTA. Heating at 60° or treatment with papain impaired agglutination, further indicating that the receptor site was a protein, at least in strains C67-1, Ingbritt, and C-67-25 (107). Immune serum inhibits dextran- or sucrose-induced agglutination on strain OMZ 176. Immune serum against the glucosyltransferase had no effect on agglutination (108) lending further support to the distinction between agglutination and adhesion.

Agglutination of \(S. \text{sanguis}\) strains Blackburn, 10556, 10557, and M5 (109) could not occur with saliva or concanavalin A but could if both were present and working together. Other strains, \(S. \text{sanguis}\) HTPR3 and F90A, \(S. \text{mitis}\) 26, and \(S. \text{salivarius}\) 6S2R and H6, showed an indirect
response to Con A. Saliva agglutinated them, and NaCl (1 mM) reduced this effect, but Con A added to the system reversed the NaCl effect (110). When these bacteria were compared in the same individual over a 150-day period, radical differences were noted. Over a three-day period the titer of the agglutination ability of the IgA from the pooled saliva did not change much (110).

The preceding has explained that *S. mutans* and probably *S. sanguis* cause dental caries. Only these bacteria are able to selectively colonize the tooth surface due to their ability to form water insoluble polysaccharides. The ability to agglutinate in the presence of dextran enables more than one cell layer to accumulate. The ability to synthesize bacteriocins that are lethal to other streptococci also helps to select which bacteria will be in the oral cavity. Now that the bacteria are attached to the tooth the next step is to explain why anyone cares that they are there. Slimy bacteria can be tolerated, but these do more than just secrete a slime. They also create acid that is trapped next to the surface of the tooth. This acid is very efficient in attacking teeth and in producing pain and tooth decay.

Tanzer et al. (111) used sucrose specifically labeled in the glucosyl moiety to study the metabolic products in the nongrowing strain of SL-1. The results were those
expected from a typical homofermentative microorganism.
Part of the glucosyl portion was incorporated into both the soluble and the insoluble glucan. The rest was fermented. This means there must be another mechanism for splitting sucrose besides the polysaccharide sucrases. Some sort of invertase or phosphorylase must be present so all of the sucrose can be utilized without wasting half of it on glucans and fructans. At the concentrations which were tested, only 18% of the glucosyl residues were incorporated into glucan. All the rest were consumed to acid (111). The fructose was not wasted. When acid production ceased the yield per mole of sucrose was the same as that expected from two hexose units, according to Robrish and Krichevsky (112).
The strains that were studied were SL-1, IB 1600, 104400, and 21 Typ. Of the total acid produced, 85% could be identified as lactic acid. This work agrees with that above (111), that if dextransucrase were necessarily linked to the use of sucrose for cellular transport, then only two lactates per mole of sucrose would be observed. Instead they found four moles were generated from sucrose. Less than 15% was found in the polymer. Visual inspection of the culture might indicate more was diverted into polysaccharide but this was just an illusion (112).

Tanzer (113) observed that increasing levels of sucrose in the culture medium restricted the growth rate and the
growth yield in strain SL-1. As the sucrose concentration was increased, a lower percent conversion to lactate was observed. At 1 mM concentrations virtually 100% was metabolized to acid. At 100 mM concentrations only 50% was metabolized and the rest formed polysaccharide that agglutinated the cells and caused them to stick to the vessel.

This observed shift to polysaccharide production (112, 113) deluded investigators into the misconception that S. mutans grew slower in the presence of sucrose than it did in glucose. Turbidity was a convenient tool to assess bacterial growth. Because the cells clumped in large masses, they scattered proportionally less light, artificially reducing the apparent absorption. The suspensions seemed less turbid and therefore apparently grew slower. Daneo-Moore et al. (114) actually determined the increase in DNA, RNA, and protein using radioactive precursors. They determined that the growth rate was identical in both media. After 7 hr in sucrose, an optical artifact was produced due to the cellular aggregates. The cells did not grow linearly in sucrose but exponentially as was observed in glucose. If there was a mixture of glucose and fructose in the medium the cells would more readily incorporate the glucose into the acid soluble cellular components than it would the fructose by a factor of three (115). If, for example the concentration of glucose was a quarter of that of fructose,
the fructose incorporation would be inhibited 85%.

The solubility of the dextrans is closely related to their content of linear α(1→3) linked glucose residues (116-118) and not to their molecular weight or degree of branching (118). In strain Kl-R the enzyme gets trapped in the insoluble matrix and if not released with clinical dextran or other soluble dextrans, it eventually becomes inactive (117).

This water insoluble glucan acts as a skeletal polysaccharide (119) and as a diffusion barrier to the metabolically produced acid (71, 119, 120). It prevents diffusion of the dextran-like glucans and other dialyzable carbohydrates (119). This was illustrated with the cariogenic strain PK1 and a mutant which produced less insoluble glucan. Cells were deposited on pH electrodes and dipped in medium from a continuous culture. The pH of the medium was also monitored. The pH of the normal plaque bacteria dropped to 4.0 while that of the medium was 6.0. The pH of the mutant cells, which produced a more fragile plaque, was the same as that of the surrounding medium, pH 5.5. When both are grown in a cell suspension on glucose there is no difference in the ability of each to produce lactic acid (120).

Since *S. mutans* needs sucrose for dextran agglutination and adherence and uses sucrose, glucose, and fructose for acid production, possibly other sugars may be substituted
that are not cariogenic. Xylitol at a concentration of 1% to 5% retards cell growth in the strain Ingbritt. Sorbitol and mannitol had a weaker effect (121). The cells grew at about a half the normal rate on sorbitol and showed no growth on xylitol. These seemed promising. Mäkinen (121) pointed out that these were not noncariogenic sugars since in time the microorganisms became adapted to the sugar. They could then produce acids and extracellular polysaccharides at the same rate as before on glucose, fructose, or sucrose. The time of adaption required 4-5 months.

Other treatments were sought to prevent tooth decay. One helpful treatment was the incorporation of fluoride into tooth pastes, drinking water, or special fluoride treatments. Fluoride inhibits bacterial glycolysis (122), prevents glycogen synthesis (122, 123), alters the uptake of glucose by intact cells (123) by interacting with the cell membrane, and causes increased resistance of tooth enamel to acids (124). Fluorine is incorporated into the mineral enamel structure and is deposited as a fluorapatite \((\text{Ca}_5\text{F}(\text{PO}_4)_3)\). As little as 1 ppm will cause a 50% reduction in carious lesions. Fluorine at a concentration of 0.2 ppm reduces the rate of acid dissolution of the calcium phosphates and protects the tooth down to a pH of 2.0. This procedure is helpful but it does not work for everyone.
Extraction of the plaque deposits with water yielded soluble materials which were dextran-like (\(\alpha(1\rightarrow6)\) glucan). This led to the assumption that plaque consisted mainly of \(\alpha(1\rightarrow6)\) linked glucans (119) and the incorporation of dextranase into the diet (89, 118, 125, 126), drinking water (89), and mouth washes (126) was all that was necessary to prevent plaque and control dental caries. This treatment seemed to be effective in hamsters but not in white rats (89). Strain E49 produces a glucan in hamsters that contains 69\% \(\alpha(1\rightarrow6)\) bonds (127). This percentage may be high enough for dextranase to be effective. Gnotobiotic rats infected with strain PK1 and kept on a cariogenic diet including 200 U/g dextranase showed decreased incidence of dental caries after 60 days. This was repeated with 400 U/g dextranase with less encouraging results (125). Dextranase seemed to be somewhat effective when it was incorporated into the sucrose containing diet (118, 126). In mouthwashes it was less effective in removing mature plaque (126). Chewing gum with dextranase incorporated into it did not prevent the development of marginal gingivitis (128) but did decrease it some. Amyloglucosidases were ineffective (126). The effect in human subjects who have higher proportions of linear \(\alpha(1\rightarrow3)\) linkages in their oral glucans was very slight (71). Strain 6715 produces both a soluble dextran which can be hydrolyzed with dextranase and an insoluble glucan which is resistant to
dextranase (129). Inhibition of the insoluble glucan synthesis rather than the soluble was sufficient to inhibit plaque formation (130). Dextranase from Actinomyces israelii and Bacteroides ochraceus had an effect on the sucrose dependent adherence of these nongrowing cells onto glass both before and after initial attachment. So there may be some partial regulation of S. mutans in the oral cavity due to interactions with other indigenous oral bacteria and their dextranases (131).

More recently the possibility of using antibodies has been investigated. The submandibular region of gnotobiotic rats have been injected with killed whole cells from strain 6715. IgA antibodies were produced which reduced caries in the buccal surface of the molars and the sulcal areas when compared to control animals. Caries were more extensive on the developing teeth (132). Thus conflicting data were found. Antibodies to the glucosyltransferases of strain HS6 inhibited insoluble glucan synthesis in group c by 45-60%, in group b by 70%, and in group a and d by 90% (130). Adherence of heat killed cells to smooth glass surfaces was also inhibited in some cases. In others it was increased. When total glucan synthesis was examined, only serotype a was inhibited. Insoluble synthesis decreased in serotype d and soluble synthesis was increased. There was no effect on the soluble synthesis in serotype b. Serotype c showed
a 30-38% increase in soluble glucans. Linzer and Slade (130) explained this as any α(1→3) polymers that were synthesized were rapidly incorporated into insoluble polymer by the enzyme. In the presence of the antiglucosyltransferases, this enzyme was inhibited and a soluble polymer was recovered. In general, results with crude enzyme preparations used as immunizing agents in animals have not been conclusive (130).

In 1974 Fukui et al. (133) reported that native or denatured human salivary immunoglobulin A or colostrum IgA accelerated the rate of synthesis of dextran by strain HS-6. The former stimulated synthesis by 3-fold and the latter by 7.3-fold (133). This might indicate some carbohydrate on the immunoglobulin may be responsible for the stimulation. A later report by Cole et al. (134) could not confirm these results using the same strain. They could only see about 36% stimulation in their polymer assay. Perhaps Fukui et al. (133) were observing a different phenomenon in their reducing value assay.

Mouthwash with chlorhexidine gluconate (Figure 16) in it did prevent plaque formation and development of gingivitis (128). It is bacteriocidal to 9 strains of S. mutans E-49-2, Ingbritt-1600, Berergren, SL-1, FA-1, BF-1, OMZ 61, and NCTC 100449) and Actinomyces viscosus T6-1600-6. It either absorbs to the microorganisms (135) directly or it absorbs
Figure 16. The structure of chlorhexidine
to substances in the mouth (136). The prolonged bacteriocidal effect would then be due to the slow desorption from the mouth and absorption onto the organism.

It has been mentioned above that in order to account for the total metabolism of sucrose, an invertase should be present. Those that have been studied the most have been shown to be β-fructosidases. These consist of strain GS-5 (137), 5 other S. mutans strains (138), and a strain of S. salivarius (138). The latter two groups possessed invertase activity both intra- and extracellularly and the quantity of enzyme present depended on the carbohydrates in the medium. The invertase from strain GS-5 was located intracellularly and its concentration was inversely proportional to the sucrose concentration (139). Growth in glucose or in fructose increased its specific activity. Its concentration was equivalent in both the log and stationary growth stages (137). No sucrose phosphorylase was present. Fructose was a competitive inhibitor. Phosphate stabilized its activity on heating. It was sensitive to p-chloromercuribenzoate but not to iodoacetate or to EDTA. Invertase is believed to be present in strains SL1, IB1600, and 21 Typ (138). Invertase was found in strains AHT (140), HS-6 (141), and 6715 (142). The enzyme is constitutive and its activity could be increased by treatment with toluene (142) which makes the cells impermeable to sucrose and increases the yield 10-fold over
glucose grown cells (142).

Up until now, I have just mentioned the polysaccharides present in dental plaque. The soluble glucan called dextran helps the cells agglutinate and its active synthesis helps cell adhere to smooth glass surfaces. The insoluble glucan is necessary for adherence to glass and tooth surfaces. Cells no longer possessing the ability to synthesize it are no longer cariogenic. Guggenheim and Haller (118) proposed the word mutan, from the species S. mutans that produces it, be used when referring to this insoluble glucan. Another polysaccharide exists. This consists of fructosyl residues bound covalently via β-linkages. For now, this will be called levan and later it will be qualified. Guggenheim (72) observed that the acellular portion of dental plaque is 31–41% carbohydrate which is mostly glucan. Levan consists of 1–2% of the total dry weight. Active dental plaque was able to produce acid 2–6 hr after exposure to sucrose. This suggested the presence of a carbohydrate storage mechanism. Guggenheim observed that levan was completely hydrolyzed by an enzyme he called fructan hydrolase since it also cleaved inulin. This enzyme was also extracellular and inducible by growth in levan, inulin, or sucrose containing medium. Further plaque studies revealed that about half of the extractable polysaccharides were hydrolyzed, indicating dextran must be cleaved also. Dextranase was
found in 3 strains of *S. mutans* (143). An exodextranase from *S. mitis* strain 439 (144-146) and from *Arthrobacter globiformis* T6 (147-149) have been useful in dextran structure work. They remove a single glucose unit and an isomaltose unit, respectively, bound in α(1→6) bonds. Other dextranases are found from strains isolated under sugar cane waste piles (150). None of these dextranases act on mutan. It appears that both levan and dextran can serve as sources of energy when other carbohydrates are not available to the microorganism.

The polyfructan mentioned above was termed levan to illustrate the point I wish to make here. Levan is a polyfructan linked β(2→6) with β(2→1) branch linkages. It is a high molecular weight polymer of about 2 x 10^6 D (151). Inulin also is a polyfructan but it is linked opposite to that of levan. The chain is linked β(2→1) and it has no branch points at β(2→6). The molecular weight is low, only about 5000 D (152). Levan has always been isolated from bacteria like *Bacillus*, *Leuconostoc*, *Corynebacterium*, *Pseudomonas*, and *Xanthomonas* while inulin was isolated strictly from higher plants like *Dahlia variabilis* and *Heliathus tuberosus* (153). It was therefore natural to assume that bacterial fructans of high molecular weight were levans.

Carlsson in 1970 (154) also assumed this when he pub-
lished the purification and properties of a levansucrase from strain JC-2. The yield from hydroxylapatite chromatography and isoelectric focusing was 10%, with a 200-fold purification. The fructan had a molecular weight of $25 \times 10^6$ D. The optimal temperature was $40^\circ$ and the optimal pH was 6.0. EDTA inhibited it but iodoacetamide, cysteine, tris, and NaF had no effect. He stated that the polyfructan ought to be levan but this was not yet established (154). Avigad (152) discussed levans and listed several ways they could be distinguished from inulin; infrared spectroscopy, double diffusion precipitation plates, partial acid hydrolysis, and others (152). In 1973 Kawai et al. (155) isolated a polyfructan from the nongrowing fungal conidia of *Aspergillus sydowi* IAM 2544. This was synthesized from sucrose and had a molecular size of $20 \times 10^6$ D. Upon partial acid hydrolysis and comparison to levan and inulin standards, they found that the fructan was more inulin-like than levan-like. The backbone linkage was $\beta(2\rightarrow1)$ (157). Baird et al. (156) discovered the polyfructan from strain Ingbritt A was also inulin-like (156). This fructan consisted of 57% of the total polysaccharide synthesized. Garszczynski and Edwards (151) then showed that the polyfructan ($2 \times 10^6$ D) produced by *S. salvarius* SS2 was a levan. The levansucrase was a constitutive cell bound enzyme with a pH optimum of 5.6. This levansucrase was independent of
divalent cations. The primary linkage, $\beta(2\rightarrow6)$, was determined by isoprecipitation bands against concanavalin A and infrared spectral analysis. The SS2 fructan was similar to known levans. The *S. salivarius* SS2 levansucrase was inhibited by tris and could be released from the cell by trypsin but it was no longer active. This levan was not a storage polysaccharide since the organism was unable to degrade it.

In 1974 Carlsson's (154) assumption that JC-2 fructan formed was levan was proven incorrect when Rossel and Birkhed (157) published the structure of the polyfructan from strain JC-2. Methylation, hydrolysis, treatment with NaBD$_4$, acetylation and gas-liquid chromatography-mass spectroscopic (GLC-MS) analysis revealed the fructan to be primarily linked $\beta(2\rightarrow1)$ in an inulin-like structure.

In 1975 Ebisu et al. (153) compared several *S. mutans* and *S. salivarius* strains. They found *S. mutans* BHT with an average repeat of 8 sugar residues (153, 158), JCl with an average repeat of 27 sugar residues (153, 158), AHT, and CHT were all inulin-like with a backbone of $\beta(2\rightarrow1)$ linkages (153). *S. salivarius* HHT was levan-like with a backbone of $\beta(2\rightarrow6)$ linkages and an average repeat of 9 sugar residues (153). The determinations were performed by GLC-MS as above. They also used inulinase II, an enzyme that only cleaved inulin. They found that the inulin-like polysac-
charides were less cold water soluble than the levans. Solution could be attained by warming to 60°. They therefore expect to find these insoluble fructans in dental plaque (153). *S. salivarius* ATCC 13419 (159) elaborates a levan (31.5 x 10⁶ D). The rate of synthesis is higher than that for dextran and the rate of degradation is also high. Hancock et al. (160) studied the polyfructan elaborated from *S. salivarius* strain S1 and found it to be a levan with at least four fructose units per branch.

From the data listed above six different *S. mutans* strains, Ingbritt A, JC-1, JC-2, BHT, AHT, and CHT, were shown to elaborate an inulin-like fructan and four different strains of *S. salivarius*, SS2, HHT, S1, and ATCC 13419, were shown to elaborate levan-like fructans. It would be easy to generalize that all fructans from *S. mutans* are inulin-like and all fructans from *S. salivarius* are levan-like. That may not be wise in light of previous generalizations.

The next topic to be discussed is the purification of the glucosyltransferases, or loosely speaking dextran-sucrases, from several *Streptococci*. Strictly speaking dextranucrase is the enzyme which produces a water soluble dextran-like product. Mutansucrase then produces a water insoluble mutan-like product. Most investigators have not made this distinction so the following will reflect the terms used by those investigators. Glucan is a general enough
term and will be used most often.

Rossel and Birkhed (157) analyzed the ethanol insoluble glucan produced by strain JC-2 and found it contained 10% α(1→3), 46% α(1→6), 22% branched and 22% nonreducing ends. The analysis was by GLC-MS.

Baird et al. (156) analyzed the water soluble and water insoluble glucans in strain Ingbritt A by GLC and periodate. Of the polysaccharides formed, 43% are glucans and of those, 25% are insoluble in 0.1 N NaOH. Analysis of the water insoluble glucan revealed 24% α(1→6) linkages, 52% α(1→3) linkages, 12% branches, and 12% nonreducing ends. Analysis of the water soluble glucan revealed 66% α(1→6) linkages, 3% α(1→3) linkages, 16% branches, and 15% nonreducing ends. They theorize that the soluble glucan may be a precursor of the insoluble one.

Robrish et al. (139) studied the enzymes from strains SL1, IB1600, and 21TYP. They compared the total activities found when the pH was maintained at 6.8 or allowed to drop to various levels. They found no differences. They investigated the intracellular activity and found none. They did observe the cell free enzyme, when subjected to sonic treatment or agitated with glass beads, became inactive when used as controls in their breakage experiments.

Inoue et al. (140) purified an enzyme from strain AHT with 5.3% yield by ammonium sulfate precipitation and
isoelectric focusing. The enzyme formed an insoluble glucan.

Chludzinski et al. (161) purified an enzyme 1500-fold from strain 6715 by ammonium sulfate precipitation, hydroxylapatite chromatography, and isoelectric focusing. They obtained a protein with an isoelectric point of 4.0, pH optimum of 5.5, temperature optimum of 34-47°C, Km of 3 mM for sucrose, and a molecular weight of 94,000. Fukui et al. (83) suggested that this value may be underestimated since they used a Bio-Gel P-100 column and their enzyme eluted near the void volume. Polyacrylamide gel electrophoresis revealed 10 bands in the hydroxylapatite fraction and four in the isoelectric fraction. Fructansucrase activity was lost at the ammonium sulfate step. DTT at 100 mM inhibited the enzyme 41% and 1 mM EDTA had no effect. Dextran stimulated the enzyme.

Scales et al. (162) purified the enzyme from strain FA-1 by ammonium sulfate precipitation, polyacrylamide gel electrophoresis, DEAE-cellulose chromatography, and agarose gel filtration. The dextransucrase was contaminated with a fructansucrase. Purification was 353- and 500-fold, respectively, and the ratio of the two remained constant. Both sucrases had a pH optimum of 6.0, a Km of 55 mM, and an isoelectric point of 3.7 for the dextransucrase and 4.6 for the fructansucrase. The enzymes were inactivated by freezing and thawing but still retained partial activity after heating
at 100°. The enzymes were glycoproteins and did not need primers. The enzymes easily aggregated and precipitated from solution during concentration in a positive pressure cell. The precipitate could not be dissolved by 0.8 M urea or 1.0 M NaCl and was unable to enter a polyacrylamide gel.

Fukui et al. (141) purified the glucosyltransferase activity from strain HS-6 with Sepharose 6B, DEAE-cellulose, and hydroxylapatite chromatography. The glucansucrase that made insoluble polysaccharide was not recovered but the enzyme forming soluble polysaccharide and invertase were separated at least partially. The dextransucrase still possessed invertase activity, but it did not cross react with antiserum against invertase. The pH optimum of dextransucrase was 5.25 and the molecular weight was 160,000 D. The pH optimum for invertase was 5.75 and the molecular weight was 170,000 D. The dextran polymer consisted of 94% \( \alpha(1\rightarrow6) \) linkages (140).

Kuramitsu (163) studied the cell associated dextran-sucrase activity of strain GS-5 and found two forms. One could be removed from the cell with hypertonic salt washes and was sensitive to proteolytic enzymes. This existed in an aggregated form since it eluted at the void volume of Bio-Gel A-15m column. The second form was intracellular and was not susceptible to proteolytic enzymes. This form
eluted as two peaks on Bio-Gel A-15m. Most of the activity was retained by the gel. Both formed insoluble glucans. Cellular adherence was destroyed by pronase treatment (163). The extracellular glucosyltransferase of this strain was also passed over a Bio-Gel A-15m column (164). The void volume peak consisted of highly aggregated enzyme that formed both insoluble and soluble glucans. The nonoverlapping retarded peak formed only soluble glucan. The activity distribution of the peaks depended on the growth medium. Todd-Hewitt medium favored the void volume peak. The retarded peak was favored when invertase or a mixture of dextranase and mutanase was added to the medium. Both had identical pH optima, temperature optima, and Km values for sucrose. The retarded fraction was an aggregate of the basic 45,000 D enzyme. Throughout the purification procedures, aggregation progressively became more of a problem. Kuramitsu (164) suggested that the soluble α(1-6) linked glucans were synthesized to serve as acceptors for insoluble glucan formation. Radioactively labeled product from the retarded peak was purified and added to the void volume peak in the presence of sucrose. The insoluble product became labeled.

An enzyme purified from *S. sanguis* 804 is included for comparison (165, 166). It was isolated from the glucose containing broth and purified by hydroxylapatite chromatography and isoelectric focusing. The enzyme precipitated
during dialysis unless 2 M urea was added. The enzyme was stable from pH 5.2-8.5 with an optimum of pH 5.2-7.0. There was no metal ion requirement. The water insoluble product contained 50% α(1→6) linkages (166). The purified enzyme, with an isoelectric point of 7.9, was tested for reversibility. None was observed. The product obtained from the culture supernatant and sucrose contained 83.4% α(1→6) and 2.4% α(1→6) bonds. The product from this purified enzyme contained 52.2% α(1→6) and 17.7% α(1→3) bonds (165).

A question of interest is whether or not these organisms require a carbohydrate primer for activity. The following will present what has been reported on this subject. A more critical review can be found in the Discussion section of this part.

Strain 6715 enzyme loses activity on storage (167). This activity can be recovered by incorporation of dextran into the assay procedure. The enzyme purified by hydroxylapatite was totally dependent on dextran for activity. The half maximal activation concentration was 2-3 μM. The purified enzyme was supposedly homogeneous and contained 32-58% carbohydrate. As pointed out earlier in this Introduction, Fukui et al. (83) suggested that the enzyme actually ran at the void volume of a Bio-Gel P-100 column, so it may even be an aggregate. Other saccharides could not substitute for dextran. Glycogen, amylose, inulin, and isomaltose
were tried (167). The mode of action was studied. Dextran of 10,000 D or 2,000,000 D on a weight percent basis were equally efficient as activators. Treatment with NaBH$_4$ did not decrease this activation. The authors assumed that dextran acted as a primer and, since primers act either from the nonreducing end or the reducing end, concluded the direction of synthesis was from the nonreducing end. Synthesis could not occur from the reducing end since NaBH$_4$ reduced dextran was equally active. Two substrates which differed in molecular weight by a factor of 200 should also differ. On a weight percent basis, the number of available reducing ends would also differ by a factor of 200. The nonreducing ends are identical in both cases. Both substrates were equally effective so the investigators concluded that the priming action must come from the nonreducing end (168). This logic is valid only if their first assumption, viz. that dextran activates by a priming mechanism, is valid. The authors show that growth in a fructose medium results in an enzyme which is more primer dependent. Presumably that grown in glucose, which is not a competitive inhibitor of the enzyme, allowed any stray sucrose in the medium to form enough endogenous dextran to act as a primer. Dextran activated this enzyme 1.0-1.5-fold. Fructose is a competitive inhibitor and growth in this medium should be primer free. Dextran activated this enzyme more than 10-
fold. Fructose also caused an increase in the amount of extracellular enzyme. Glucose cultures contained enzymic aggregates that were stable in 3 M NaCl. These aggregates were due to the dextran present causing some dextran induced agglutination which complicated the purification steps. Elution of the fructose grown enzyme over Bio-Gel A-1.5m column resulted in a jagged up and down line. One of the up jags corresponded to where a 40,000 D protein might elute. This was assumed to be the base peak from which everything else aggregated (168). The elution profile they show is not very convincing. Growth of this strain on a chemically defined medium showed similar growth and 17-fold more enzyme that can be activated 11-fold by dextran. This enzyme could be dissociated by 1 M salt (169).

Kuramitsu (164) states that soluble glucan synthesis by the void volume peak is activated 2-fold by dextran. Synthesis by the retarded peak is activated 12-fold by dextran. The void volume peak was able to use nigeran, glycogen, or dextran as acceptors for insoluble glucan formation. Low molecular weight dextran ($10^4$ D) is a poor acceptor for insoluble glucan formation since it caused a shift in the production to low molecular weight species.

Linzer and Slade (130) observed that low molecular weight dextran in strain HS6 enzyme stimulated both soluble and insoluble glucan synthesis. Carter et al. (117) observed
the results of adding clinical dextran to sucrose and the enzyme from strain Kl-R. In the absence of dextran, the enzyme becomes entrapped in the insoluble polysaccharide matrix and is inactivated. Addition of clinical dextran results in more soluble dextran being formed and prolonged activity of the enzyme. They state that the exogenous dextran is not a good primer for synthesis but that it simply acts as a soluble acceptor for glucosyl transfer. They find that the total polysaccharide synthesized is independent of the concentration of dextran acceptor if the time of reaction is extended proportional to the concentration of the dextran acceptor. This does not agree with the results on strain 6715 above (167-169). Solubility of the glucan increases as the content of $\alpha(1\rightarrow6)$ linkages increases (116).

Smith (170) studied a system of glucosyltransferases from Leuconostoc mesenteroides NRRL B-1299 which makes both a soluble and an insoluble glucan. The enzyme is structure bound and not soluble. Radioactively labeled soluble dextran was added to the cell suspension in sucrose solution to see if it acted as a precursor to insoluble polysaccharide. No incorporation was observed. A $^{14}\text{C}$ pulse to the enzyme was followed by washing away the soluble products and reactants. Addition of fresh cold sucrose resulted in radioactive, soluble dextran. He concluded that the insoluble polysac-
charide is a precursor of the soluble polysaccharide. Several low molecular weight acceptors were added to see if the soluble dextran was covalently attached to the insoluble enzyme causing it to appear insoluble in the assay procedure. No acceptors were found that were able to release soluble dextran (170). There appears to be a difference in opinion concerning a primer requirement.

What follows now is a survey of the literature concerning the enzymes from *S. mutans* OMZ 176, the strain that I have been working with. Much of the enzymic work has been done by Guggenheim (72), Guggenheim and Newbrun (171). He finds three principle isoelectric focusing bands with isoelectric points at 4.25, 5.00, and 5.65. There are a maximum of 5-7 bands. The Km for sucrose ranges from 0.98-7.23 mM. Each bond produced insoluble glucan and separated into several bands on polyacrylamide gel electrophoresis. The initial purification involved hydroxylapatite chromatography (171). The pH optimum ranged from 5-7. No glucan primer was necessary for activity. Purification was more reproducible when the pH was controlled. There was no metal ion dependency in one of the hydroxylapatite fractions. No inhibition was observed at 0.1 mM concentrations of Hg^{++}, Ni^{++}, Mg^{++}, Pb^{++}, Ag^{+}, Cu^{++}, Fe^{++}, or KSCN. Guggenheim (172) stated that based upon his observations of *S. sanguis* 804, which has equal amounts of α(1→6) and α(1→3) linkages, in-
solubility is dependent on the content of α(1→3) linkages and not on molecular weight. Analysis of the Smith degradation products gives 15.9% glycerol from glucose and 84.1% free glucose. It appears most of the glucose is in branch points or attached via α(1→3) linkages that are resistant to periodate. The polysaccharide formed from the enzyme with an isoelectric point of 5.0 contained 94% α(1→3) linkages, 3% α(1→6) linkages, and 3% nonreducing ends; that from the enzyme with an isoelectric point of 5.6 contained 49% α(1→3) linkages, 40% α(1→6) linkages, 5% nonreducing ends, and 5.4% branches (173).

Guggenheim and Burckhardt (174) isolated a dextranase from strain OMZ 176 that had about 4% activity on mutan. The dextranase was very closely associated with the glucosyltransferase activity and he believed the structures were related. This lends further support to the argument that soluble glucans may be reserve polysaccharides. The dextranase itself has a pH optimum of 4.5-5.0 and a temperature optimum of 37-40°. The Km is 0.967 mM. Again they find no metal ion dependency for this enzyme. No glucose is found as product and very little isomaltose. The products consist of higher molecular weight oligomers and suggest the enzyme works via an endomechanism. About 40% of the total glucose present in the plaque is in a low molecular weight soluble form indicating dextranase is active in plaque.
Dewar and Walker (143) also studied the dextranase from strain OMZ 176 and 24 other strains and found eight had dextranase activity. Some were exolytic, others were endolytic, and some contained both activities (143). Kuramitsu (175) observed that heat killed cells from OMZ 176, HS-6, and FA-1 could adhere to dextran previously synthesized on a glass surface by extracellular glucosyltransferase and sucrose.

Plaque formation may be studied by deposition of polysaccharides on a glass wire. A dextranase preparation from the culture liquor of Spicaria violacea strainIFO 6120 can inhibit formation of plaque. It was only able to hydrolyze the alcohol precipitable polysaccharide from S. mutans between 13-36% with little isomaltose release. The OMZ 176, MT 615R, and HS-6 enzyme contained little c(1\to3) hydrolase activity. Like in previous animal studies there was no significant change in previously deposited plaque. It was effective in rats and in hamsters. This suggests the c(1\to6) linkages are important to cellular adherence (176).

Ebisu and Misaki (177) studied the mutan produced by strain OMZ 176. The enzyme from a Flavobacterium was able to cleave c(1\to3) linkages and release nigerose. Mutan was isolated in the presence and in the absence of a dextranase. The product in the absence was sticky and in the presence was not sticky. The native mutan contained 50.5% c(1\to3) linkages,
20.9% \( \alpha(1\rightarrow6) \) linkages, 14.6% nonreducing ends, and 14.0% branch points. The average repeat unit was seven sugar residues. The dextranase modified mutan contained 94.5% \( \alpha(1\rightarrow3) \) linkages, 1.9% \( \alpha(1\rightarrow6) \) linkages, 1.9% nonreducing ends, and 1.7% branch linkages. The polysaccharide product isolated after Smith degradation on the native mutan was prepared in 63% yield and was no longer sticky. No \( \alpha\)-glucosylglycerol was detected in the supernatant which would come from runs of \( \alpha(1\rightarrow6) \) and \( \alpha(1\rightarrow3) \) linkages. Therefore it was concluded that the molecule consists of a backbone of \( \alpha(1\rightarrow3) \) linked glucose residues with short isomaltodextrins attached via an \( \alpha(1\rightarrow6) \) branch onto the main chain (158, 177). The insolubility of mutan apparently is due to the \( \alpha(1\rightarrow3) \) runs of glucose units in the molecule and the stickiness is due to the \( \alpha(1\rightarrow6) \) linked branches (158, 169).

It was postulated that the simplest way to synthesize these polysaccharides was to have initially two enzymes. One enzyme would be a true dextranucrase, synthesizing a water soluble \( \alpha(1\rightarrow6) \) linked polysaccharide. The other enzyme would be a mutansucrase, synthesizing a water insoluble \( \alpha(1\rightarrow3) \) linked polysaccharide. I began my research on the strain OMZ 176 in the hopes of purifying the dextranucrase (used in the sense of a general glucansucrase) and then determining some of its properties. I wanted to examine
the possibility of two different enzymes as mentioned above and not in the context that Guggenheim (72) found. He observed isozymes that made basically the same product. I wanted to determine the identity of the fructansucrase, whether it synthesized levan or inulin, and I wanted to determine whether or not dextran was required as a primer. This research covered a period from January 23, 1975 to June 20, 1977.
II. MATERIALS AND METHODS

A. Materials

The following is a list of materials used in Part II that were not already listed in Materials of Part I.

The following are a list of materials obtained from Sigma Chemical Company, St. Louis, MO.

- Dextranase (endo)
- Dextran, T$_{17.7}$ and T$_{72.6}$
- Concanavalin A
- Coomassie Brilliant Blue G-250

The following is a list of materials obtained from Fisher Scientific Co., Chicago, IL.

- Cellulose tubing for dialysis
- Sucrose
- Orcinol monohydrate
- Barium oxide
- Dowex 50
- Molecular sieves

The following is a list of materials obtained from Calbiochem, Los Angeles, CA.

- Chelex
- Pronase
The following is a list of materials obtained from Difco Laboratories, Detroit, MI.

Bacto-tryptose
Bacto-casamino acids
Bacto-yeast extract

The following is a list of materials obtained from J. T. Baker Chemical Co., Phillipsburg, NJ.

Chlorotriphenylmethane, Triphenylmethyl chloride (trityl chloride)
Sodium hydride

The following is a list of materials obtained from Amersham/Searle, Arlington Heights, IL.

2,5-Diphenyloxazole, (Scintillation Grade), (PPO)
1,4-bis(2-(5-phenyloxazolyl))benzene, (Scintillation Grade), (POPOP)

The following is a list of materials obtained as a gift from T. F. Walseth, Iowa State University, Ames, IA.

D_2^-dextranase from *Arthrobacter globiformis* T6
Dextranase from *Leuconostoc mesenteroides* NRRL B-512
Purified dextran from *Leuconostoc mesenteroides* NRRL B-512
Isomaltodextrins
The following is a list of materials and from where they were obtained.

Trifluoroacetic acid (TFA), Pierce Chemical Company, Rockford, IL.

Dextran from *Leuconostoc mesenteroides* NRRL B-1299, gift from B. K. Kimble, Iowa State University, Ames, IA.

Dextran from *Leuconostoc mesenteroides* B-1254, B-1415, and B-1422, gift from A. Jeanes, Northern Regional Research Laboratory, Peoria, IL.

Levan from *Aerobacter levanicum* NRRL B-1678, gift from J. N. Zikopoulos, Iowa State University, Ames, IA.

Inulin, Matheson, Coleman and Bell, Norwood, OH.

$^{14}$C-(U)-Sucrose, $^{14}$C-Sucrose-[(U)-glucose], $^{14}$C-Sucrose-[(U)-fructose], New England Nuclear, Boston, MA.

Nigerose, gift from J. Nordin University of Massachusetts, Amherst, MA.

2,3,4,6-tetra-O-methylglucose, gift from P. A. Rebers, National Animal Disease Laboratory, Ames, IA.

Bio-Gel A series, Bio-Rad Laboratories, Richmond, CA.

Amberlite MB-3 resin, Malinckrodt Chemical Co., St. Louis, MO.

A Bio-Fiber 80 miniplant, Bio-Fiber 80 beaker, Bio-Fiber dialyzer minibaker, a vacuum/pressure regulator, and a pump module from Bio-Rad Laboratories, Richmond, CA.
Mutanase, Novo Industries, Copenhagen, Denmark.
Ethylene glycol-bis(aminooethyl)tetraacetic acid (EGTA),
The G. Frederick Smith Chemical Co., Columbus, OH.
Raffinose, Nutritional Biochemicals Corporation,
Cleveland, OH.
Triisopropylbenzenesulfonyle chloride (tripsyl chloride),
Aldrich, Milwaukee, WI.
Organism: Lyophilized powder of *Streptococcus mutans*
OMZ 176, gift from B. Guggenehim, Dental Institute,
University of Zurich, Switzerland.
Amicon ultrafiltration cell, model 52 and Amicon B-15
minicon concentrator were obtained from Amicon Cor­
poration, Lexington, MA.
Darco G-60, Matheson Coleman and Bell, Norwood, OH.

B. Methods

1. Polysaccharide purification

Crude polysaccharide is first dialyzed continuously
against running distilled water to remove any unreacted
substrates and products. Pronase (1 mg, 45 PUK units) (178)
is then added to the 20 mM β-glycerophosphate (pH 7.0) buf­
fered polysaccharide and digestion is allowed to proceed at
37°C for 24 hr. This is then dialyzed for two days against
running distilled water and lyophilized. Pronase is capable of hydrolyzing 60-90% of protein with the end products primarily amino acids (178).

2. Agar purification

The method is essentially that described by Kabat and Mayer (179). Bacto-Agar (30 g) is dissolved in 1 l hot water and CaCl$_2$ is added to effect precipitation of some impurities. This is then filtered hot through glass wool and the cooled agar is cut into chunks. These are then washed in running distilled water for three days. To calculate the percentage of agar present, a 10 g representative chunk is dried and reweighed. After water removal the percentage of dry agar is calculated per weight of the chunk. The chunks prepared contained 2.43% agar (w/w). These are stored in the refrigerator.

3. Bio-Gel chromatography

Bio-Gel A-1.5m, A-5m, A-15m, A-50m columns are prepared as described by the manufacturer. The preswollen gels are washed by swirling in three changes of buffer. After each wash, the gel is allowed to settle and the supernatant buffer and any fines are poured off. The column is packed in the cold room via a funnel reservoir and with a pressure head not exceeding 50 cm. Buffer is allowed to flow through the gel 24 hr before use to stabilize the gel.
The column is run at a flow rate not exceeding 20 ml/hr and is maintained by adjustment of the pressure head. Sodium azide (0.05%) is washed through the column for storage.

4. Partial acid hydrolysis of glucans

Glucan samples were dissolved in 0.5 N TFA and hydrolyzed in a sealed 2 ml ampule for 10-15 min at 121° in an oil bath. The samples were cooled in an ice bath and concentrated under vacuum at 40° in a test tube evaporator, Rotary Evapo-mix, Buchler Instruments, Fort Lee, NJ.

5. Protein determination

Protein was determined by a modification of the method of Bradford (180) using Coomassie Brilliant Blue G-250. The brown dye binds quantitatively to protein to form a blue complex. It is this shift in color that is used as a basis of this method. The absorbance maximum of the unreacted dye is 465 nm and the absorbance maximum of the bound dye is 595 nm. The absorbance spectrum of the unreacted dye has a slight overlap into the 595 nm region resulting in a relationship between absorbance at 595 nm and amount of protein that is not strictly linear. A standard curve can be obtained with samples of known protein concentration from which the unknown concentrations are determined. The reagent is prepared by dissolving 100 mg Coomassie Brilliant Blue
G-250 in 50 ml of 95% ethanol and adding 100 ml of 85% (w/v) phosphoric acid. This is diluted to 1 l with distilled water. The assay consists of 0.2 ml standard BSA solutions containing 10-200 μg/ml, 0.3 ml buffer, and 1.5 ml reagent solution. The absorbance is read at 595 nm 5-20 min after mixing. The unknown protein solutions, 0.1-0.5 ml, are added with 0.4-0 ml buffer, respectively, and 1.5 ml of the protein solution. The protein concentration is determined by comparing the absorbance of the unknowns with that on the standard curve and making any necessary adjustments due to dilution.

6. Gel electrophoresis

   a. Separating gel (3.75%) - separation at pH 8.0.
Reagent A: 48.0 ml 1 N HCl + 6.85 g tris + 0.46 ml TEMED,
diluted to 100 ml with distilled water (181).
Reagent B: 15.0 g recrystallized acrylamide + 1.2 g bis,
made up to 100 ml with distilled water (182).
Reagent C: 4.0 mg riboflavin made up to 100 ml with dis­tilled water (181).

One part reagent A : 2 parts reagent B : 1 part reagent C :
4 parts distilled water (181). The reagents are mixed as
indicated, deaerated in the dark, and added to 105 x 6 mm
columns. Water (4 mm) is carefully layered over the gel and
the gels are photopolymerized within 30-45 min.
b. Spacer gel - concentration at pH 7.0.

Reagent D: 39.0 ml of 1 M \( \text{H}_3\text{PO}_4 \) + 4.95 g tris + 0.46 ml TEMED, diluted to 100 ml with distilled water.

Reagent E: 10.0 g recrystallized acrylamide + 2.5 g bis, made up to 100 ml with distilled water.

One part reagent D : 2 parts reagent E : 1 part reagent C : 4 parts distilled water. The reagents are mixed as indicated (182), deaerated in the dark, and 0.2 ml added to the top of the separating gel after removal of the water layer. Water (4 mm) is carefully layered onto the spacer gel and the gel is photopolymerized within 30-45 min.

c. Electrode buffer is made up to 1 l with distilled water and contains 6.0 g of tris and 28.8 g of glycine (182).

d. The enzyme sample is added to the top of the spacer gel after the water has been removed. The enzyme solution (0.1 ml) contains 2 drops of 0.5% bromophenol blue in 60% glycerol. The electrode buffer is carefully layered over this.

e. Electrophoresis is complete in 1-2 hr at 2.5 ma per tube. A 7 mm length of copper wire is inserted in the middle of the dye front for reference.

f. Protein bands are determined by staining with Coomassie Brilliant Blue R-250 (see section 1c under Methods in Part I), or are more quickly determined by the less sensitive Coomassie Brilliant Blue G-250 (183a). The dye was
prepared at a concentration of 0.04% (w/v) in 3.5% (w/v) perchloric acid solution. This solution is added to the gel and blue bands are visible against an orange background.

g. Enzymatic activity is determined by initially pre-soaking the gel in 7.5% acetic acid and 5% methanol for 30-45 sec and incubating the gel overnight in 0.1 M sucrose in 20 mM β-glycerophosphate (pH 7) or in 0.1 M raffinose in 20 mM β-glycerophosphate (pH 7).

h. Distinction between dextranasesucrase or levansucrase activity and mutansucrase activity may be seen visually. Dextranasesucrase forms dextran from sucrose, which appears faintly cloudy and renders the gel stiff in the region. The gel will also be swollen in the region. Levansucrase forms levan from sucrose or raffinose, which appears of a different refractive index from the rest of the gel. Mutansucrase forms mutan from sucrose, which is completely white and opaque. These polysaccharides can also be distinguished by their chemical properties. Periodate will cleave dextran's soluble α(1→6) linked glucose units and those of levan. Periodate will not cleave the insoluble mutan which is predominantly α(1→3) linked. The procedure is as follows:

Staining is done as described by Maurer (182).

(a) The gels are immersed in 1% periodic acid in 3% acetic acid and incubated 50 min.
(b) The gels are washed overnight with a few changes of distilled water.

(c) The gels are immersed in fuchsin-sulfate stain in the dark for 50 min. Fuchsin sulfate is prepared by the method of McGucken and McKenzie (183b). A solution is prepared in 2 l distilled water with the addition of 16 g potassium metabisulfite and 21 ml of concentrated HCl. Basic fuchsin (8 g) is then added and dissolved over a 2 hr period at room temperature to a faint pink color. This is allowed to stand another 2 hr after which Darco G-60 charcoal is added and filtered after 15 min. The solution is stored at 4°.

(d) The gels are washed with freshly prepared 0.5% metabisulfite (3 times for 10 min each, with a volume of 25-50 ml/gel).

(e) They are then washed overnight continuously against running distilled water.

(f) The gels are stored in 3-7.5% acetic acid. Glycoproteins and periodate susceptible polysaccharides are stained pink against a colorless background.

7. Total carbohydrate analysis

The method employed was that of orcinol-sulfuric acid as adapted for use on the Technicon AutoAnalyzer (184, 185). Orcinol (0.5 g) is dissolved in 1 l 70% (v/v) sulfuric acid. This is stored in a brown bottle at 4°.
8. Purification of sucrose

Sucrose (10 g) is dissolved with stirring in the minimum amount of hot methanol under reflux. The solution is filtered and the filtrate is concentrated on a rotary evaporator until slight cloudiness appears. The suspension is then cooled at 4° for 12-16 hr and then at -10° for 4 hr. The clear supernatant is decanted and the crystals are again dissolved in a minimum amount of hot methanol as above.

After filtering, concentrating, cooling and decanting, the crystals are dissolved in 100 ml of 20 mM pyridine acetate (pH 5.0) buffer and 1 mg of mutanase and 1 mg of dextranase S are added. Reaction is allowed to proceed at 37° for 16 hr. The solution is concentrated by rotary evaporation on the Buchi Rotary Evaporator (Swissco Instruments, Greenville, IL.), and the residual water is removed by azeotroping it with ethanol. The solid is then recrystallized as above. The purified crystals are dissolved in distilled water and the sucrose concentration is determined by orcinol-sulfuric acid total carbohydrate analysis using sucrose as a standard. The sucrose was diluted appropriately, buffered and 14C sucrose was added. This purified sucrose was used when a polysaccharide free sucrose was required.
9. Detection of contaminating enzymatic activities

The enzyme solution (100 μl) was added to 100 μl of the best solutions a-d below and incubated for various times at 37° in 20 mM β-glycerophosphate buffer (pH 7). Aliquots are removed and spotted on a 23 x 57 cm Whatman 3 MM paper. The paper is developed in ethyl acetate : pyridine : water (10:4: 3) in descending chromatography at 37° for 16-20 hr. Examination of the products formed, if any, indicates the presence or absence of contaminating enzymatic activities. Products are found by the silver nitrate dip procedure.

(a) Incubation with sucrose (0.2 M) is a test for dextran-sucrase, mutansucrase, and levan- or inulinsucrase. Release of fructose or glucose indicate the presence of a glucan synthetase or a fructan synthetase, respectively. If phosphate is added to this system, a white spot of glucose-1-phosphate indicates the presence of a sucrose phosphorylase.

(b) Incubation with raffinose (0.3 M) is a test for levan-sucrase or inulinsucrase and invertase. Release of melibiose indicates the presence of a fructansucrase. Release of melibiose and fructose indicates an invertase or a fructansucrase and a fructanase. Release of galactose, glucose, and fructose indicates a galactosidase and an invertase.

(c) Incubation with levan or inulin (50 mg/ml) is a test
for levanase or inulinase respectively, if fructose is released.

(d) Incubation with dextran (50 mg/ml, 50 mM pyridine acetate buffer, pH 5) is a test for dextranase. Appearance of glucose, isomaltose, tri-, tetra-, or pentasaccharides indicates the presence of a dextranase. Release of fructose indicates the presence of a fructanase.

10. Growth of the organism Streptococcus mutans OMZ 176

The lyophilized organism was grown on an undialyzed liquid medium that is a modification of that described by Carlsson et al. (166). Bacto-tryptose (100 g), Bacto-casamino acids (25 g), Bacto-yeast extract (50 g), and K$_2$HPO$_4$ (15 g) were dissolved in 4 l water and autoclaved at 121° for 20 min. Glucose or fructose solutions (1 l, 5% w/v) were autoclaved for 20 min at 121°.

For the primer experiments, Bacto-tryptose (100 g), Bacto-casamino acids (25 g), and Bacto-yeast extract (50 g) were dissolved in 1 l water and the pH was adjusted to 4.5 with H$_2$SO$_4$. A 10% solution (500 ml) of the sugar was prepared and the pH was also adjusted to 4.5. Invertase was added to the concentrated medium (10 mg) and to the sugar solution (2 mg), and the solutions were incubated overnight at 55°. The solutions were cooled to room temperature and the pH adjusted back to 6.8 with NaOH (1 N). K$_2$HPO$_4$ (15 g)
was then added to the medium and it was diluted to 4 l.
The sugar solution was diluted to 5% and both were autoclaved for 20 min at 121°. After cooling, the medium and sugar solutions are mixed and a 10% (v/v) inoculum, obtained after 3-4 transfers through increasing volumes of the above medium, was added to the culture medium. Cells were allowed to grow 20 hr at 37° in a 6 l flat bottom boiling flask. The medium which by now was at pH 4.2 was readjusted with 1 N NaOH to 6.8 and the cells were removed by centrifugation for 35 min at 4° at an average of 11,000 x G. This supernatant is the crude culture supernatant.

11. Enzyme purification

a. The crude culture supernatant is adjusted to 40% saturation with ammonium sulfate by slow addition of the crystals with stirring in the cold room. To 1 l of the culture supernatant, 2530 g of ammonium sulfate is added. The solution is allowed to precipitate overnight and the precipitated enzyme is collected by centrifugation at 4° for 30 min at 11,000 x G. The supernatant is further fractionated with the addition of 735 g of ammonium sulfate, resulting in a solution that is at 50% saturation. The solution is allowed to precipitate overnight and the precipitate is collected by centrifugation as above. Similarly, addition of 816 g gives a precipitate that formed between 50 and 60% saturation, and addition of a further 1405 g gives a pre-
cipitate that forms between 60 and 75% saturation. Each precipitate is dissolved in 20 mM β-glycerophosphate (pH 7) buffer and dialyzed against this buffer for two days with three changes of 2 l each in the cold. All purification steps were carried out at 4°C.

b. The crude culture supernatant is concentrated 10-fold through use of the Bio-Fiber 80 miniplant, the accessory pump, and the accessory vacuum/pressure regulator. The enzyme solution was pumped through the miniplant fibers at a flow rate of 150 ml/min and a vacuum was maintained outside the fibers at 50 cm Hg. Ultrafiltration was complete when the enzyme solution was totally contained within the fibers and the tubes leading to and from the fibers. The ultrafiltrate outside the fibers was replaced with the above pH 7 buffer using negative pressure. The tubes to and from the pump were closed off and positive pressure was applied into the chamber outside the fibers. The pressure differential forces buffer to enter the fibers, dislodging any molecules entrapped within the pores of the fibers. The concentrated enzyme is collected in a volumetric cylinder and the backwash is continued until a tenth of the starting volume has been collected. Most of the enzyme is thus recovered. Further backwashing releases a minor amount of enzyme. A further 10-fold concentration can be achieved by replacing the Bio-Fiber miniplant with the Bio-Fiber
80 beaker. The flow rate is reduced to 4 ml/min by replacing the drive head on the pump.

The molecular weight cut off for the Bio-Fiber 80 fiber bundle was 30,000 D. Concentration of column effluents was attained through the use of the Amicon ultrafiltration cell, model 52, using UM-30 membrane and an operating pressure up to 75 psi (N₂), Bio-Fiber 80 beaker, Bio-Fiber dialyzer minibaker, or Amicon B-15. The latter devices were used according to the manufacturer's recommendations.

12. Enzyme assays
   a. Continuous reducing value assay. This was performed identically to that in section 3a of the Methods, Part I.
   b. ¹⁴C total polysaccharide assay. This contained 1 part enzyme solution, 1 part sucrose solution, and (depending on the assay this may not be included) 1 part test solution. The test solution may be dextran, a metal ion solution, a buffer, etc. Assays are initiated at 1 min intervals and aliquots are thus removed for spotting on papers at 1 min intervals. The concentration of the sucrose was 0.275 or 0.2 M, dissolved in 20 mM β-glycerophosphate buffer (pH 6.8). ¹⁴C-(U)-sucrose was added to give a specific activity of 30-40,000 cpm/mg. The assays were performed in an AutoAnalyzer disposable cup in a water bath at 37°C. Aliquots
(30 µl) were removed with time and spotted on a 1.5 x 1.5 cm filter paper square that had been marked at the origin of a 23 x 57 cm Whatman 3 MM paper. These spots were blown dry within 45 sec by a hair dryer. The paper was developed in a descending direction at 37° in ethyl acetate : pyridine : water (10:4:3) solvent for a minimum of 8 hr. Within this time, all mono- and disaccharides are washed from the origin and only polysaccharides remain. After drying the chromatogram, the 1.5 x 1.5 cm squares are cut from the chromatogram and placed in a counting vial (186) containing 10-15 ml of a toluene cocktail containing 5 g PPO and 0.3 g POPOP per liter of toluene, modified from (187). These are then counted in the Packard Tri-Carb model 2405, model 3310, or model 3003 liquid scintillation counter.

c. 14C polysaccharide assay for determining soluble and insoluble polysaccharides. This consists of 0.1 ml enzyme solution, 0.1 ml sucrose solution, and 0.1 ml dextran solution. Each time point was performed in its own Auto-Analyzer cup. The reaction was stopped by adding 0.7 ml of 0.1 M sodium carbonate solution. The insoluble polysaccharide was obtained by filtering through 2.4 cm glass fiber filter discs. The filtrate was collected in a 15 ml centrifuge tube. Washes (4 x 0.5 ml each) removed the soluble products from the filter pad and were collected and
combined with the initial filtrate in the centrifuge tube. The soluble polysaccharide was precipitated with 3 vols of ethanol and cooled for 12 hr at -10°. The water insoluble polysaccharide was further washed 10 times with 0.5 ml water and twice with 95% ethanol. The polysaccharide containing filter pad was dried and placed in a counting vial as above, and counted.

d. 14C levansucrase assay. Aliquots were collected either as in the 14C polysaccharide assay in section 13b or 13c in the Methods section. Values were compared using 14C-(U)-sucrose, 14C-sucrose-[(U) glucose], or 14C-sucrose-[(U) fructose].

e. Methanol assay. This is a modified assay of Germaine et al. (188). Aliquots from the 14C total polysaccharide assay were spotted on 1.5 x 1.5 cm Whatman 3 MM filter paper squares and immersed in a beaker containing methanol and a magnetic stir bar. The papers are protected from mechanical disruption by the stir bar by interposition of an nonmagnetic wire meshed dome covering the bottom of the beaker. The methanol wash continues 15 min after addition of the last paper square. The methanol is replaced with fresh methanol and washing is continued another 15 min. The methanol washes are repeated two more times. The squares are then dried, immersed in counting fluid, and counted as above. The methanol washes remove oligosac-
charides up to the pentasaccharides. Higher oligomers remain and are counted. This assay was mainly used for one point assays after one hr of incubation of column eluant fractions to find the enzymatically active peaks.

13. Fructans and their partial acid hydrolysis

Levan from *Aerobacter levanicum* NRRL B-1678 was isolated by Mr. John Zikopoulos and purified as described earlier (see section 1 in Methods).

Levan was prepared from *Leuconostoc mesenteroides* by incubating 10 U of enzyme (an 80% saturated ammonium sulfate precipitate from the crude culture supernatant), obtained from Mr. Tim Walseth, with 100 ml of 100 mM raffinose dissolved in 20 mM \( \beta \)-glycerophosphate pH 7.2. Incubation proceeded for eight days with stirring at 25\(^\circ\). The levan was precipitated with three volumes of ethanol, centrifuged, redissolved, and reprecipitated. This levan was then dialyzed continuously three days against running distilled water and then treated with 1 mg dextranase S and 1 mg mutanase at pH 5.0 in 20 mM pyridine acetate buffer for 24 hr at 37\(^\circ\). The pH was adjusted to 7 with 1N NaOH and 50 mM \( \beta \)-glycerophosphate pH was added. Pronase (1 mg, 45 PKU units) was added and incubated overnight at 37\(^\circ\). This was then dialyzed continuously for four days against running distilled water and lyophilized.
The unknown fructan from *Streptococcus mutans* OMZ 176 was prepared by incubating 0.25 U of enzyme (the 40-50% ammonium sulfate saturation cut from the crude culture supernatant grown with fructose as the carbohydrate source) with 2.3 l of 0.1 M raffinose dissolved in 20 mM β-glycerophosphate, pH 7.2, buffer for two days. This was then concentrated to 800 ml and precipitated with three vol of ethanol. The precipitate was dialyzed continuously against running distilled water and lyophilized. Solutions of each of the above fructans and inulin were prepared at a concentration of 50 mg/ml. To 4.5 ml of polysaccharide solution, 0.5 ml of 0.1 M H₂SO₄ was added and the solutions were incubated for 20 min at 70°. The reaction was stopped by cooling in an ice bath and with the addition of 0.6 ml of 0.2 M NaOH. Each was then desalted by passage through its own 1 x 7 cm Amberlite MB-3 column. Eluents (50 ml) are collected and concentrated to dryness on a rotary evaporator. Each is then dissolved in 0.5 ml distilled water and 1 ml ethanol. Aliquots (1-2 μl) are spotted on paper for paper chromatography and detected by silver nitrate dip on 23 x 57 cm Whatman 3 MM paper (see section 5 in Methods, Part I). Descending chromatography is run in ethyl acetate : pyridine : water (10:4:3) at 37° for 16-20 hr. The oligosaccharides are analyzed as $R_{fru}$ values by comparing their mobility with that of fructose. Ascending chromatography is run on 23.5 x 33
cm Whatman No. 1 filter paper in a stainless steel tank at room temperature until the solvent reaches the top of the paper (13 hr). The solvent system is n-butanol : pyridine : water (6:4:3). The chromatogram is subjected to 4 ascents. The oligosaccharides are analyzed by the method of French and Wild (189). Log $\alpha' = R_f/(1-R_f)$ where $R_f$ is that value for one ascent. It is calculated from the apparent value $R_f^a$ after 4 ascents by the equation $(1-R_f)^4 = 1-R_f^a$. Log $\alpha'$ is plotted against the degree of polymerization of the oligosaccharides. The Texas Instruments SR-56 was programmed to calculate the log $\alpha'$ given the $R_f^a$. The linear regression and correlation coefficient was also calculated using one of the prewritten programs provided with the instrument.

14. Double diffusion agar plates

The method described is a modification of Goldstein and So (190). Commercial concanavalin A (Con A) was lyophilized in salt so that 223.5 mg of the powder dissolved in a 1 ml solution results in 25 mg/ml of Con A in half-saturated NaCl. The buffer used contained 10 mM acetate and 0.003 M CaCl$_2$ at pH 5.3. Dilutions of the stock solution were accomplished in similar buffer containing in addition half-saturated NaCl. Purified agar (100 g) is diluted with 124 ml of buffer of 0.2M phosphate, 1.77% NaCl, 2 mM CaCl$_2$ (pH 7.2)
to a 1% solution by heating. This is filtered through glass wool and 22 ml of warm water is used to wash the rest of the agar from the glass wool. This solution and petri dishes are autoclaved 20 min at 121° and 25 ml is added to the bottom dish. Wells are formed in the cool hardened agar by using a No. 3 cork borer to remove 7 mm diameter by 3 mm deep agar plugs. The bottom of the well was formed by adding 2 drops of hot agar to each well. The well could contain about 90 µl of solution. Con A was added to a center well and the test solutions to outer wells placed radially around it such that the distance between the inner wells and outer wells did not exceed 5 mm. Con A was effective at 3-25 mg/ml. Test solutions contained 1-50 mg carbohydrate per ml. After filling all wells, a piece of Whatman No. 42, 11.0 cm paper was moistened and placed inside the top petri dish to create a water saturated atmosphere inside the petri dish chambers to prevent evaporation from the wells. Precipitin bands formed within 12-24 hr at 25° between the well containing Con A and those wells containing test solutions that give a positive reaction with Con A.

15. Dextran modifications

Dextran with an average molecular weight of 17,700 was used in this part.

a. Trityl derivative. The method of Holló et al. (191) was used. Dextran (1 g, which had been dried for 2 days at
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105°) was dissolved in 20 ml anhydrous formamide (dried 2 days over molecular sieves) and 1 g of triphenylmethyl chloride (trityl chloride), dissolved in 2 ml of pyridine (dried 2 days over potassium hydroxide) was added. This was allowed to react in a sealed ampule for 2 hr at 121°. After cooling, the derivatized product remained in solution and the unreacted trityl chloride was filtered away (ultra-fine sintered glass funnel). The dextran was precipitated with ethanol and washed with benzene and acetone and dried. Alternatively, the method of Rees et al. (192) was used. All materials were dried as above. Dextran (2 g) was dissolved in 25.2 ml of DM80 (dried 1 day over molecular sieves and stored over barium oxide) at 50° and cooled. Dry pyridine (25.2 ml, freshly distilled and dried) was added followed by 6.92 g trityl chloride. A control dextran was treated the same except for the trityl chloride addition. Reaction was allowed to proceed for 4, 11, and 24 hr. The reaction was stopped by heating in 3 vol of methanol. The derivatized dextran was further precipitated with 3 vol of methanol and cooled. The product was washed with 1 l of methanol.

b. Analysis of the extent of tritylation. The gravimetric method described by Holló et al. (191) was used. Tritylated samples (100 mg) and the control were added to 2 ml concentrated sulfuric acid and stirred until
totally dissolved. The trityl carbinol was precipitated with water and collected by centrifugation. The dried product was then weighed. The method of Rees et al. (192) and Duffield and Nussbaum (193) was also used. Tritylated samples and the control (1 mg) were dissolved in a 1:1 mixture of sulfuric acid and methanol. The yellow color of the derivative had the same spectrum as that reported by Duffield and Nussbaum (193). From the published extinction coefficient, the moles of trityl carbinol per mole of dextran derivatized were calculated.

c. Tripsyl derivative. The above method of Rees et al. (192) was modified. The same procedure was followed except that the trityl chloride was replaced with 5.19 g of triisopropylbenzenesulfonyl chloride (tripsyl chloride). This is a more specific reagent for primary hydroxyls (194) than is trityl chloride (192). This orange mixture was allowed to react 4 days. The product was precipitated with 6 vol ethanol, filtered, washed with 300 ml of ethanol and washed with acetone. The product was dialyzed continuously against running distilled water for 2 days, further washed with ethanol and methanol, and lyophilized.

d. Limit-dextran-dextrin. Dextran or tripsyl dextran was treated extensively in a dialysis bag with G_2-dextranase from *Arthrobacter globiformis* T6 (147, 149). Isomaltose was continuously removed from the solution by dialysis.
Treatment was at pH 5.0 and 37° in 20 mM pyridine acetate buffer. The product was further hydrolyzed with fresh G₂-
dextranase (300 mU were added each time) and the dialysis buffer changed twice a day, until no further release of iso-
maltose was observed. All of the dialysate was pooled and concentrated by rotary evaporation. Isomaltose released and limit dextran remaining was determined by total carbo-
hydrate analysis using orcinol-sulfuric acid (see section 7 under Methods). The final limit dextran was dialyzed against running distilled water overnight and lyophilized. This was analyzed for complete reaction by testing the polysaccharide with fresh G₂-dextranase and underivatized dextran with G₂-dextranase. Aliquots were removed and spotted at the origin of a 22 x 22 cm Whatman 3 MM paper. This was developed by ascending chromatography in ethanol : nitromethane : water (46:35:18) at 65° for 2 hr until the solvent reached the top of the chromatogram. The dried chromatogram was developed by the silver nitrate dip.

16. Metal ion removal

a. Electrodialysis. The procedure and instrumentation is similar to that described by Stein et al. (195). Electrodialysis was performed in the cold room on a sample (enzyme concentrated by the Bio-Fiber 80 beaker) that had been previously dialyzed against the electrodialysis solution
(distilled water, 1 mM or 2 mM β-glycerophosphate, pH 6.8). The dialysis solution was pumped upward through the electrode chambers washing out secondary products of electrodialysis at about 10 ml/min. The current was kept below 10 ma with the voltage increased as allowed by the current decrease. The enzyme solution was in the inner chamber separated from the outer electrode chambers by a piece of dialysis membrane that had been leached of ions by boiling water. Protein may eventually precipitate out but it may be solubilized by buffer.

b. Dialysis against 10 mM EDTA, 10 mM EGTA, or Chelex (10 ml/50 ml buffer) was attempted.

c. Dialysis of the enzyme concentrated by the Bio-Fiber 80 beaker, against 10 mM EDTA, 20 mM β-glycerophosphate buffer (pH 7.0) was performed with 4 changes of 2 l each, every 12 hr, of the EDTA containing buffer. Half (1.5 ml) of the enzyme was removed and stored at 4° (EDTA treated enzyme). The other half was further dialyzed against buffer without EDTA. The buffer was changed every 12 hr, 4 times, 2 l each time. The enzyme was stored at 4° (EDTA removed enzyme).

17. Metal analysis

This was performed by Mr. Walter Sutherland of the Ames Laboratory ERDA, Iowa State University, Ames, IA. Samples (600 µg) from electrodialysis treatment (see section 16 under
Methods) were dialyzed continuously against running distilled water and lyophilized. These were then placed into 10 ml volumetric flasks that had been pretreated with nitric acid for 5 days to leach out any metal ions that would leach out. The flasks were washed twice with distilled water and twice with doubly distilled nitric acid. The samples were then dissolved in 1.2 ml of a 6:1 mixture of doubly distilled nitric acid and perchloric acid at room temperature for 30 min. Heat from a hot plate was slowly increased over a 3.5 hr period over which time the nitric acid was evaporated and the fumes of perchloric acid became apparent. This procedure destroyed the organic materials and any lipid material, leaving any metal ions in the remaining perchloric acid. A blank was also run that had been treated the same as the sample. The volume of the samples was then diluted to the 10 ml mark with deionized distilled water and these samples were submitted. The procedure that was followed was that described by Winge et al. (196) using pneumatic nebulization on the QVAC 127.

18. Determination of pH profile

The pH profile was determined in the buffer described by Campbell (197) that he used for a-amylase analysis. This Universal buffer consists of 200 mM sodium dibasic phosphate, 200 mM acetic acid, and 200 mM boric acid. The pH is
adjusted to the desired values using 1 N NaOH or 1 N HCl. The final concentration is adjusted to 100 mM of each of the above buffer species. Since the enzyme was already buffered to pH 7.0 by 20 mM β-glycerophosphate, the assay pH was determined for each stock buffer using 1 part stock buffer, 1 part enzyme buffer, and 1 part distilled water. The pH of these solutions was measured and used as the working pH in the enzyme assays. The assay was the 14C total polysaccharide assay (see section 12b under Methods) where the test solutions used were the different stock solutions of the Universal buffer.

19. Periodate analysis

Periodate analysis of carbohydrates was performed as described by Dyer (198). Sodium arsenite (0.0506 M) was prepared by adding 5.0034 g of arsenious oxide, that had been dried for 3 hr at 105°, to a 1 l volumetric flask. Sodium hydroxide (10 g) in 50 ml of boiled water was added to dissolve the arsenious oxide. Distilled water was added to bring the volume to about 500 ml. This was then saturated with carbon dioxide by adding dry ice and the solution was brought to volume with distilled water. This stock solution was diluted to 0.01 M for use.

Iodine solution (0.1 N) was prepared by dissolving 6.5 g iodine and 12 g potassium iodide in 10 ml of distilled
water. After dissolution, the volume was brought to 500 ml and stored in a dark bottle. This must be standardized each time before use against the sodium arsenite solution. Saturated sodium bicarbonate solution was made by preparing about 1.3 M and adding crystals until no further dissolution occurred. Potassium iodide solution was prepared by dissolving 20 g in 100 ml of saturated sodium bicarbonate.

Starch solution was prepared by suspending 1 g of soluble starch in 5 ml cold water and adding this to 95 ml of boiling water containing 3 g boric acid. This was boiled 10 min and cooled to room temperature and the volume was adjusted to 100 ml with distilled water.

Carbohydrate (100 mg) was treated with 50 ml of 0.05 M sodium periodate in the cold. Aliquots (1 ml) were removed and pipetted into a 50 ml flask. Quickly, 10 ml of saturated sodium bicarbonate, 5 ml of 0.01 M sodium arsenite and 1 ml of 20% potassium iodide were added. The indicator starch solution (1 ml) was added and titrated with the freshly standardized iodine solution via a 10 ml microburet. Formic acid was determined by adding 1 ml of ethylene glycol to 5 ml of the reaction mixture to neutralize the excess periodate. After 5 min, this was diluted with 10 ml water and titrated with standardized 0.0105 N sodium hydroxide (standardized with potassium acid phthalate) to the phenolphthalein end point. A blank was run to determine the
spontaneous loss of periodate with time.

Periodate will cleave an α(1→6) linked glucose residue twice, releasing one mole of formic acid and consuming 2 moles of periodate per residue. Periodate will cleave a fructosyl residue linked via the 1 and/or 6 position, consuming one periodate per residue. A glucosyl residue linked through the 2 or 4 carbon position will be cleaved once, consuming one periodate molecule per glucose residue. A glucose residue linked through the 3 position or both the 3 and the 6 is resistant to periodate.

20. Methylation analysis

Methylation was performed by the procedure of Sandford and Conrad (199). Methylsulfinyl anion was prepared by adding 4.5 g sodium hydride (50%, coated with mineral oil) into a 150 ml three necked flask fitted with a rubber serum cap and containing a magnetic stir bar. The oil was removed from the hydride by stirring 4 times and decanting off 50 ml portions of n-pentane. The flask was then fitted with a thermometer and stoppered condenser and the pentane removed by evacuations interrupted by regassing with nitrogen through an 18-gauge needle. The stopper was removed and nitrogen passed continuously, through the needle fitted into the serum cap, into the flask. Dimethyl sulfoxide (45 ml) that had been redistilled over calcium hydride under reduced
pressure and stored over molecular sieves, was added and the mixture heated at 50° for 2 hr until the solution was clear and green. The anion concentration was about 0.23 N. This can be stored frozen for several months if mineral oil is layered over it (200).

The polysaccharide (50mg) was placed in a vacuum drying oven at 60° overnight. This was dissolved at 60° in 5 ml of dry DMSO in a 50 ml microapparatus resembling that used for the preparation of the methylsulfinyl anion. Nitrogen was passed through this also. After 2 hr, 1 ml of the methylsulfinyl anion was added and allowed to react 4 hr. The reaction mixture was cooled in an ice bath to 20° and 0.3 ml of methyl iodide was added slowly such that the temperature did not exceed 25°. After 30 min, the mixture was dialyzed against running distilled water for 1 day, lyophilized and dried overnight in a vacuum oven at 60°. This process was repeated. The dried polysaccharide was dissolved in 5 ml methyl iodide and silver oxide was added. Reaction continued 0.5 hr at 50° (201). The methylated polysaccharide was extracted with chloroform (202) and evaporated to dryness at 40° via a rotary evaporator. The dried product was dissolved in ether and filtered to remove insoluble material (199). Hydrolysis was performed according to Lindberg (200) by dissolving the methylated polysaccharide in 20 ml of 90% formic acid and heating this 2
hr at 100°. Saran wrap covered the tubes. This was evaporated to dryness by rotary evaporation and further hydrolyzed in 0.25 M H₂SO₄ (10 ml) at 100° for 12 hr. The reaction vessel was again covered by Saran wrap. The acid was neutralized with barium carbonate, centrifuged, and the precipitate extracted twice with distilled water.

The solutions were then concentrated by rotary evaporation at 40° and reduced with 100 mg sodium borohydride in 30 ml of distilled water for 2 hr at 25°. Excess sodium borohydride was removed by addition of Dowex 50 resin. Boric acid was removed by 6 x 2 ml of TFA-acidified methanol codistillations. The alditols were then acetylated with 3 ml of redistilled acetic anhydride, stored over molecular sieves, for 1 hr at 100°. This was followed by further treatment for 3 hr at 121° (203). The acetic anhydride was evaporated by rotary evaporation at 40° with the aid of toluene additions. Toluene forms an azeotrope with acetic anhydride (200). The product was then dissolved in water (20 ml) and extracted 3 times with 20 ml ethyl acetate. The extracts were concentrated and injected into the GLC for analysis. The instrument used was a Packard Instruments Becker Delft type 409 gas chromatograph containing twin 6 foot x one-eighth inch inside diameter glass columns packed with 3% ECNSS-M and gas chrom Q solid of 100-200 mesh. Two columns were run to correct for column leakage. Air flow
was at the rate of 1.75 kp/cm², hydrogen flow was at a rate of 1.3 kp/cm², and the carrier gas, nitrogen, flow was at a rate of 2.8 kp/cm². One microliter samples were injected. The standard, 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol, was prepared by reduction and acetylation of 2,3,4,6-tetra-O-methylglucose. Lindberg (200) published retention times on this column compared to this standard. These values were used to identify the peaks. The peak areas were evaluated by triangulation.

21. Preparation of mutan and soluble polysaccharide

A 0.1 M sucrose (6.1 l) solution was incubated with 120 U of lyophilized crude culture supernatant of Streptococcus mutans OMZ 176 (as determined by the continuous reducing value assay, see section 12a under Methods). The flask was stoppered with a cotton plug. After seven days, the mixture was filtered through a glass wool mesh in a Buchner funnel and the filtrate was precipitated with 3 vol of ethanol. The alcohol precipitated polysaccharide was then added to the water insoluble polysaccharide and both were dissolved in 4 l of 1 N NaOH. This was then neutralized and refrigerated. The water insoluble fraction was removed by filtration and the filtrate was again precipitated with 3 vol of ethanol. The water insoluble polysaccharide was redissolved in 1 N NaOH and neutralized. The insoluble
polysaccharide was centrifuged and the supernatants were combined with the ethanol precipitated polysaccharide above. This was concentrated by rotary evaporation. The water insoluble polysaccharide was again redissolved, neutralized and concentrated. Both the water soluble and water insoluble polysaccharides were dialyzed five days against running water. The water soluble polysaccharide was precipitated with 3 vol ethanol. Both were then treated with 10 mg pronase in 20 mM β-glycerophosphate (pH 7), overnight with stirring. Pronase (5 mg) was then added twice more at 12 hr intervals. The soluble polysaccharide was then precipitated with three vol ethanol and the insoluble one dissolved and neutralized twice more with 1.0 N NaOH. Both the soluble and insoluble were dialyzed continuously against running distilled water. The soluble was lyophilized. The insoluble was treated with ethanol to remove the water. Both were then dried in acetone. The insoluble weighed 56.4 g and the soluble, 14.4 g.

22. Calculation of enzymatic activity in U/ml with $^{14}$C assay

$$\text{dil. factor} \times \frac{\text{ml assay}}{\text{ml aliquot}} \times \frac{\text{cpm aliquot}}{\text{sucrose corr. fact.}} \times \frac{\text{labeling factor}}{\text{S.A. + cpm/mg sucrose}} \times \frac{\mu \text{molar product factor}}{\text{portion factor}} = \text{U/ml}$$
dilution factor - dilution of enzyme added to assay
ml assay - total volume of assay
ml aliquot - volume of aliquot spotted for counting
cpm aliquot of polysaccharide - cpm as determined by counting origin where polysaccharide runs
sucrose correction factor - % of counts present in substrate as sucrose (purity of radioactive substrate)
specific activity - specific activity of uniformly labeled sucrose; sucrose concentration as determined on a weight basis from AutoAnalyzer using weighed sucrose as a standard
cpm as determined, spotting sucrose and counting sucrose; cpm above was of polymerized product; remaining at the origin; not counting decrease in actual sucrose
labeling factor - the mg sucrose is actually determined as the total sucrose concentration: The weight of the labeled sucrose is \( \frac{1}{2} \) that of total sucrose when \(^{14}\text{C}-\text{sucrose-}\[(\text{U})\text{glucose}\] or \(^{14}\text{C}-\text{sucrose-}\[(\text{U})\text{-fructose}\] is used, since only \( \frac{1}{2} \) of molecule is labeled: This factor is 1 for \(^{14}\text{C}-\text{(U)}\text{-sucrose}\) and \( \frac{1}{2} \) for \(^{14}\text{C} \) sucrone-[(U)-one monosaccharide]
\(\mu\)molar product factor - \( \frac{(1000 \, \mu\text{g sucrose/mg sucrose})}{(180 \, \mu\text{g/}\mu\text{ mole monosaccharide from sucrose})} \)
portion factor - ml enzyme used in assay volume
units are \( \mu \text{mole monosaccharide from sucrose} \) \( \frac{\text{ml enzyme}}{\text{ml}} \) : An activity is expressed as \( \mu \text{mole monosaccharide polymerized per ml of enzyme from sucrose.} \)
III. RESULTS AND DISCUSSION

Gel electrophoresis was run on concentrated crude culture supernatant. Figure 17 is a typical example of the information that can be obtained from the gels by various treatments. Gel A has not been treated with sucrose but gels B, C, and D have been. Gel A has been treated with periodate and basic fuchsin (see section 6h under Methods). Only glycoproteins are visible. Two bands are visible at the dye front in the vicinity of levansucrase migration. A band can be seen at the interface of the separating gel and the spacer gel. This is due to protein that is too large to enter the gel. A strong band can also be seen at the top of the spacer gel. This is due to protein that is too large to enter this gel. The fact that any protein is visible in these top two positions is an artifact of the gel electrophoretic procedure. Water is placed on the top of the unpolymerized gel to provide a flat gel surface after polymerization. Without the water, a curved surface would result and the migrating protein bands would also be curved. The layering process mixes the water with the acrylamide solution and dilutes this out. After polymerization the percentage of acrylamide on the surface is actually lower than in the rest of the gel. Protein then begins to enter the top of the gel but is prevented from penetrating further by
Figure 17. Gel electrophoresis of lyophilized dextransucrase

A. Electrophoresed gel of the enzymes subjected to periodate treatment and basic fuchsin

B. Electrophoresed gel of the enzymes incubated in sucrose

C. Electrophoresed gel of the enzymes incubated in sucrose. The gel has been treated with periodate and basic fuchsin. The gel is photographed with the aid of transmitted light accentuating those areas that are susceptible to periodate treatment which appear dark here.

D. Electrophoresed gel of the enzymes incubated in sucrose. The gel has been treated with periodate and basic fuchsin and is identical to that in C except it has been photographed with the aid of stray light, with the gel against a dark background as in B. Those bands that are not susceptible are visible in white.

The dye front, bromophenol blue, is indicated by the wires.
the higher acrylamide concentration. It can be demonstrated that this top glycoprotein is enzyme by incubating a fresh gel in sucrose. Gel B has been incubated in sucrose (see section 6g under Methods). The top spacer gel is not present. Swelling is present in the top part of the separating gel. Here aggregated enzyme is present causing dextran to be formed. It is this large local concentration of dextran absorbing water that is responsible for this swelling phenomenon. Increasing the enzyme concentration added to the gel increases this lip shaped area causing it to appear mushroom-shaped. Even higher amounts of enzyme have been responsible for pressure build up within the system causing the gel to rise out of the test tube with the puffed up portion resting on the top of the test tube. Enzyme activity (see section 6h under Methods) is observed in the upper half of the gel. This portion is hard and stiff with all the acrylamide pores filled with dextran. The gel is contracted in the middle where both dextranucrase and mutansucrase activities are present. Mutansucrase forms the bands that are nontransparent in the center of the gel. Gels C and D are identical. They have both been incubated with sucrose and then treated with periodate and basic fuchsin. Gel C is photographed with transmitted light and gel D is photographed with stray light against a dark background. The periodate sensitive dextran regions are recognizable in gel C as the darkened
areas. The white areas in D are not attacked by periodate and remain nontransparent. This is the mutan region. Faint dextran bands are present in the middle and just below the mutan region. The cloudy band in the top sixth of the gel in B is periodate sensitive. Gels that have been incubated with raffinose show activity at the dye front.

It appears that gel electrophoresis may be a valuable tool for studying this system. The results indicate that true dextran-, mutan-, and levansucrases exist. At least four dextransucrase bands, one levansucrase band, and three or more mutansucrase bands are discernable. Extraction of enzymes from polyacrylamide gels has not been possible. The banding pattern changes throughout purification steps. Bands that were originally present with high mobility show a shift towards the upper part of the gel. The enzymes aggregate and create new bands. Removal of the glycerol sample layer on top of the gel after electrophoresis and addition of sucrose to this solution resulted in the production of polysaccharide by that enzyme, which was so highly aggregated that it could not enter the spacer gel. This decreases the value of gel electrophoresis as a diagnostic tool. Coomassie Brilliant Blue G-250 stains those bands seen in gel A. The more sensitive Coomassie Brilliant Blue R-250 stains the mutansucrase protein bands. Stain is also present throughout the top half of the gel where the dextransucrase is
located. Initially when the lower protein activities were added to the gel, it was felt the dye was not being destained in this region since the area did not visibly produce any polysaccharides from sucrose. The dye was staining the dextranucrase enzyme that smeared throughout the whole top half of the gel.

All of the enzyme preparations were tested for the presence of contaminating activities (see section 9 under Methods). No sucrose phosphorylase activity was observed. All preparations contained varying amounts of levansucrase. The relative proportion of this enzyme compared with the glucansucrases increased with successively higher ammonium sulfate saturation cuts (see section 11a under Methods) or with decreased molecular weight fractions from the Bio-Gel A-5m columns. Only the most concentrated samples show a slight release of fructose from raffinose, when large quantities of melibiose are released. If there is an invertase present it must be in very low concentrations. Fukui et al. (83) state that they have found an invertase in OMZ 176 but that is most likely intracellular. If it does exist, it is most probably intracellular. A few cells lysed during growth might release their invertase and yield the observed results. It is more likely that the fructose is not directly released from raffinose but is cleaved by a fructanase, from the levan that is forming. Incubation with
exogenous levan or inulin results in the release of fructose equally well from both substrates. Dextran obtained from Sigma from cultures of *Leuconostoc mesenteroides* B-512 is contaminated with small amounts of levan. Incubation with this dextran results in release of fructose. Guggenheim (72) has shown a fructan hydrolase is present in OMZ 176. Dewar and Walker (143) and Guggenheim and Burckhardt (174) have shown OMZ 176 contains an endodextranase. It has maximal activity at pH 5. Small amounts of isomaltose with larger amounts of the higher oligomers are the products. Both of these investigators grew the cells in a medium in which the pH was controlled so it did not drop below 6.8 (175) or 6.0 (143). After 20 hr the pH of my medium was 4.2. It was probably below pH 6.0 for most of the time. G. J. Walker has stated in a personal communication (FASEB meeting, Chicago, IL, 1977) that the dextranase is active when the pH is not allowed to drop below 6.0. This makes the enzyme easier to purify, since it does not have attached glucans that cause aggregation. If the pH drops below 6.0, as in an uncontrolled system, similar to that which I employed, then the dextranase is not active. It appears that incubation at this low pH eliminated dextranase as a contaminant in my system but may have caused the aggregation problems observed in gel electrophoresis. Dextranucrase is not stable to pH 4.2 indefinitely. A portion of the culture supernatant was
not neutralized but merely stored at pH 4.2 at 4°. After a week no activity remained when adjusted to pH 6.8. It is possible that some enzyme was lost during growth of the cells, as the pH fell in the medium.

Several different purification techniques were attempted. Before the Bio-Rad miniplant was available, the mildest technique for concentrating large volumes of enzyme was lyophilization. Gel electrophoresis indicated enzyme bands could still be separated and no activity was lost. The band location had changed due to aggregation. Triton X-100 (0.1-2.6%), BSA (400-800 μg/ml), phosphate (0.5-1.0 M), 10 mM mercaptoethanol, 10 mM EDTA, NaCl (50-6330 mM), 10% glycerol and pH incubation (pH 3.3 and 4.0) were used to disaggregate the enzyme. Each was ineffective. Urea (1.0-4.55 M), succinylation, maleylation and SDS (0.01-5%) inactivated the enzyme.

Isoelectric focusing in gels showed enzyme that focused into more than 8 bands but aggregation was still a complication. This was readily apparent when isoelectric focusing was tried in a preparative column. The voltage was not sufficient to focus the protein, which merely dropped to the bottom of the column, giving the impression that it focused at pH 3.3-4.5. The focusing data was received by personal communication with Elva Wohlers, August 1975, at Iowa State University.
Hydroxylapatite and acetone precipitations were tried on freshly produced enzyme. No fraction seemed to be enriched in dextranucrase or in mutansucrase. Acetone precipitates were no longer completely soluble in water resulting in insoluble, active aggregates. Less than 10% yield was recovered by acetone precipitation. The hydroxylapatite fractions were also aggregated such that no activity migrated into gels during electrophoresis.

Kuramitsu (164) and Germaine and Schachttele (168) have reported that the materials commonly used in preparing media contain significant amounts of sucrose. The active enzymes use this to form a limited amount of polysaccharide that causes the enzymes to aggregate. Kuramitsu (164) incorporated invertase or a mixture of dextranase and mutanase into the culture medium. This resulted in a shift in the migration of the enzymes from predominantly the void volume position (peak A) to the retarded position (peak B). Germaine and Schachttele (168) observed that fructose was a competitive inhibitor for dextranucrase activity and used this as a carbohydrate source instead of glucose, which does not affect the activity. This resulted in an enzyme preparation that did not aggregate as much as the glucose grown one. If fructose inhibits the enzyme, preventing the endogenous synthesis of polysaccharides, then studies can be performed on this enzyme to evaluate the mechanism of activation by
exogenous polysaccharides on the enzyme system. Table 5 contains the activity analysis of various ammonium sulfate precipitations of the crude culture supernatant from cells grown in glucose or fructose medium. Data concerning the protein content of each fraction have not been included in the table since the method employed for this analysis was not suitable. Freezing and thawing experiments performed earlier indicated that activity was lost and aggregation was increased by this process. Therefore, the enzyme preparations were stored at 4°C in thymol (saturated solution). At the time of analysis, I was not aware of the fact that thymol interfered with protein determination by the method of Brewer et al. (204). I was unable to repeat the protein analysis on all the fractions with the improved method described in section 5 under Methods.

Table 5 shows that the type of carbohydrate source seems to affect the enzyme that is produced. Most of the enzymatic activity is isolated with the first ammonium sulfate cut in the glucose-grown samples. Most of the remaining recovered activity is present in the second fractionation. The fructose-grown enzyme shows a shift in the amount of enzyme isolated at the various ammonium sulfate cuts. Very little enzyme is recovered in the first 40% saturation cut. Most of the activity is recovered in the second fractionation with substantial amounts still present in the third fractionation.
Table 5. Purification of dextran sucrase from *Streptococcus mutans* OMZ 176 by precipitation with ammonium sulfate

<table>
<thead>
<tr>
<th>Fraction %{(NH4)2SO4}</th>
<th>vol ml</th>
<th>Buffer mU/ml total yield units</th>
<th>Dextran 400 μg/ml total yield mU/ml</th>
<th>Activationa</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>glucose</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>crude</td>
<td>1056</td>
<td>11.6</td>
<td>12.3</td>
<td>100</td>
</tr>
<tr>
<td>0-40</td>
<td>29</td>
<td>138</td>
<td>4.0</td>
<td>33</td>
</tr>
<tr>
<td>40-50</td>
<td>44</td>
<td>44.8</td>
<td>2.0</td>
<td>16</td>
</tr>
<tr>
<td>50-60</td>
<td>50</td>
<td>4.0</td>
<td>0.2</td>
<td>1.6 n.d. b</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>50.1</td>
</tr>
</tbody>
</table>

| fructose              |       |                               |                                     |             |
| crude                 | 5586  | 9.6                           | 53.3                                | 100         |
| 0-40                  | 100   | 21.1                          | 2.1                                 | 4.0 121     |
| 40-50                 | 152   | 171                           | 26.0                                | 48.8 304    |
| 50-60                 | 232   | 33.9                          | 7.9                                 | 14.8 32.7   |
| Total                 |       |                               |                                     | 67.6        |

a Ratio of activity in presence of dextran to the activity in absence of dextran.

b n.d. = not determined.
A differing response is also noted in the effect exogenous dextran has on the enzymes. Dextran present at a concentration of 400 μg/ml results in slight differences in the activity of the glucose-grown enzyme. The maximal effect is 40% stimulation of activity in the 0-40% saturation ammonium sulfate cut. The effects on the fructose-grown enzyme are more apparent. About 70% more activation is observed in the crude enzyme from the fructose-grown cells than that observed in the glucose grown cells. The most striking development occurs in the 0-40% precipitate of the fructose-grown enzyme. Here the final activity in the presence of exogenous dextran is 5.7 times that in the absence of exogenous dextran.

Germaine and Schachtele (168) obtained activation of dextran-sucrase by dextran and attributed this to a primer requirement. Since their experiments did not answer all possible questions, it seemed advantageous to extend their experiments to ascertain whether or not their conclusions were valid. If the medium used does contain endogenous sucrose, then treatment with a competitive inhibitor may not be sufficient over a 20 hr period to completely stop all enzymatic synthesis. If some polysaccharides were produced they might be present in a concentration sufficient to partially mask the true primer requirement. The media used in both of the cases listed in Table 5 was pretreated with invertase (see section 10 under Methods) to eliminate sucrose. Assuming the enzyme
was effective, no sucrose remained in either the glucose grown or fructose containing medium. Endogenous polysaccharide was not responsible for the activity present in either. An enzyme possessing a true primer requirement (205-207) is inactive in the absence of polysaccharide. The ends of the polysaccharide serve as required sites onto which new glucose units are added one at a time. This means that, without polysaccharide present that can be covalently attached to additional glucose residues, no synthesis can occur. The conditions of the experiment with the invertase pretreatment make it difficult to understand the activation of the fructose-grown cells compared to the glucose-grown cells solely on the basis of the former being polysaccharide free and the latter containing sufficient endogenous primer. Neither one should contain polysaccharide primer synthesized from endogenous sucrose.

All future experiments were performed on the 0-40% saturated ammonium sulfate precipitated fructose-grown enzyme and primer free sucrose (see section 8 under Methods) as substrate. Okada and Hehre (208) observed that ordinary commercial sucrose contains trace amounts of dextran, starch, and other polysaccharide components that may make the study of primer requiring enzymes more complicated by introducing exogenous polysaccharides that were not anticipated.

Figure 18 shows the relationship between exogenous
Figure 18. Total dextranucrase activity of the 40% saturated ammonium sulfate precipitate of the fructose-grown enzyme plotted as a function of the exogenous dextran concentration.
dextran concentration and resultant enzyme activity in mU/ml. In the absence of exogenous dextran the enzyme contains about 30 mU/ml. The protein concentration for this enzyme was 440 μg/ml so the specific activity was 58 mU/mg. The assay used was the 14C total polysaccharide assay (see section 12b under Methods) and the test solution was various concentrations of exogenous dextran. Between 0 and 200 μg of added dextran per ml of reaction volume, there is a rapid increase in activity. This rate of increase is less from 200 to 600 μg/ml. A steady increase in the activity occurs up to a concentration of about 1600 μg/ml after which there is little change. After 17,000 μg/ml there is a notable decrease in the apparent activity. This may be real or artificial. If the decrease in activity is real, this may be due to increased viscosity of the dextran solution or increased competition of the dextran and the sucrose for the same or nearby binding sites. If the apparent decreased activity is not real, it may be due to the large mass of dextran coating the filter paper square. Some of the radioactivity may be washed off during development in the solvent system or there may be self-absorption of the disintegrations in the scintillation vial by the dextran itself. A linear regression analysis on the data points, with the concentration expressed as a logarithm, accentuates this apparent downward activity plunge at high dextran values, as can be seen in Figure 19.
Figure 19. Total dextranucrase activity of the 40% saturated ammonium sulfate precipitate of the fructose-grown enzyme plotted as a function of the logarithm of the exogenous dextran concentration
$r = 0.977$

$r = 0.884 (-)$
It is also apparent that the relationship between the activity and the log of exogenous dextran concentration is linear. The point of intersection of the two lines indicates that concentration of dextran (4,500 μg/ml) where maximal stimulation of the enzyme occurs. This assumes the decrease in activity with increased dextran concentrations above 4,500 μg/ml is real. If this is an artifact then this value is only an apparent optimal dextran concentration.

Addition of clinical dextran increases the amount of soluble dextran produced in strain K 1-R (117) and decreases the amount of insoluble dextran produced which decreases carious lesions in hamsters (209). Figure 20 shows the relationship between the concentration of added dextran and the types of polysaccharide products (soluble, insoluble, and total) produced as described in section 12c under Methods. The activity responsible for the formation of insoluble product rapidly increases and parallels the total enzymatic activity between 0 and 500 μg/ml. After this value, the rate of increasing velocity slows compared to the total. The activity responsible for insoluble polysaccharide production reaches a maximum around 2000 μg/ml and then begins to lose activity. The activity responsible for soluble polysaccharide production is still increasing at this point, which accounts for the optimal dextran concentration of 4-5,000 μg/ml for total
Figure 20. Dextranucrase activity (formation of soluble and insoluble product) of the 40% saturated ammonium sulfate precipitate of the fructose-grown enzyme plotted as a function of the exogenous dextran concentration.

...Δ.... (total polysaccharide) enzyme activity
--- 0 --- (insoluble polysaccharide) enzyme activity
--- x --- (soluble polysaccharide) enzyme activity
polysaccharide production.

A large range of dextran concentrations was used and in order to represent all the data points on one graph in a meaningful manner, several breaks in the abscissa were required in addition to the scale change. From the graph, the point of crossover between the insoluble and soluble products was observed at 10,000 μg/ml. After this point the insoluble polysaccharide produced decreases to 3% of the total polysaccharide produced at 173,000 μg/ml and the soluble increases to 97% of the total (see Table 6). If this figure with the incongruent abscissa is not aesthetically appealing, Figure 21 contains the same data points with linear regression analysis where appropriate for the relationship between the polysaccharides formed and the logarithm of the exogenous dextran concentration. This logarithm treatment compressed the abscissa to a manageable scale that still allows the increases in activity at the lower concentrations to be readily apparent. This figure demonstrates the linearity in the initial activation of insoluble polysaccharide synthesis and how it parallels total synthesis. During this initial stage, that activity responsible for formation of the soluble polysaccharide is almost trivial. It becomes significant only after the insoluble polysaccharide begins to decrease. Table 6 lists the percent distribution of the activity responsible for the insoluble and soluble polysaccharide production. The
Table 6. Distribution of activities between the soluble and insoluble polysaccharide at various exogenous dextran concentrations

<table>
<thead>
<tr>
<th>Dextran sample (µg/ml)</th>
<th>% of Total Activity</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Insoluble</td>
<td>Soluble</td>
</tr>
<tr>
<td>0</td>
<td>80.5</td>
<td>14.2</td>
</tr>
<tr>
<td>0</td>
<td>88.8</td>
<td>11.2</td>
</tr>
<tr>
<td>50</td>
<td>97.0</td>
<td>3.0</td>
</tr>
<tr>
<td>150</td>
<td>94.4</td>
<td>5.4</td>
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</tr>
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<td>3.9</td>
<td>96.0</td>
</tr>
<tr>
<td>173,000</td>
<td>3.0</td>
<td>97.0</td>
</tr>
</tbody>
</table>
Figure 21. Dextranucrase activity (formation of soluble and insoluble product) of the 40% saturated ammonium sulfate precipitate of the fructose-grown enzyme plotted as a function of the logarithm of the exogenous dextran concentration.

--- Δ --- (total polysaccharide) enzyme activity
--- 0 --- (insoluble polysaccharide) enzyme activity
--- x --- (soluble polysaccharide) enzyme activity
$r_A = 0.980$

$r_o = 0.993$
sum of the number may not necessarily be 100. Each activity is calculated by linear regression analysis of the cpm at each time point. If the distribution at a particular time point is unexpected but the total activity is normal, then the deviant points will affect the calculated slope more for the insoluble or soluble but not for the total. The initial enzyme activity using $^{14}$C-(U)-sucrose was compared to $^{14}$C-sucrose-[(U)-fructose] as described in section 12c and 12d under Methods. The distinction between soluble and insoluble activities was not performed. Both the glucans and levans were precipitated with alcohol and counted. Total levan activity was 7.4% of total polysaccharide activity. Table 6 indicates about 11-14% of the total polysaccharide activity is in the soluble portion. This means about 4-7% of the initial activity is true dextranucrasase activity and about 81-89% is true mutansucrase activity. The assumption is made that dextran only affects these latter two activities and that levansucrase is not affected. The value of the insoluble portion in going from 0 µg/ml to 50 µg/ml increases and the resulting soluble decreases. This may substantiate the above assumption. If the same levansucrase activity is present at all dextran concentrations and if the activity in both the glucansucrases doubles, then the resulting percentage of activity present in insoluble should increase to 89% or 92% (if the percentage of the dextran-
sucrase is 7 or 4% respectively and the rest is mutansucrase and levansucrase). If the insoluble is activated more than the soluble, then the number would be even greater than 92%. The data indicate an increase of at least this much and more (97%). No $^{14}$C-sucrose-$(U)$glucose] was available to accurately determine the amount of true dextranase present initially. The data of Table 6 and Figure 20 and 21 could be explained by dextran causing an initial activation of mutansucrase which increases to a maximum followed by slow inactivation as is often observed with substrate inhibition. It would then have to activate the 4 to 7% dextranase present from a calculated value of about 1.5 mU/ml (30 mU x 5%) up to about 200 mU/ml (Figure 18). This is an activation of 132-fold. The activation observed in the insoluble did not reach seven-fold. This explanation is complicated and tends to favor dextranase as the primary enzyme target for dextran activation. The products, then, are a result of simple enzyme catalysis.

Normal product inhibition is readily apparent from a Lineweaver-Burk plot of the data. As the 1/S values approach 0 the 1/V values deviate from linearity. There is an upwards curve as 1/V becomes larger than expected due to decreased activity. Figure 22 contains these curves for the soluble and the insoluble activities. Chludzinski et al. (210) have published a figure identical to this except their
Figure 22. Lineweaver-Burk plot for the insoluble and the soluble product formation by the 40% saturated ammonium sulfate precipitate of the fructose-grown enzyme

--- O --- (insoluble polysaccharide) enzyme activity

--- X --- (soluble polysaccharide) enzyme activity
data were based on the total polysaccharide produced. Their data produced a curve that was biphasic and unexplainable. The curve is biphasic because two types of products are being produced by two types of mechanisms. Figure 21 shows no apparent substrate inhibition in the concentrations plotted. The Lineweaver-Burk plot resulting from data obtained as total polysaccharide production (not shown) does not curve upward as in product inhibition but curves downward. This is explained by this figure where the component parts are examined. The activity responsible for the insoluble polysaccharide formation is monophasic with an x-intercept of $-8.3 \times 10^{-3} \text{ ml/\mu g} = 1/\text{Km}$, indicating the Km value is 120 $\mu$g/ml. The slope is 0.030 $\mu$g/ml/(cpm/min) = Km/v. Therefore the maximum velocity is 4100 cpm/min. The activity responsible for soluble polysaccharide formation is also monophasic with an x-intercept of $-3.4 \times 10^{-4}$, indicating the Km value is 2900 $\mu$g/ml. The slope is 1.3 $\mu$g/ml/(cpm/min). So the maximum velocity is 2200 $\mu$g/ml.

The observed maximal activity for the insoluble is around 3800 $\mu$g/ml which corresponds well to that predicted. The observed maximal activity for the soluble is around 3500 $\mu$g/ml. This is above that predicted by Michaelis-Menten kinetics. Chludzinski et al. (210) state that the biphasic nature may have something to do with branching. The proposed mechanism involved a growing dextran polymer and
another dextran molecule. At high dextran to sucrose ratios more enzyme is involved with forming branches than in normal elongation. In this situation the enzyme exists in a different catalytic state (210). Another mechanism is also proposed but this does not seem as plausible. This branching explanation is very similar to that demonstrated by Robyt and Taniguchi (211) for the mechanism of dextran branching; they incubated low molecular weight dextran with insolubilized enzyme that was radioactively labeled via a short exposure to $^{14}$C-sucrose with a high specific activity. Before this incubation was performed the beads onto which the enzyme was attached were washed extensively to remove sucrose. After incubation the radioactivity was released from the enzyme by the acceptor dextran which became more highly branched due to transfer of the radioactive chains from the enzyme molecule onto the acceptor dextran. The radioactive label was attached to the acceptor dextran via $\alpha(1\rightarrow3)$ linkages. Robyt and Taniguchi (211) expanded the proposed mechanism of Robyt et al. (212) for dextran biosynthesis, to explain branching (Figure 23). The hydroxyl on carbon atom three of any of the glucosyl residues makes a nucleophilic attack onto the potential reducing end of the enzyme-carbohydrate complex forming a new covalent bond and forcing the site to react with sucrose, if available. One site of dextransucrase is proposed (212) to consist of a single
Figure 23. Mechanism of alteration of product formation by the 40% saturated ammonium sulfate precipitate of the fructose grown cells

Ø reducing end of dextran

Ø potential reducing end of growing polymer chain attached covalently to the enzyme

0 glucose residue in dextran α(1→6) linked

Ø glucose residue in the growing polymer chain - probably α(1→3) linked

HO - C3 - OH on dextran

The bracket represents the active site of the enzyme molecule
199
glicosyl residue and the other site a growing dextran chain. Presumably these two sites are identical and there is transfer back and forth from site to site during polymerization. A similar mechanism has been proposed for rat liver fatty acid synthetase (213); two identical sites are involved with the fatty acid chain being transferred from one site to the other and back again as acetyl CoA units are added.

The following explains the shift in product formation from insoluble to soluble glucan. This is more probable than the explanation discussed earlier involving a 132-fold dextranucrase activation. Figure 23 explains how the Robyt and Taniguchi (211) branching mechanism might apply to the OMZ 176 system when incubated with dextran. Let us assume the systems are similar. Mutan contains \( \alpha(1 \rightarrow 3) \) glucosidic linkages which render the polysaccharide insoluble (116-118). There is competition at the active site between the normal sucrose, which results in synthesis of polysaccharide, and an occasional acceptor polysaccharide that removes the growing chain.

There should be a shift in the proportion of time that the enzyme participates in synthetic and acceptor reactions. If the concentration of acceptor increases, then the intervening length of time spent in synthesis should decrease, resulting in proportionately shorter times of synthesis and thus shorter chains. The length of the chains become shorter.
and the relative proportion of transferred α(1→3) bonds becomes less. This results in increased solubility of the resulting dextran-mutan hybrid. Eventually, only very short chains are transferred and 97% of the activity is expressed as soluble product.

The acceptor reactions of the last paragraph could also explain the increased activity of the enzyme responsible for synthesis of insoluble polysaccharide, that maximizes at an exogenous dextran concentration of 2000 μg/ml. Several investigators have observed (99, 214) that enzyme activity decreases as synthesis proceeds. The growing chain creates a certain drag on the forward reaction, towards synthesis, resulting in a decreased apparent enzyme activity. If these chains are displaced, the nascent enzyme is free to proceed at its own natural potential until the length of the chains builds up again. A steady release of these growing chains would apparently increase the observed enzymatic activity. On the molecular level the enzyme is simply allowed to fulfill its potential without environmental restraints. The dextran is not stimulating activity, it is merely removing natural constraints that reduce the apparent activity. If the acceptor reaction is not too frequent, sufficient insoluble polysaccharide may be formed, such that the acceptor is unable to solubilize the product. Thus, activation of the enzyme responsible for synthesizing insoluble product is the
This explanation would be sufficient if only similar activation were present in the ammonium sulfate cuts of the remaining fructose-grown enzyme and of the glucose-grown enzyme. Table 5 lists the resultant activation for these enzyme preparations. Ostensibly, there is no reason that the acceptor mechanism, just explained, should not show a similar effect with these enzymes. Presumably the same shift from insoluble to soluble product should be observed with these enzymes, as exogenous dextran concentrations increase. An alternative explanation may be necessary for the initial rapid activation of the enzyme responsible for synthesis of the insoluble product. Schachtele et al. (115) have observed that glucose is utilized more readily by the growing cells than fructose. Cultures grown on glucose or on fructose gave glucansucrases that had different physical properties, although both synthesized the same glucans. Table 5 shows the differences between enzyme produced in fructose- and glucose-grown cultures. These differences are apparent from the precipitation data and from the dextran activation response. An alternate explanation assumes that the glucose-grown enzyme is complete, and that the fructose-grown enzyme is missing something which can be remedied by addition of exogenous dextran. The effect of exogenous dextran (400 μg/ml) on dextranucrase activity was manifested most
extensively (5.7) with fructose-grown enzyme that was precipitated with 40% saturated ammonium sulfate, whereas fructose-grown enzyme precipitated between 40 and 50% saturated ammonium sulfate was only activated 1.8 times as compared with 1.4 times for the 40% saturated ammonium sulfate precipitated glucose-grown enzyme. The activation may be at a site other than the active site resulting in stimulation of activity through some sort of allosteric effect. This activation would be actual stimulation of otherwise deficient enzymatic activity. This is not the secondary activation observed with acceptor reactions. This theory of activation includes the origin of the enzyme in its explanation. Hill plots were published (168, 210) comparing activity to the log of the dextran concentration. The slope with dextran was 0.77 while that with sucrose was 1.1 in the presence of dextran and 1.7 in its absence. They proposed 2 binding sites. In the absence of dextran, sucrose occupies a donor and an acceptor site. In the presence of dextran, sucrose is in the donor site and dextran is in the acceptor site. My calculated slope for total polysaccharide synthesis was 0.68 which was similar to the published value. As in Figure 19 and Figure 21, a descending slope was also present with an observed value of -0.69. The descending slope was the inverse of the ascending slope. To the closest integer, 0.77 value means that there is one binding site for
dextran (168, 210). Better values may be obtained when the Hill plots are calculated for only that enzyme involved with insoluble polysaccharide synthesis vs. dextran. Figure 24 shows the Hill plot obtained for the enzyme that produces insoluble polysaccharide. The ascending slope is 0.93 or almost 1. The descending slope is -1.45 which differs radically from that observed in the total system. Figure 21 shows that during the drop in activity of the enzyme forming insoluble product, there is an increase in the soluble product formed. This is responsible for decreasing the slope from -1.45 to -0.68 by rotating the far right hand points up and about the point of intersection of the two lines.

If the Hill plot really indicates a binding site for the dextran molecule, then it should be possible to perform binding study experiments. These were performed according to the method described by Paulus (215) using \(^{14}\)C-(U)-dextran prepared as described by Robyt and Taniguchi (211). As probably should have been expected, saturation kinetics were not obtained. The binding had the same shape as that in Figure 19. A Scatchard plot showed a large scatter with the best fitting line parallel to the abscissa, indicating an infinite number of binding sites. Maybe dextran is not bound to the enzyme and that is why I could not determine a binding constant or number of binding sites. If dextran is truly a primer, it should be bound to the enzyme if the enzyme is
Figure 24. Hill plot for the activity producing insoluble product and dextran concentration
to add additional glucosyl residues onto its ends. Earlier in this discussion, primer was defined. The question of the existence of the primer requirement will be further explored now that the background information has been presented and explained.

Data have been published concerning the relationship between the length of the dextran chains and their degree of dextran sucrase stimulation (167, 210). The same curve was presented but it was labeled as having originated from strain GS-5 (167) or 6715 (210). No dextran sucrase stimulation occurred until D.P. 8 at which point it was only minimal. Stimulation increased up to D.P. 32. Maximal stimulation occurred at 3.8 times the dextran free system. Unhydrolyzed dextran gave a 3.4-fold stimulation. Germaine and Schachtele (168) showed that priming did not originate from the reducing end. Since only one end was left for priming to occur at, the nonreducing end, it was assumed to be the site at which priming occurred. The question of whether activation occurred by priming or some other mechanism was not addressed (167, 168, 210).

If priming occurs at the nonreducing end, whose chain lengths must be at least 8 glucosyl residues long for minimal stimulation of activity, and it is the concentration of these nonreducing ends that is important, then altering the number of nonreducing ends should alter the stimulation. A
molecule containing more nonreducing ends should stimulate more and one containing fewer nonreducing ends should stimulate less than B-512 dextran on an equal weight basis.

B-1299 contains about 50% non-α(1→6) linkages (216) and shows no activation. B-1254 contains 7% non-α(1→6) linkages (216) and activates the enzyme 7.4 times. B-1415 contains 11% non-α(1→6) linkages (216) and activates the enzyme 7.3 times. B-1422 contains 26% non-α(1→6) linkages (216) and activates the enzyme 4.6 times. Dextran T17.7 at this concentration activates the enzyme 6.5 times. These data indicate that increased numbers of nonreducing ends do not necessarily result in proportionally increased activation. An alternative to using dextrans with increased numbers of nonreducing ends is to take dextran and decrease this number.

Tritylation occurs most readily at the primary hydroxyls (192). Two methods of tritylation were attempted (see section 15a under Methods). The extent of derivatization was then calculated (see section 15b under Methods) for both methods. No detectable tritylation occurred in formamide at 121° during 2 hours by the method of Holló et al. (191). Three time samples were recovered using the method of Rees et al. (192). The 4 hours sample contained 7.0 moles by gravimetric method and 6.3 moles by spectrophotometric, or an average of 6.7 moles of trityl derivatized residues per
mole of T₁₇.7 dextran (average molecular weight 17,700 D, D.P. 109). The 11 hour sample contained 10.6 and 11.9 moles, respectively, giving an average of 11.2 moles of trityl derivatives per mole of dextran. The 24 hour sample contained 12.8 and 14.2 moles, respectively, giving an average of 13.5 moles of trityl derivatives per mole of dextran. Native dextran contains 5% non-α(1→6) linkages. Acid hydrolysis cleaves α(1→3) bonds 2-3 times (217) faster than α(1→6) bonds, so during the commercial preparation of T₁₇.7, the number of nonreducing ends may have decreased from 5%. With 5% branching, dextran would then contain a maximum of 6.5 nonreducing ends per mole (D.P. 109, (109 x .05) + 1 = 6.5). The 4 hour sample indicates that all are derivatized. Rees et al. (192) observed over-derivatization of dextran due to side reactions with the secondary hydroxyls. The 11 and 24 hour samples must therefore have a significant number of these side reactions to achieve 11.2 and 13.9 moles of trityl groups per mole of polysaccharide. These latter two samples were insoluble in water. The 11 hour sample absorbed water, forming a gum, but the 24 hour sample absorbed none and was completely insoluble. The 4 hour sample still contained nonreducing ends as determined by G₂-dextranase treatment (see section 15d under Methods) and release of isomaltose.

Tripsylation (see section 15c under Methods) was then
tried. This yielded a derivative after 4 days that released less isomaltose when treated with G2-dextranase. A 12-day treatment still released some isomaltose, indicating incomplete derivatization of the nonreducing ends. If reaction with tripsyl chloride is incomplete then the use of G2-dextranase would remove any untreated nonreducing ends or at least reduce them to 1-2 glycosyl units such that the length is no longer sufficient to act as a primer (167, 210). Dextran itself (T17.7 and T72.6) was also treated (see section 15d under Methods) to yield a limit-dextran-dextrin with nonreducing ends too short to act as primers. The extent of reaction with G2-dextranase was tested on the purified, modified dextrans by fresh incubation with G2-dextranase and analysis of products. Unmodified dextrans were also tested for comparison. The latter served as controls with fully accessible nonreducing ends from which isomaltose units could be removed. If these ends are accessible for G2-dextranase then they would be accessible for the priming reaction. If no isomaltose is released then no priming reaction is possible. Figure 24 shows these results. Tripsyl T17.7 still contains accessible nonreducing ends but are, on an equal weight basis, much less than the unmodified T17.7. During preparation of this limit dextran 20% was released as isomaltose. This compares with the release of 50% by the unmodified dextrans. Thus at least 60% of the
Figure 25. G₂-dextranase test for availability of non-reducing ends for enzymatic action on dextrans and modified dextrans

\[ T_{17.7} \quad T_{72.6} \] dextrans with average molecular weight of 17,000 D and 72,000 D

L.D.D. \quad G₂-dextranase limit dextran

Tripsyl \[ T_{17.7} \] tripsylated derivative of \[ T_{17.7} \] dextran
nonreducing ends were derivatized.

The limit dextrans release insignificant amounts of isomaltose, when treated with G₂-dextranase, indicating they would be unable to act as primers. The activation effects of these modified dextrans are tested compared with unmodified dextran. If primer reactions were responsible for the activation by exogenous dextran, then unmodified dextran should show full enzyme activation, and the tripsylated T₁₇.₇ should show significantly reduced enzyme activation. The limit-dextran-dextrins should show no activation if the mechanism of activation is due to priming activity, because of their very short outer chains. These results are listed in Table 7. Two concentrations of activators were used, 300 and 1000 µg/ml. At 300 µg/ml the tripsyl derivative exerts an activating effect (4.7) which is slightly lower than that observed with the unmodified T₁₇.₇ (5.4). G₂-dextranase treatment of this and T₁₇.₇ causes no decrease in activation (4.7 and 4.8, respectively). At 1000 µg/ml no significant differences are seen in any of the modified or unmodified T₁₇.₇ dextrans (6.0, 6.6, 6.0, and 6.4). T₇₂.₆ and its limit-dextran-dextrin at both concentrations (6.3 and 5.6 at 300 µg/ml and 7.5 and 6.5 at 1000 µg/ml) have somewhat higher activations than the T₁₇.₇ dextrans as also do the native polysaccharides from OMZ 176 (5.9 at 300 µg/ml and 8.3 at 1000 µg/ml) and B-512 (6.0), which are possibly due
Table 7. Activation of 40% saturated ammonium sulfate precipitated fructose-grown enzyme by dextrans and modified dextrans

<table>
<thead>
<tr>
<th>Type of dextran added</th>
<th>Activation at two concentrations of dextran</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>300 µg/ml</td>
</tr>
<tr>
<td>T₇₂.₆</td>
<td>6.3</td>
</tr>
<tr>
<td>sol. glucan OMZ 176</td>
<td>5.9</td>
</tr>
<tr>
<td>Native B-512</td>
<td>6.0</td>
</tr>
<tr>
<td>T₇₂.₆ L.D.D.</td>
<td>5.6</td>
</tr>
<tr>
<td>Tripsyl T₇₂.₆ L.D.D.</td>
<td>4.7</td>
</tr>
<tr>
<td>Tripsyl T₁₇.₇ L.D.D.</td>
<td>4.7</td>
</tr>
<tr>
<td>Tripsyl T₁₇.₇</td>
<td>4.8</td>
</tr>
<tr>
<td>T₁₇.₇ L.D.D.</td>
<td>5.4</td>
</tr>
<tr>
<td>T₁₇.₇</td>
<td>4.7</td>
</tr>
</tbody>
</table>

aDextran with average molecular weight of 17,700 D or 72,600 D.

bThe numbers in the columns represent the activation, ratio of the activity of the enzyme in the presence of activator to that in the absence of activator.

cL.D.D. = limit-dextran-dextrin of the polysaccharide by G₂-dextranase.

dNative soluble glucan (see section 21 under Methods).

eNative B-512 dextran.

fN.D. = not determined.
to the higher molecular weight of these materials. If a primer reaction were the cause of the activation of dextran-sucrase, the modified dextrans would not have been expected to give the degree of activation observed. In fact, the G2-dextranase modified dextrans produced significant activation, indicating that a primer reaction mechanism is not involved in the exogenous dextran activation of dextran-sucrase. The activation is, therefore, not by a primer mechanism but probably by one or both of the proposed mechanisms mentioned earlier, i.e., activation by acceptor mechanism giving branch linkages and activation by allosteric effects. Guggenheim and Newbrun state (171) that no glucan primer is necessary to initiate synthesis with this enzyme.

Attempts to physically determine the extent of derivatization by tripsylation proved futile. Partial carmelization occurred during the reaction, which resulted in degradation materials absorbing at 280 nm where the tripsyl group absorbs. Base and acid hydrolysis, NaBH4 treatment, and methylation analysis were not conclusive.

Low molecular weight acceptors, maltose, α-methyl-D-glucoside and α-cyclodextrin, at various concentrations, did not activate the enzymatic synthesis of polysaccharides.

A portion of the introduction discussed the identification of the type of fructan elaborated by various Streptococci. Throughout this discussion the terms levan and
levansucrase have been used. Evidence will now be presented proving that the fructan elaborated by *Streptococcus mutans* OMZ 176 is a levan. Section 13 under Methods describes the purification and partial acid hydrolysis of known levans from *A. levanicum* NRRL B-1678 and *L. mesenteroides* B-512, the unknown fructan from *S. mutans* OMZ 176 and a commercial inulin. The results for descending chromatography are presented in Figure 26 and Table 8. The oligosaccharides are easily distinguished within each series.

Table 8. Descending chromatography of the partial acid hydrolysates of fructans

<table>
<thead>
<tr>
<th>D.P. a</th>
<th>R_{fru} values b</th>
<th>levan c</th>
<th>OMZ 176</th>
<th>inulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.68</td>
<td>0.67</td>
<td>0.60</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.37</td>
<td>0.36</td>
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</tr>
<tr>
<td>4</td>
<td>0.21</td>
<td>0.21</td>
<td>0.24</td>
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</tr>
<tr>
<td>5</td>
<td>0.13</td>
<td>0.12</td>
<td>0.16</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>0.07</td>
<td>0.06</td>
<td>0.11</td>
<td></td>
</tr>
</tbody>
</table>

aD.P. - degree of polymerization.

bR_{fru} values - distance moved by the oligomer divided by the distance moved by fructose.

cLevan - levan from *A. levanicum*. 
Figure 26. Descending chromatogram of the partial acid hydrolysates of fructans

L levan from *A. levanicum*
I inulin
S *S. mutans* OMZ 176 unknown fructan
Fru fructose

2-6 degree of polymerization of the oligo-saccharides
The mobilities of the corresponding oligosaccharides are different in the levan series and the inulin series. Comparison of the unknown fructan oligosaccharides with the oligosaccharides from inulin and levan reveals that the unknown is probably a levan. Table 8 lists the relative mobilities for the oligosaccharides. Comparison of these values reaffirms the observation by descending chromatography that the unknown is similar to a known levan. Figure 27 shows the ascending chromatogram that was developed in a different solvent system (see section 13 under Methods). The interpretations are the same as in the descending system. The unknown is more similar to the levan than to the inulin. The data are plotted on a French-Wild plot (189) and represented in Figure 28. The inulin curve is linear as expected. The levan curve shows a break between D.P. 1 and D.P. 2 due to the presence of $\beta(2\rightarrow6)$ linkages in levan that lock the reducing end into a furanose or open chain form. Fructose, D.P. 1, is free to migrate as the open chain, the furanose, or the pyranose form. Since furanose sugars have higher mobilities than the pyranose, the oligosaccharides migrate proportionately further than expected (189).

An alternative method for distinguishing between levan and inulin is by their interaction with concanavalin A (Con A). Levan and Con A form precipitin-like bands in agar gel double-diffusion plates (see section 14 under Methods).
Ascending chromatogram of the partial acid hydrolysates of fructans

L  levan from *A. levanicum*
I  inulin
S  *S. mutans* OMZ 176 unknown fructan
Fru fructose
2-6 degree of polymerization of the oligosaccharides
Figure 28. French-Wild plot of the partial acid hydrolysates of fructans

- O oligosaccharides from levan from *A. levanicum*
- X oligosaccharides from unknown fructan *S. mutans* OMZ 176
- 0 oligosaccharides from inulin
- I error bars from 4 determinations
$r(o) = -0.997$

$r(x) = -0.999$
Inulin does not form these bands. Figure 29 shows an agar double-diffusion test for levan and inulin. Inulin shows no reaction with Con A at 5, 10, or 25 mg/ml. Both of the known levans and my unknown interact with Con A to form precipitin bands which curve into each other, representing the identity reaction. This shows that all are very similar and levan in nature. Thus, levan is the fructan elaborated by OMZ 176. Levan has been identified in 4 S. salivarius strains (151, 153, 159, 160). Inulin has been identified in 6 S. mutans strains (153, 155, 157, 158). Until now, it has not been shown that any strain of S. mutans produces a levan.

Ammonium sulfate precipitation of either the glucose-grown or the fructose-grown enzyme did not separate the mutansucrase from the dextran sucrose activity. Bio-Gel A-1.5m chromatography or DEAE-cellulose chromatography on any of these precipitates failed to separate the two activities. Aggregation occurred during ammonium sulfate precipitation and the enzymes in these aggregates were not separated by chromatographic means. A milder method for concentration by the Bio-Fiber 80 miniplant did not cause extreme variations in salt concentration since the ultrafiltration process maintains the same ionic strength as was originally present in the culture supernatant. A portion of this miniplant concentrate was then further concentrated by
Figure 29. Agar gel double-diffusion test for interaction of fructans with Con A

A - inulin 50 mg/ml
B - S. *mutans* OMZ 176 fructan 50 mg/ml
C - A. *levanicum* levan 50 mg/ml
D - L. *mesenteroides* levan 50 mg/ml
1 - Con A 5 mg/ml
2 - Con A 10 mg/ml
3 - Con A 25 mg/ml
the Bio-Fiber 80 beaker.

The miniplant concentrated enzyme (10 ml) was then passed over Bio-Gel A-1.5m, A-5m, A-15m, and A-50m columns. Little separation of the two enzymes was achieved by Bio-Gel A-1.5m chromatography. Both fructose-grown and glucose-grown enzymes were used. All the enzymatic activity (see section 12e under Methods) of the glucose-grown enzyme eluted as a sharp, narrow, peak at the void volume. Only slight tailing was observable on the descending side of the peak. The fructose-grown enzyme eluted as two overlapping, poorly separated peaks, the first peak was at the void volume, while the second peak was partially retarded. This indicates that the fructose-grown enzyme preparation is not as highly aggregated as the glucose-grown preparation. Bio-Gel A-5m chromatography of the glucose-grown enzyme and the fructose-grown enzyme was very similar. A peak occurred at the void volume which contained primarily dextranucrase. Testing each fraction with sucrose however, showed low levels of mutansucrase activity spread throughout the fractions, beginning in the middle of the void volume peak. Mutansucrase and levansucrase activities were located halfway between the void volume peak and the thymol peak. Bio-Gel A-15m chromatography of the glucose-grown enzyme and the fructose-grown enzyme revealed slight differences. For both preparations, nearly insignificant amounts of mutansucrase
were spread throughout the column eluent. The dextranucrase void volume peak is contaminated with low levels of mutansucrase. A second peak contains mutansucrase and levan­sucrase which are partially separated; the major part of the mutanase is in the front part of the peaks. The major part of the levan­sucrase is in the back part of the peak. About 50% of the activity added to the column was recovered in these two peaks. The amount of mutansucrase is greater in fructose-grown preparations than in glucose-grown preparations while the levels of levan­sucrase remain the same.

Only the fructose-grown preparation was purified by Bio-Gel A-50m chromatography. Figure 30 shows the elution pattern.

Visual observation indicated no enzymatic activity was present between fractions 56 and 82. No significant counts were obtained when the fractions were analyzed with $^{14}$C­-sucrose (see section 12e under Methods) between fractions 60 and 80. The figure indicates where the fractions were pooled. The Bio-Fiber 80 beaker concentrate of the Bio-Fiber 80 miniplant concentrate was also eluted from this Bio-Gel A-50m column. The eluent has the same $A_{280}$ absorption and $^{14}$C activity distribution as in Figure 30, but as judged by the formation of insoluble polysaccharide a significant amount of mutansucrase was now present in the void volume, indicating aggregation of mutansucrase during concentration.
Figure 30. Bio-Gel A-50m purification of the miniplant concentrate of the fructose-grown enzyme preparation
The concentrated enzyme was treated overnight with 10 mM DTT and elution repeated on a column that had been equilibrated with 20 mM mercaptoethanol. This treatment did not change the elution behavior nor the total enzymatic activity. The enzyme was too aggregated to allow separation of dextran-sucrase and mutansucrase.

Peak B (Figure 30) was concentrated by the Amicon ultrafiltration cell model 52 (see section 11 under Methods). Peak A and peak B were analyzed by DEAE-cellulose chromatography. The columns were eluted with buffer (20 mM β-glycerophosphate, pH 6.0), then with 50 mM NaCl-containing buffer, and then with 500 mM NaCl-containing buffer. No further isolation of the different activities was achieved. This is because peak A was already an aggregate and concentration of peak B resulted in its aggregation. Concentration by Bio-Fiber 80 beaker also aggregated the pooled peak B. The enzymes passed straight through the DEAE-cellulose column without binding.

Table 9 lists the purification data obtained. About 50% of the enzyme was recovered. The miniplant concentrate lost activity on storage. Most of the activity present in the miniplant concentrate is recovered on further concentration with the beaker. Most of the enzyme added to the Bio-Gel A-50m column can be recovered in the A and the B peaks. The specific activity of the fractions does not increase much.
Table 9. Purification of the fructose-grown enzyme preparation

<table>
<thead>
<tr>
<th>Fract #</th>
<th>vol ml</th>
<th>U/ml</th>
<th>Total U</th>
<th>µg/ml</th>
<th>U/mg</th>
<th>% yield</th>
<th>Purification factor</th>
<th>Date assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>culture super.</td>
<td>6000</td>
<td>.27</td>
<td>1620</td>
<td>380</td>
<td>.710</td>
<td>100</td>
<td>1</td>
<td>12/9/76</td>
</tr>
<tr>
<td>miniplant conc.</td>
<td>630</td>
<td>1.27</td>
<td>800</td>
<td>600</td>
<td>2.12</td>
<td>49</td>
<td>3</td>
<td>12/9/76</td>
</tr>
<tr>
<td>miniplant conc.</td>
<td>500</td>
<td>.804</td>
<td>402</td>
<td>600</td>
<td>1.34</td>
<td>(49)a</td>
<td>(3)b</td>
<td>1/22/77</td>
</tr>
<tr>
<td>beaker conc.</td>
<td>77</td>
<td>4.56</td>
<td>351</td>
<td>1060</td>
<td>4.30</td>
<td>(43)</td>
<td>(9.6)</td>
<td>1/22/77</td>
</tr>
<tr>
<td>miniplant conc.</td>
<td>10</td>
<td>.72</td>
<td>600</td>
<td>1.2</td>
<td>(49)a</td>
<td>(3)b</td>
<td></td>
<td>4/23/77</td>
</tr>
</tbody>
</table>

Bio-Gel A-50m

<table>
<thead>
<tr>
<th></th>
<th>g.s. c</th>
<th>l.s. c</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>22</td>
<td>0.037</td>
</tr>
<tr>
<td>B</td>
<td>19</td>
<td>0.36</td>
</tr>
</tbody>
</table>

a Since miniplant lost activity on storage % yield for each set has been corrected as if all done on one day.

b Since miniplant lost activity on storage purification factor for each set has been corrected as if all done on the same day.

c Glucosyltransferase activities, glucansucrase (g.s.) and levansucrase (l.s.).
The A peak is almost entirely dextran sucrase. Levansucrase may be present at 1% but this is insignificant. The B peak contains both mutan sucrase and levansucrase. Only 40% is present as mutan sucrase.

The miniplant concentrate, Bio-Gel A-50m peak A and Bio-Gel A-50m peak B (Figure 30) were subjected to pH profile analysis as described in section 18 under Methods, using $^{14}$C-(U)-sucrose for the miniplant concentrate and peak A, and $^{14}$C-sucrose-[(U)glucose] and $^{14}$C-sucrose-[(U)fructose] for peak B. The results are given in Figure 31. Figure 31a shows the pH profile of the miniplant concentrate, which has a broad pH maximum plateau, from pH 5 to pH 6.5. Figure 31b shows the pH profile of the Bio-Gel A-50m peak A (dextran sucrase). It also has a broad pH maximum plateau from pH 5.7 to 7.2. Figure 31c shows the pH profile of the Bio-Gel A-50m peak B (mutan sucrase) obtained by using $^{14}$C-sucrose-[(U)glucose]. It has an extremely broad pH maximum plateau from 4.5 to 7.5. Figure 31d shows the pH profile of the Bio-Gel A-50m peak B (levan sucrase) obtained by using $^{14}$C-sucrose-[(U)fructose]. It has a skewed peak with a relatively narrow pH optimum of 5.3 to 6.3. Using a pH value of 6.0 would insure assay of all enzymes. Borate present in the buffer does not interfere with the assay. Campbell (197) used borate in his α-amylase analysis. Robrish et al. (139) used it with several S. mutans strains.
Figure 31. Analysis of the pH-profile of the purified enzymes and the miniplant concentrate

A - miniplant concentrate
B - dextranucrase
C - mutansucrase
D - levansucrase
Tris cannot be used since 5 mM inhibits the activity 50%. NaHCO₃ cannot be used since it completely inhibits the enzyme at 20 mM.

The Bio-Fiber 80 beaker concentrate was tested for metal ion effects. Duplicate enzyme dilutions were made, one with buffer (20 mM β-glycerophosphate), and the other with 10 mM EDTA in the buffer. After overnight incubation, each was then tested for activity in the absence and presence of exogenous metal ions. Table 10 lists the results. EDTA inhibits the enzyme 85%. This activity may be completely restored by Ca²⁺, Mn²⁺, Fe²⁺, or Co²⁺. Other ions (Zn²⁺, Ni²⁺, Hg²⁺, and Cu²⁺) added at the same concentration as EDTA restore varying amounts of the activity. At higher concentrations as in the column headed EDTA, these ions almost completely destroy the enzymatic activity. It may be possible that they restore activity by chelating the EDTA and not by the addition to the enzyme itself. Sn²⁺ treatment seems to activate the enzyme slightly. This may be due to the possibility of oxidation-reduction reactions with the metal. Dialysis of the enzyme against Chelex did not cause inhibition of activity. EGTA or EDTA inhibit the enzyme 90 and 88%, respectively at pH 7.3. Adjustment of the pH to 6.0 restores activity to 49 and 44%, respectively. This may be due to the chelator releasing metal ions at the lower pH which is less favorable for chelation.
Table 10. Divalent metal ion effects on the Bio-Fiber 80 beaker concentrate

<table>
<thead>
<tr>
<th>Metal ion added&lt;sup&gt;a&lt;/sup&gt;</th>
<th>% relative activity&lt;sup&gt;a&lt;/sup&gt;</th>
<th>-EDTA</th>
<th>+EDTA&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>100 15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ca&lt;sup&gt;++&lt;/sup&gt;</td>
<td>95 98</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mg&lt;sup&gt;++&lt;/sup&gt;</td>
<td>72 58</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mn&lt;sup&gt;++&lt;/sup&gt;</td>
<td>60 93</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fe&lt;sup&gt;++&lt;/sup&gt;</td>
<td>86 86</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sn&lt;sup&gt;++&lt;/sup&gt;</td>
<td>122 44</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ba&lt;sup&gt;++&lt;/sup&gt;</td>
<td>57 35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Co&lt;sup&gt;++&lt;/sup&gt;</td>
<td>37 88</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cd&lt;sup&gt;++&lt;/sup&gt;</td>
<td>35 33</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pb&lt;sup&gt;++&lt;/sup&gt;</td>
<td>28 63</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zn&lt;sup&gt;++&lt;/sup&gt;</td>
<td>7 92</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ni&lt;sup&gt;++&lt;/sup&gt;</td>
<td>6 80</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hg&lt;sup&gt;++&lt;/sup&gt;</td>
<td>1 31</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cu&lt;sup&gt;++&lt;/sup&gt;</td>
<td>0 42</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Concentration of 3.33 mM.
Table 11. EDTA treatment and removal and its effects on the Bio-Fiber 80 beaker concentrate

<table>
<thead>
<tr>
<th>Treatment</th>
<th>pH6</th>
<th></th>
<th>pH7</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>U/mg</td>
<td>%</td>
<td>U/mg</td>
<td>%</td>
</tr>
<tr>
<td>none</td>
<td>4.45</td>
<td>100</td>
<td>3.02</td>
<td>100</td>
</tr>
<tr>
<td>none + CaCl₂&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.90</td>
<td>110</td>
<td>3.34</td>
<td>110</td>
</tr>
<tr>
<td>EDTA treated&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.32</td>
<td>54</td>
<td>.45</td>
<td>15</td>
</tr>
<tr>
<td>EDTA treated + CaCl₂</td>
<td>3.91</td>
<td>88</td>
<td>2.57</td>
<td>86</td>
</tr>
<tr>
<td>EDTA removed enzyme</td>
<td>3.13</td>
<td>70</td>
<td>3.07</td>
<td>102</td>
</tr>
<tr>
<td>EDTA removed enzyme + CaCl₂</td>
<td>3.80</td>
<td>85</td>
<td>3.90</td>
<td>129</td>
</tr>
</tbody>
</table>

<sup>a</sup>CaCl₂ concentration was 3.75 mM.

<sup>b</sup>EDTA concentration was 3.33 mM.

Another experiment was performed to study the enzymatic reaction after extensive dialysis in EDTA and then removal of EDTA (see section 16c under Methods). The results are listed in Table 11. The ratio between pH 6.0 and pH 7.0 is usually 1.5. Dialysis against EDTA drops the enzymatic activity to 15%. Adjustment of the pH to 6.0 increases the activity to 52%. Addition of Ca<sup>++</sup> to the EDTA treated enzyme at pH 6 or 7 restores activity to 88 and 86% respectively. Removal of EDTA from the system restores full activity at pH 7 and
only 70% at pH 6. Addition of Ca$^{++}$ gives a further increase in activity to 129% at pH 7 and to 85% at pH 6. If EDTA inhibition was removing metal ions from the enzyme then this complex should be dialyzed away, and dropping the pH should have no effect. If EDTA were binding amino acids directly on the protein, then it would not be washed away. Dropping the pH may change the chelation due to EDTA, becoming less effective at pH 6, or the ion charge of the enzyme may change such that there is nothing onto which the chelator can bind. The ternary complex enzyme-Ca$^{++}$-EDTA has never been observed (218). This is the complication that arises when a small molecular weight substance is added which could interact directly with the protein. Keller and Cori (219) and Martensen et al. (220) have observed EDTA inhibition of muscle phosphorylase phosphatase which is not via its metal ion binding characteristics. Dialysis against EDTA inhibited the enzyme but further dialysis to remove the EDTA restored the activity (219). 8-Hydroxyquinoline also binds metal ions but it did not decrease enzymatic activity (219). Activity is not stimulated by addition of metal ions (219). EDTA was found to be a simple competitive inhibitor with the substrate (tetradecapeptide) (220). The locus of the inhibition by metals is removed from that of the EDTA inhibition site (220). Guggenheim and Newbrun (171) found EDTA had no effect on
the S. mutans pooled 0.5 M eluate from hydroxylapatite chromatography of dextranucrase.

Electrodialysis (see section 16a under Methods) is a method that will remove metal ions from enzymes without introducing anything that will react with the enzyme. The metal ions in B. subtilis and human salivary α-amylases can be completely removed after 2-4 hr of electrodialysis during which time they lose activity. The enzymes can be reactivated 90-100% by addition of Ca^{++} (195, 218). Electrodialysis was performed against distilled water. Over an 8 hr period a drop in activity occurred that was not restored by the addition of Ca^{++}. The enzyme precipitated out but could be solubilized by buffer (20 mM β-glycerophosphate, pH 7). Electrodialysis against 1 and 2 mM buffer gave the same results. If metal ion were being removed causing the drop in activity, the addition of metal ion should restore the activity; it did not.

Metal analysis (see section 17 under Methods) was performed on each time point for electrodialysis against 1 mM buffer. No metal ion concentration dropped during the course of the experiment. Since a crude concentrate was used, some ions were present that were not removed by electrodialysis. Large amounts of Ca^{++} and P were present with lesser amounts of Al^{++}, Cu^{++}, Fe^{++}, Mg^{++}, and Zn^{++}. These concentrations were unchanged. It seems unlikely there is a metal
ion requirement. The enzyme purification procedure does not provide enough pure enzyme to do these analyses on pure enzyme.

Partial TFA hydrolysis (see section 4 under Methods) of the product formed by peak A (Figure 32) gave a glucose spot, a nigerose spot, and an isomaltose spot as the primary products of hydrolysis. Lesser amounts of the higher oligosaccharides were also produced. The partial acid hydrolysate and the soluble product formed from peak B consists of fructose and glucose. The partial acid hydrolysate of the insoluble product formed by peak B contains glucose, nigerose, isomaltose, and higher isomaltodextrins. The insoluble polysaccharide is so insoluble that it is only slowly attacked by acid. TFA hydrolysis was performed on some insoluble polysaccharide in a step-wise fashion. 0.5 N TFA was added, hydrolysis was allowed to occur 15 min at 121°C. The supernatant was removed and fresh TFA was added for 10 min at 121°C. This continued through 8 hydrolysis steps. The first two released nigerose and triose and the isomaltodextrin series. The following ones slowly released nigerose with lesser amounts of the isomaltodextrins. After 8 hydrolysis steps, considerable amounts of insoluble polysaccharide still remained. A small amount of fructose may be present.

Methylation analysis (see section 20 under Methods) was
Figure 32. Partial TFA hydrolysis of the products of the purified enzymes

S - standard isomaltodextrin series
N - nigerose
D - product from peak A, dextranucrase
L - product from peak B, soluble, levansucrase
M - product from peak B, insoluble, mutansucrase
performed on the soluble product from peak A (dextran), on the water insoluble product from peak B (mutan), and on the insoluble and the soluble glucans produced by the lyophilized crude enzyme (see section 21 under Methods). Table 12 contains these results. The mutan obtained from peak B is similar to that found by Ebisu and Misaki (177) from the total system. It is not as highly branched but the amount of \( \alpha(1\rightarrow6) \) linkages is greater. This suggests that the side chains of \( \alpha(1\rightarrow6) \) linked glucose units must be longer if the percentage of branches is less.

The peak A dextran and soluble glucan contain large amounts of \( \alpha(1\rightarrow3) \) linked residues and is highly branched. It is not very different from the peak B mutan. Periodate analysis of the soluble glucan agrees with the methylation analysis given in Table 12: Periodate gave 59\% \( \alpha(1\rightarrow6) \) (methylation gave 54.5\%) and 41\% \( \alpha(1\rightarrow3) \) (methylation gave 45.5\%) linkages. Periodate analysis of the insoluble glucan gave 37\% \( \alpha(1\rightarrow6) \) and 63\% \( \alpha(1\rightarrow3) \) linkages. Methylation of the insoluble polysaccharide did not work.

The amount of soluble and insoluble levan and soluble and insoluble glucan produced by peak B with time was analyzed. No insoluble levan was found. Some soluble glucan was produced by this fraction; the amount decreased with time in a linear fashion from 15.9\% at 1 hr to 6.7\% at 4 hr. Extrapolation to zero soluble glucan, indicates that no soluble
Table 12. Methylation data for various OMZ 176 polysaccharides

<table>
<thead>
<tr>
<th>Enzyme used to form polysaccharide</th>
<th>% glucosyl residues</th>
<th>Nonreducing ends</th>
<th>α(1→3)</th>
<th>α(1→6) branches</th>
</tr>
</thead>
<tbody>
<tr>
<td>insoluble polysaccharide (^a)</td>
<td>14.6</td>
<td>50.5</td>
<td>20.9</td>
<td>14.0</td>
</tr>
<tr>
<td>insoluble polysaccharide (^a) + dextranase</td>
<td>1.9</td>
<td>94.5</td>
<td>1.9</td>
<td>1.7</td>
</tr>
<tr>
<td>isoelectric focused (^b) at pH 5.0</td>
<td>3.0</td>
<td>94.0</td>
<td>3.0</td>
<td>0.0</td>
</tr>
<tr>
<td>isoelectric focused (^b) at pH 5.6</td>
<td>5.0</td>
<td>49.0</td>
<td>40.0</td>
<td>5.4</td>
</tr>
<tr>
<td>soluble glucan</td>
<td>13.7</td>
<td>29.3</td>
<td>40.8</td>
<td>16.2</td>
</tr>
<tr>
<td>peak A</td>
<td>14.0</td>
<td>32.4</td>
<td>37.2</td>
<td>16.5</td>
</tr>
<tr>
<td>peak B</td>
<td>5.8</td>
<td>60.7</td>
<td>26.6</td>
<td>6.9</td>
</tr>
</tbody>
</table>

\(^a\) Ebisu and Misaki (177).
\(^b\) Ceska et al. (173).

glucan would be present after 6.4 hr. The insoluble glucan increased with time, which would imply that the soluble product may be incorporated into the insoluble product.

These data suggest that the products isolated as soluble glucan (formerly called dextran) and insoluble glucan (formerly called mutan) may actually be graft polymers of the polysaccharides produced by a "true" dextran exhibiting which forms α(1→6) polysaccharides and by a "true" mutan exhibiting which
forms α(1→3) polysaccharides. Unless otherwise stated, throughout the rest of the discussion dextran refers to a polymer linked exclusively α(1→6) and mutan refers to a polymer linked exclusively α(1→3). These are products arising from the "true" dextransucrase and the "true" mutansucrase listed above, if they were isolated pure from each other. The dextransucrase and mutansucrase in the rest of the discussion refer to individual enzyme molecules that make dextran and mutan as polymers, respectively. Dextran serves as an acceptor for dextransucrase and mutansucrase. Since dextran is soluble, it serves as a better acceptor than mutan which may also serve as an acceptor, although because of its relative insolubility, mutan is much less efficient. Figure 33 indicates the possible products formed after acceptor reactions of polysaccharide on growing dextran or mutan chains. Figure 33a shows the graft polymer resulting after dextran has accepted two growing mutan chains from mutansucrase and one dextran chain from dextransucrase. Figure 33b shows the graft polymer resulting after mutan has accepted two growing dextran chains from dextransucrase. As the length of the mutan chains (v and w) increase (see Figure 33a) and the total number of mutan chains transferred to the soluble glucan increases compared to the length of the dextran chains (x and n), the resultant polysaccharide becomes less and less soluble. As the length of the dextran chains
Figure 33. Dextran and mutan acceptors forming graft polymers

A dextran as an acceptor
B mutan as an acceptor
@ $\alpha(1\rightarrow3)$ linkage
0 $\alpha(1\rightarrow6)$ linkage
A. Dextran (n residues long) as acceptor

B. Mutan (m residues long) as acceptor
(y and z) increases (see Figure 33b) and the total number of dextran chains transferred to the insoluble glucan increases compared to the length of the mutan chain (m), the resultant polysaccharide becomes more and more soluble.

This explains the similarity between the methylation analysis given in Table 12 for peak A and peak B polysaccharides. An increase in the number of \( \alpha(1\rightarrow3) \) linkages in the polysaccharide produced by peak A would shift the distribution pattern in the % of glucosyl residues observed in peak A polysaccharide towards that observed in peak B polysaccharide. If mutansucrase is incubated with two different concentrations of dextransucrase, the products formed should differ. Assuming mutansucrase forms relatively insoluble glucans that act poorly as acceptors, decreasing the amount of dextransucrase will result in longer dextran chains due to fewer acceptor reactions with the dextran which is not as readily available. Fewer branch points will result due to decreased transfer of growing chains. The final insoluble polysaccharide contains longer dextran chains and fewer branch points compared to the product formed with higher concentrations of dextransucrase which allows more chain transfer and, therefore, shorter chains. If mutan is a poor acceptor, the length of the dextran chain would increase more in low concentrations of dextransucrase than in high concentrations where more acceptor dextran is available.
to receive chains transferred from dextran sucrase. For equal rates of synthesis a long chain has only one nonreducing end where several short chains with the same amount of synthesis have more nonreducing ends. This explains why the data listed in Table 12 for peak B polysaccharide have more α(1→6) linkages (26.6%) than the insoluble polysaccharide derived from the culture supernatant enzyme which has 20.7%. The culture supernatant has more dextran sucrase present than peak B. Peak B polysaccharide has fewer branch linkages (6.9%) than the insoluble polysaccharide from the culture supernatant which has 14.0% branch linkages. This is due to decreased chain transfer in the lower dextran sucrase concentration in peak B. The amount of dextran sucrase present in peak B explains the increased number of α(1→6) linkages and branch linkages compared to the polysaccharide formed by the enzyme focused at pH 5.0 in the Ceska et al. (173) report. This apparently contains a very low amount of dextran sucrase which gives a polysaccharide containing 3.0% α(1→6) linkages and no detectable branch linkages. This polysaccharide is similar to the insoluble product formed by the culture supernatant in the presence of dextranase which has 1.9% α(1→6) linkages and 1.7% branches. Here the dextranase effectively reduces the available dextran for transfer. The extremely low amounts of effective dextran sucrase would make the transfer of chains by acceptor
reactions negligible and cause few $\alpha(1\rightarrow6)$ branches and low amounts of residues linked $\alpha(1\rightarrow6)$. The soluble glucan is partially hydrolyzed by mutanase to glucose so it must have at least 4 residues in an $\alpha(1\rightarrow3)$ linked chain (118), further substantiating transfer by acceptor reactions.

It may be possible to further separate dextranucrase from mutansucrase and also to separate mutansucrase from levansucrase. Electrophoresis may possibly be used. Two problems were encountered in the present study: Extraction of the enzyme from the gel and aggregation of the enzymes. The first might be eliminated by the use of bis-acrylylcystamine for the cross-linking reagent. Hansen (221) was able to remove ribonucleic acid from gels using this technique. After electrophoresis, addition of mercaptoethanol dissolves the gel and releases the separated substances. Reducing agents do not inactivate OMZ 176 enzymes (page 229 in Results and Discussion). The big problem that must be solved is that of the aggregation of the enzymes. Once they aggregate, they can no longer be separated. Peak B cannot be further purified since concentration causes aggregation. Incorporation of additional invertase, dextranase, and mutanase into the culture supernatant may be useful if the aggregation is caused by polysaccharides. Controlling the pH during cell growth may also be valuable. Whatever the physical reason
for the aggregation, it must be prevented before a clean separation of the enzymes is possible.
IV. CONCLUSIONS

1. The glycosylsucrases produced by *Streptococcus mutans* OMZ 176 are dextranurase, which produces a soluble glucan, primarily α(1→6) linked; mutansucrase, which produces an insoluble glucan primarily α(1→3) linked; and levansucrase, which produces a soluble fructan β(2→6) linked. These are separable on gel electrophoresis. Dextranurase has been isolated free from mutansucrase contamination and levansucrase contamination by Bio-Gel A-50m chromatography. Levansucrase and mutansucrase have not been separated.

2. The nature of the fructan was determined by a paper chromatographic analysis of the partial acid hydrolysis of the fructan, known levan, and inulin. The oligosaccharides produced were identical to those produced from levan. Agar gel double diffusion tests with concanavalin A indicated that the fructan was levan, since both it and known levan formed a precipitin reaction with the lectin and inulin did not.

3. Dextranurase, mutansucrase, and levansucrase have different pH optima; dextranurase pH 5.7-7.2, mutansucrase pH 4.5-7.5, and levansucrase pH 5.3-6.3.

4. Addition of 3.3 mM EDTA inhibits the enzyme 85% which is reversed by dialysis. The enzyme probably does not
possess a divalent metal ion requirement.

5. Dextran stimulates the activity of the glucansucrase obtained from fructose-grown culture after ammonium sulfate precipitation up to 6-fold. Increasing concentrations of dextran stimulate the enzymatic activity up to a maximum which is accompanied by a shift in products produced. Initially the predominant product is insoluble but with increasing concentrations of exogenous dextran this disappears and soluble polysaccharide is produced.

6. The glucansucrase does not possess a primer requirement. This has been established through the use of modified dextrans (tripsyl derivative and G2-dextranase limit-dextran-dextrins of the tripsyl derivative and two dextrans) which activated the enzyme to nearly the same extent as unmodified dextran. The modified dextrans should not be activating by a primer reaction because the nonreducing ends were not available for reaction.

7. Aggregation of the enzymes causes complications in the purification and analysis of enzymatic properties.
LITERATURE CITED


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