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*Loyola University Chicago*

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PURIFICATION, PROPERTIES, AND CARBON-13 NMR STUDY  
OF THE PRIMER MODIFICATION REACTIONS  
OF GLUCOSYLTRANSFERASES FROM  
STREPTOCOCCUS SOBRINUS

by

Mark E. Churchill

A Dissertation Submitted to the Faculty of the Graduate School  
of Loyola University of Chicago in Partial Fulfillment  
of the Requirements for the Degree of  
Doctor of Philosophy

March

1989

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Finally, the author would like to dedicate this work to his parents, who have given him their constant support and encouragement throughout his education.

## VITA

The author, Mark Edward Churchill, is the son of Layman Churchill and Arlene (Venske) Churchill. He was born August 26, 1960, in Joliet, Illinois.

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## CHAPTER I

### INTRODUCTION AND STATEMENT OF PURPOSE

Human dental caries, commonly called "cavities," form when a pathological erosion of the tooth surface enamel occurs. It is well established that caries are caused in human beings by bacterial colonization of the tooth surface (Frank and Brendel, 1966; deStoppelaar et al., 1969). Of the hundreds of bacterial species that have been isolated from the human oral cavity, only a few species of streptococci (Michalek et al., 1975; Druker et al., 1984a, 1984b) and lactobacilli (Fitzgerald et al., 1966; Fitzgerald, 1968) are able to cause caries when inoculated into the germ-free mouths of rats. In human beings, only S. mutans and S. sobrinus are cariogenic enough, and are isolated from carious lesions frequently enough, to be considered important in initiating dental caries (Krasse et al., 1968; Huis-in't-Veld et al., 1979; Alaluusua, 1983).

Since bacterial colonization is a critical step in caries formation, mechanisms of bacterial attachment to teeth have been extensively investigated. Some of these mechanisms involve specific binding reactions. For example, S. sanguis and S. mitis bind to the acquired enamel pellicle (AEP), which is a thin layer of salivary glycoproteins that surrounds teeth (Lie, 1977), largely through lectin-like



interactions between the bacterial cell surface and the pellicle (Gibbons and van Houte, 1980) as well as by ionic (Gibbons, 1984) and hydrophobic (Nesbett et al., 1982) interactions. Other bacteria can then bind to the cell surfaces of these organisms through specific coaggregation reactions (McIntire et al., 1978; Cisar et al., 1979; Cisar, 1982).

However, the binding of S. mutans and S. sobrinus to the AEP does not appear to be as dependent upon such specific interactions (van Houte and Green, 1974). The loose binding of these bacteria to the AEP is strengthened by the production of water-insoluble polyglucose polymers from dietary sucrose (Gibbons and Nygaard, 1968; Staat et al., 1980). These polymers, called dextrans or glucans, are synthesized from sucrose by enzymes called glucosyltransferases (GTF's), which are secreted into the extracellular medium by S. mutans, S. sobrinus, and other oral bacteria. Crude GTF preparations from both S. mutans and S. sobrinus are capable of producing insoluble glucan, which has been shown to play an important role both in bacterial accumulation on the tooth surface and in cariogenicity for both species (Staat et al., 1980; Gibbons, 1983). The glucans form the matrix of the sticky substance called dental plaque, to which the cariogenic streptococci bind very tightly (Hamada and Slade, 1980). The plaque matrix allows efficient proliferation of these cariogenic bacteria on the

tooth surface (Staat et al., 1980; Hamada, 1983). The formation of insoluble glucan by GTF is largely responsible for the increased incidence of caries when a high sucrose diet is consumed (Newbrun, 1982).

Once a stable colony is present on the tooth surface, the plaque pH drops quickly in the presence of fermentable dietary carbohydrates (Schachtele and Jensen, 1982), due to the production of organic acids, especially lactic acid, by the plaque organisms (Minah and Loesche, 1977; Chassy, 1983). This acidic environment further encourages the growth of acid-resistant bacteria, such as S. mutans, S. sobrinus, and lactobacilli, at the expense of other plaque organisms (Brown et al., 1976). Lactobacilli, although important as acid producing bacteria in the progression of carious lesions, are apparently too dependent upon an acidic environment to be important in initiating caries in nearly neutral saliva (Ikeda et al., 1973; Milnes and Bowden, 1985). When the plaque pH falls to 5.0 - 5.5, demineralization of the tooth surface begins (Jenkins, 1963). If this process continues without interference, dental caries will result.

Because of the importance of the GTF enzymes in the formation of dental plaque and caries, many investigators have attempted to purify the enzymes in order to study their catalytic and physical properties (reviewed in Montville

et al., 1978 and Ciardi, 1983). However, the enzymes are difficult to purify for several reasons, including:

1. the small quantities of the enzymes produced by S. mutans and S. sobrinus using standard culturing techniques;

2. the multiplicity of forms of GTF that synthesize different types of dextran products; and

3. the tendency of the enzymes to aggregate and to bind to the bacterial cell surface, particularly in the presence of dextran.

Thus, no completely satisfactory purification scheme has yet been reported, and the lack of sufficiently purified preparations has hampered enzymatic studies of GTF.

It is of particular interest to study the structures of the glucan products that GTF synthesizes from sucrose, since these products form the dental plaque matrix. However, the lack of purified enzyme preparations has also hindered the study of products synthesized by the different forms of GTF. Particularly, the modification of pre-formed "primer" dextran, which stimulates GTF activity markedly and acts as a second substrate, has been largely uninvestigated.

In view of these deficiencies in GTF enzymatic studies, I developed the following specific aims for this dissertation:

1. To develop a novel purification scheme which allows isolation of highly purified GTF's, and which solves

many of the problems associated with previous purification schemes reported in the literature;

2. To study, using these highly purified enzyme preparations, some basic properties of the GTF's, including:

- a. physical properties, such as molecular weight and pI;
- b. the catalytic behavior of the enzymes toward the substrates sucrose and dextran;
- c. the dependence of catalytic activity on pH; and
- d. the effect of the aggregation of GTF on its catalytic activity;

3. To use the purified enzymes to study, by Carbon-13 Nuclear Magnetic Resonance (NMR) spectroscopy, the structures of the dextran products of GTF catalytic activity, especially to determine the means by which the enzymes modify primer dextran.

## CHAPTER II

### REVIEW OF THE RELATED LITERATURE

#### A. The Dextran-Synthesizing GTF's as a Class

The cariogenic oral streptococci are not the only bacteria that produce dextran-synthesizing GTF enzymes. Dextrans are produced by the GTF enzymes of several bacterial species belonging to the genera Lactobacillus, Leuconostoc, and Streptococcus, of the family Lactobacillaceae (Sidebotham, 1974). Dextrans are defined as glucose homopolymers that contain an  $\alpha$ -1,6-linked backbone, with branches that may be  $\alpha$ -1,2,  $\alpha$ -1,3, or  $\alpha$ -1,4-linked, depending on the species producing the dextran. Occasionally, linear  $\alpha$ -1,3 residues are found within the  $\alpha$ -1,6 backbone. The GTF enzymes that produce dextrans all polymerize the glucose moiety of the natural substrate sucrose to form the dextran products. These extracellular enzymes are secreted into the bacterial culture medium, and are constitutive in lactobacilli and streptococci, but inducible by the substrate sucrose in Leuconostoc species (Neely and Nott, 1962). The structures of the dextran products produced vary widely with regard to percentages of linkage types and degree of branching, and depend on the species and even on the strain of the species being studied. Although some investigators have attempted to develop a general GTF polymerization mechanism

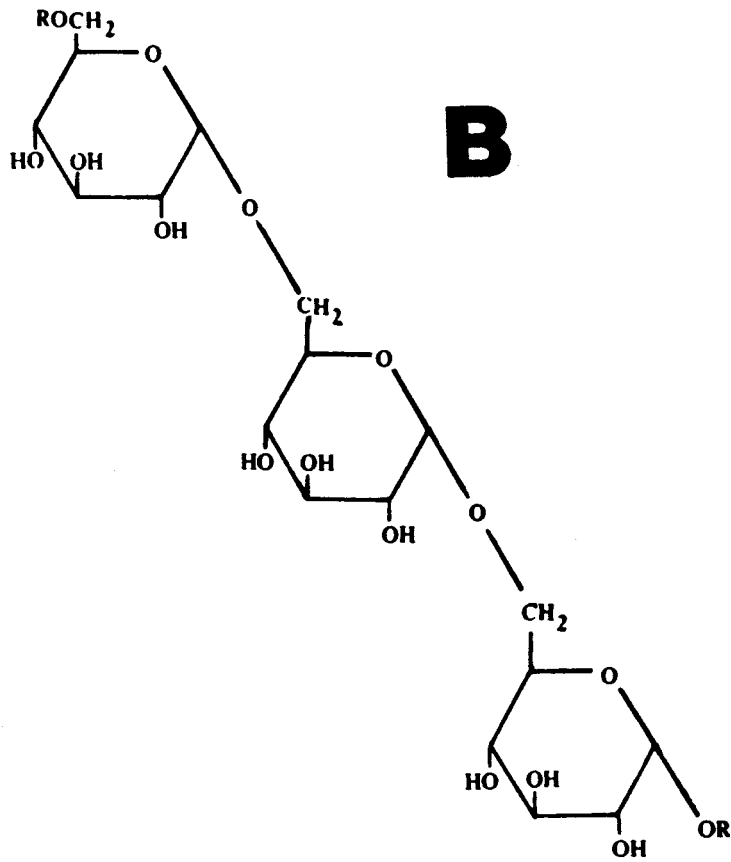
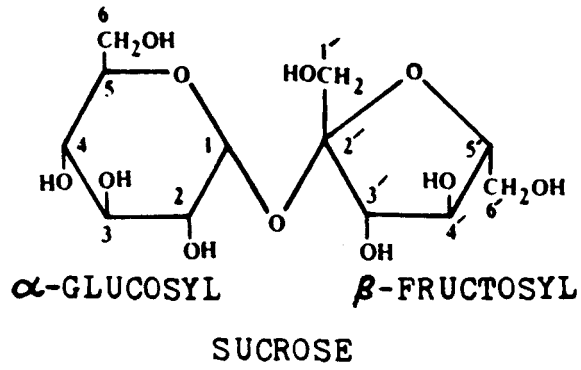
(Robyt, 1983; Ditson and Mayer, 1984), the variety of products produced and the poor characterization of most of the enzymes make any generalizations premature.

However, the GTF's isolated from several species of oral streptococci have been relatively well-studied because of their postulated role in the formation of dental plaque and caries (Gibbons and Nygaard, 1968; Gibbons, 1983). Although some differences in physical and catalytic properties between the GTF's from oral streptococci exist, these enzymes appear to be similar enough to be productively treated as a group. Thus, in the following discussion, general properties of the oral streptococcal GTF's will be described, followed by a discussion of their individual differences.

#### B. The Synthesis of Dextran by Oral Streptococcal GTF's

The principal, physiologically important reaction of the GTF enzymes is the synthesis of dextran from the substrate sucrose, the structure of which is shown in Figure 1A. The numbering system for the carbon atoms of glucose and fructose is indicated in the figure. The GTF's polymerize the glucose moiety of sucrose into dextran, with the release of free fructose. The GTF's of all oral streptococci studied to date synthesize dextrans consisting exclusively of  $\alpha$ -1,6 and  $\alpha$ -1,3 linkages between glucose residues (Hare et al., 1978; Colson et al., 1979; Trautner et al., 1982). Like other dextrans, oral streptococcal

Figure 1. Structures of sucrose and linear,  $\alpha$ -1,6-linked dextran. Single hydrogen atoms on the sugar rings are omitted for clarity. A: The sucrose molecule, with the numbering system for the carbon atoms of glucose and fructose indicated. B: A section of  $\alpha$ -1,6-linked dextran backbone. The symbol "R" represents the rest of the dextran chain, which extends in both directions.

**A**



dextran synthesized by crude GTF preparations contain stretches of dextran backbone, that is,  $\alpha$ -1,6-linked glucose residues in a chain (Figure 1B). Also found in these dextrans are  $\alpha$ -1,3 branches, which consist of glucose residues attached to the 3-hydroxyl groups on the backbone, and linear  $\alpha$ -1,3 residues, which are attached in the chain by their 3-hydroxyl, rather than their 6-hydroxyl, groups (Figure 2).

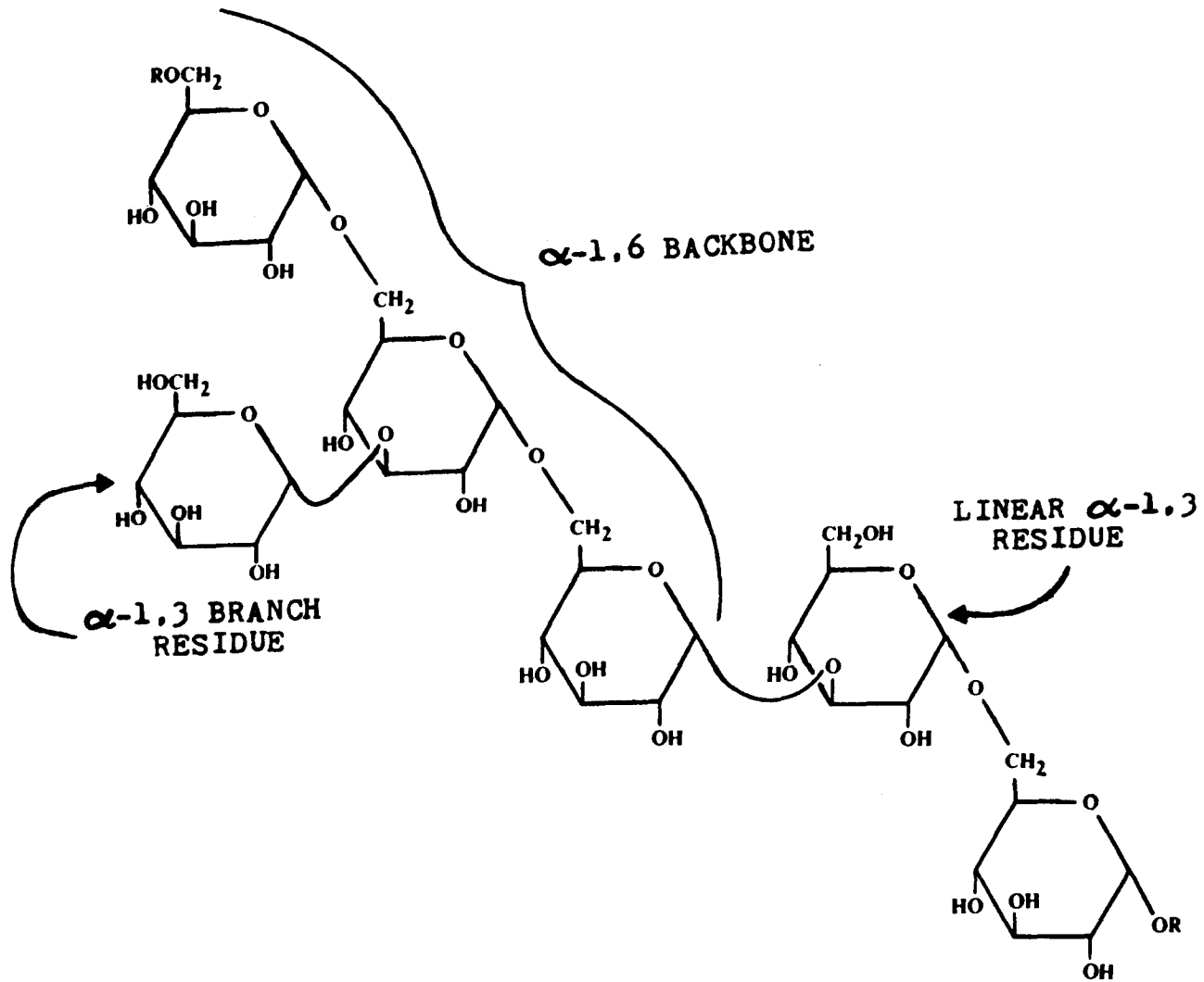
Almost all crude GTF preparations from oral streptococci synthesize from sucrose both water-soluble and water-insoluble dextrans, which contain the structural features described above. Since the water-solubility of dextrans generally decreases with increasing proportions of  $\alpha$ -1,3 linkages (Sidebotham, 1974), the soluble dextrans always contain a greater proportion of  $\alpha$ -1,6 bonds (Usui et al., 1975; Colson et al., 1979; Trautner et al., 1982).

The GTF-catalyzed synthesis of such complex dextran mixtures, consisting of products varying in both chemical and physical properties, cannot be accounted for by the action of a single enzyme. Thus, multiple forms of GTF synthesizing different kinds of products have been isolated from crude GTF preparations.

#### C. The Two Types of GTF: GTF-S and GTF-I

Two general types of GTF's have been identified, one of which synthesizes water-soluble products (GTF-S) and the other, water-insoluble products (GTF-I). Although the

Figure 2. Schematic representation of types of glucose structures that occur in dextrans synthesized by GTF's from oral streptococci. The dextran backbone sections can have  $\alpha$ -1,3-linked branches attached, and can be interrupted by linear  $\alpha$ -1,3 residues. The symbol "R" represents the rest of the dextran chain, which extends in both directions.



physical properties of each of these different enzymes vary somewhat between species, as discussed later, the enzymes from different species are catalytically very similar. GTF-S enzymes generally synthesize from sucrose, highly branched dextrans containing 65 - 80%  $\alpha$ -1,6 linkages and 20 - 35%  $\alpha$ -1,3 linkages (Shimamura et al., 1982; Tsumori et al., 1983b; Kumada et al., 1987). GTF-S is often called "dextransucrase" in the literature. GTF-I generally synthesizes from sucrose, essentially 100% linear,  $\alpha$ -1,3-linked polymers (Mukasa et al., 1985; Tsumori et al., 1985a), which are often called "mutans." In general, the rate of polymer synthesis by both types of GTF increases markedly in the presence of added  $\alpha$ -1,6-linked dextran (Figure 1B), which is therefore called "primer" dextran. As will be discussed extensively later, primer dextran acts by accepting glucosyl residues from sucrose, and so, acts as a "foundation" for the polymerization reaction. Thus, the GTF's are really two-substrate enzymes, with the substrates being sucrose and pre-formed dextran. However, the structures of the branches added to primer dextran by the GTF's have not been determined, and the determination of these structures forms a major part of this dissertation.

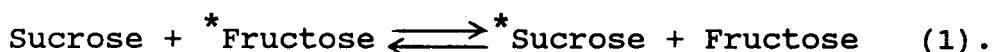
Thus, the complexity of dextran products synthesized by crude GTF preparations from the oral streptococci can be explained by the concerted action of the two enzymes GTF-S and GTF-I.

#### D. Reactions of GTF

As a preliminary to the following discussion of the reactions of GTF, it should be noted that, except for the structures of the products synthesized, GTF-S and GTF-I appear to be very similar with respect to the types of reactions they catalyze. Thus, the enzymes will be discussed together, and important differences will be pointed out where appropriate.

Although polymerization of glucose is the primary GTF reaction, the enzymes also catalyze other side reactions. One of these is an "invertase" reaction, that is, the hydrolysis of sucrose to form glucose and fructose (Mooser et al., 1985). This reaction occurs when a GTF transfers a glucosyl residue to a water molecule acceptor rather than to an  $\alpha$ -1,6-linked dextran primer. However, water is a very inefficient acceptor, and the hydrolysis reaction is essentially completely suppressed in the presence of added dextran (Mooser et al., 1985).

GTF's also catalyze a fructose-exchange reaction (Mayer et al., 1981; Mooser et al., 1985) in which labeled, free fructose in solution is incorporated into unlabeled sucrose:



This reaction is, in essence, a transfer of a glucosyl residue to the acceptor fructose, specifically, to the anomeric hydroxyl of fructose.

A third reaction is the autopolymerization of glucose residues from sucrose alone. The products of this reaction are said to be synthesized de novo from sucrose, that is, in the absence of primer dextran. All GTF's reported to date have some capacity for autopolymerization, although the rate is generally very slow compared to primer-stimulated polymerization (Chludzinski, 1976; Furuta et al., 1985).

A fourth reaction, the acceptor reaction, in which glucosyl residues from sucrose are transferred to a second substrate molecule, can utilize acceptors varying in size from monosaccharides to large dextran molecules, and some of the products of these reactions have been analyzed by chemical techniques. Fructose can accept glucosyl residues at positions other than the anomeric hydroxyl (Ono et al., 1981). The disaccharides maltose (Fukui and Moriyama, 1983) and isomaltose (Walker, 1973) can serve as acceptors. Finally,  $\alpha$ -1,6-linked glucose oligosaccharides (Walker, 1980) and high molecular weight dextrans (Chludzinski et al., 1974; Robyt and Martin, 1983) can accept glucosyl residues. In general, the larger dextrans are much more efficient acceptors than smaller molecules, with such sugars as maltose and isomaltose actually acting as inhibitors in the presence of large dextran primer (Chludzinski et al., 1976; Fukui and Moriyama, 1983).

Results from these studies of the GTF reactions indicate that GTF contains two binding sites--one for the donor

sucrose and the other for acceptor molecules. A good illustration of the presence of these sites is the behavior of fructose, which can accept a glucosyl residue either by binding in the sucrose site to reform sucrose (Mooser et al., 1985), or by binding in the acceptor site to form leucrose, that is,  $\alpha$ -D-glucose attached to the 5-hydroxyl group of fructose (Ono et al., 1981). Small molecular weight acceptors are postulated to inhibit GTF activity by competing with primer dextran for the acceptor binding site (Fukui and Moriyama, 1983).

The reverse of the GTF reaction, that is, the transfer of glucosyl residues from dextran to fructose to form sucrose, has been observed, but is very slow (Binder et al., 1983; Ditson et al., 1986). These studies also indicate that the glucosyl residues derived from dextran can be transferred to small molecule acceptors, such as maltose, but again, the rate is extremely slow.

Finally, it has been reported that the GTF-S from S. sobrinus can form branches on  $\alpha$ -1,6-linked dextran in the absence of sucrose (McCabe and Hamelik, 1983; McCabe, 1985). Presumably, this reaction involves transfer of glucosyl groups from  $\alpha$ -1,6-linked dextran to a 3-hydroxyl group of another section of  $\alpha$ -1,6-linked dextran backbone to form a branch point. This branching activity has been studied very little, and no study designed to determine its contribution

to the overall formation of  $\alpha$ -1,3 bonds by GTF-S has been reported in the literature.

#### E. The Mechanism of GTF Catalysis

It is obvious that GTF's are multifunctional enzymes that catalyze many different types of reactions. From a review of the literature on the mechanism of GTF catalysis, it can be stated that, despite some differences in physical properties, the enzymes from different oral streptococcal species appear to act by essentially the same catalytic mechanism. Furthermore, GTF-S and GTF-I enzymes, except for synthesizing different proportions of  $\alpha$ -1,6 and  $\alpha$ -1,3 linkages, also appear to be very similar mechanistically. Therefore, it is possible to formulate a descriptive, if not quantitative, mechanism that accounts for the reactions.

The observed GTF reactions can be accounted for based on the existence of a covalent glucosyl-enzyme intermediate, which forms when sucrose transfers its glucosyl moiety to a nucleophile in the GTF active site. The existence of the fructose exchange reaction (Mayer et al., 1981; Mooser et al., 1985), in which radioactive free fructose is incorporated into unlabeled sucrose, suggests that such a covalent adduct exists. The glucosyl-enzyme intermediate has been trapped by denaturing GTF under acid conditions during catalysis (Iwaoka and Mooser, 1983). The covalent complex was stable at pH 2 and 4°C, but could be hydrolyzed under alkaline conditions. When the authors compared their



kinetic hydrolysis data with those of model organic compounds and with those for a similar fructosyl-aspartate complex from the Bacillus subtilis fructosyltransferase (Chambert and Gonzyl-Treboul, 1976), they speculated that, in GTF, the active site nucleophile that binds the glucosyl residue might be a carboxylate. Another precedent for postulating that a carboxylate might bind the glucosyl residue in GTF is that a carboxyl-glucosyl intermediate has also been shown to be involved in the catalytic reaction of the enzyme sucrose phosphorylase (Voet and Abeles, 1970). The glucosyl-enzyme intermediate from this enzyme was trapped by acid denaturation during catalysis, and the nucleophile bonded to glucose was identified as a carboxyl group by chemical methods (Mieyal and Abeles, 1972). Another trapping study postulating a covalent intermediate in GTF catalysis has been reported using a Bio-Gel immobilized GTF enzyme preparation (Robyt and Martin, 1983).

The glucosyl-GTF intermediate is probably the activated form of glucose that is transferred to acceptors, and is believed to exist in the  $\beta$  configuration. Acceptor molecules attack the intermediate in an  $S_N2$  reaction to form the  $\alpha$  configuration in the product (Iwaoka and Mooser, 1983).

The mechanism by which this glucosyl intermediate participates in the autopolymerization reaction, that is, glucan synthesis in the absence of primer dextran, has been a source of controversy in the literature. There is a body

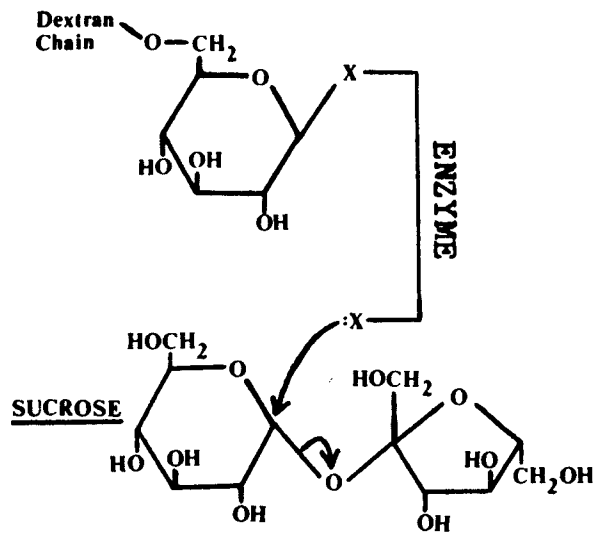
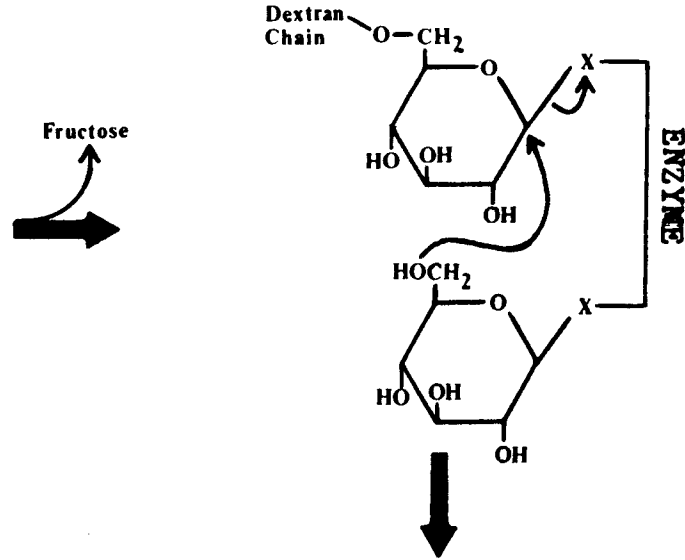
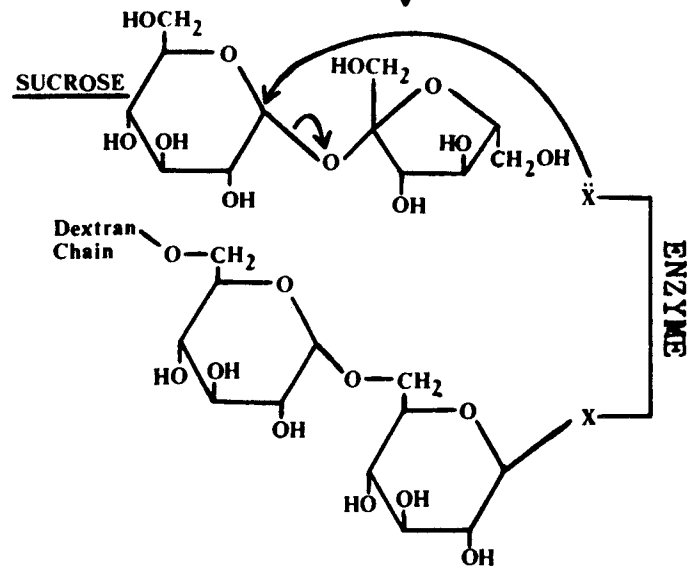
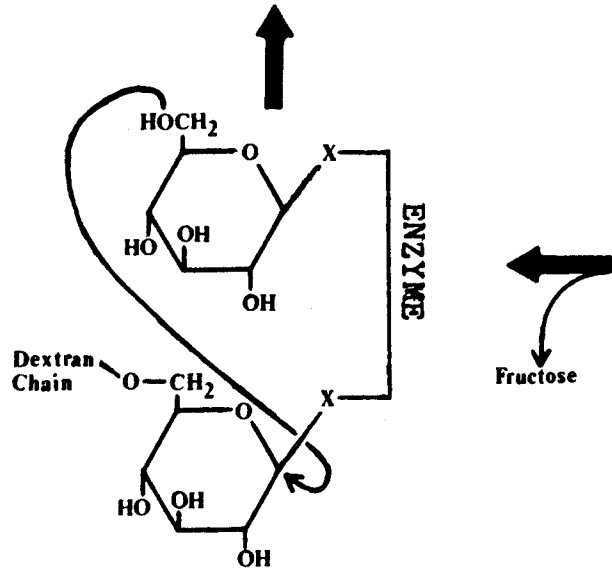
of evidence which indicates that autopolymerization occurs by the sequential transfer of glucosyl residues to non-reducing hydroxyl groups of the growing dextran chain, using sucrose or fructose as the initial acceptor. Fructose, released from sucrose by the invertase activity of GTF, is the most likely acceptor in this reaction, since glucose residues have been found attached to the non-reducing 5-hydroxyl group of fructose during autopolymerization (Ono et al., 1981; Ono and Smith, 1983). It has been shown that such glucosyl-fructose oligosaccharides, isolated chromatographically from GTF autopolymerization reaction mixtures, can themselves serve as primers for dextran synthesis (Ono and Smith, 1983). This hypothesis of the sequential addition of glucosyl residues to an initial fructose acceptor suggests that autopolymerization occurs from the non-reducing end of the growing polymer chain.

However, other studies have indicated that new glucosyl residues are added at the reducing, not the non-reducing, end of the growing oligosaccharide during autopolymerization (Robyt and Martin, 1983; Ditson and Mayer, 1984). These studies were done by immobilizing GTF and exposing the enzyme to a pulse of radiolabeled sucrose, followed by a chase of cold sucrose. During the pulse, radiolabeled dextran was formed, and during the chase, the newly added cold glucose residues were found at the reducing end of the oligosaccharides.

A mechanism accounting for this autopolymerization from the reducing end by an insertion mechanism has been proposed (Robyt, 1983). This hypothesis, diagrammed in Figure 3, states that there are two equivalent nucleophiles in the GTF active site, one of which holds a glucosyl residue derived from sucrose, while the other holds a growing dextran chain attached by the reducing end (Figure 3A). The 6-hydroxyl of the single glucose makes a nucleophilic attack on the reducing end of the growing dextran chain (Figure 3B). This frees the previously dextran-containing nucleophile to receive a new glucosyl group from sucrose (Figure 3C). Repetition of this process several times (Figure 3D, 3A, etc.) results in a new dextran molecule that has grown from the reducing end. The formation of  $\alpha$ -1,3 bonds on the  $\alpha$ -1,6-linked dextran backbone, as occurs in the GTF-S reaction, can be accounted for by conceptually adding to the active site a third nucleophile whose function is to transfer glucosyl residues to the 3-hydroxyls of the growing dextran chain. This mechanism is supported by the finding (Robyt and Martin, 1983) that covalently linked dextran chains are also bound to GTF during autopolymerization.

Whether autopolymerization occurs primarily from the reducing or non-reducing end is the most controversial aspect of the mechanism of GTF, and neither mechanism has enough experimental support to be generally accepted. Actually, the two proposed mechanisms can easily be reconciled

Figure 3. Proposed mechanism for GTF-catalyzed autopolymerization from the reducing end by an insertion mechanism. The mechanism is as proposed in Robyt, 1983. One of the two equivalent nucleophiles (represented by  $-X$ ) in the active site is bound to a glucosyl residue derived from sucrose, while the other nucleophile is bound to a growing dextranosyl chain (A). The 6-hydroxyl of the single glucosyl residue makes a nucleophilic attack on the dextranosyl chain at the reducing end to extend the chain (B). Repetition of the cycle (C, D, A, etc.) leads to growth of the chain from the reducing end. At any point, an acceptor molecule can accept a glucosyl residue or a dextranosyl chain, freeing the active site nucleophile for further catalytic activity.

**A****B****C****D**

by assuming that both occur. It can be proposed that autopolymerization from the reducing end occurs, leaving glucosyl residues and dextransyl chains attached to GTF. Fructose can then act as an acceptor and accept either a single glucosyl residue or the dextran chain, freeing the active site nucleophiles for new catalytic activity, and accounting for the presence of fructose in some autopolymerized oligosaccharides (Ono and Smith, 1983).

This mechanism can also account (Robyt, 1983) for the transfer of glucose residues to large primer dextran molecules, a process which has been shown to be very rapid and efficient in GTF catalysis (Chludzinski et al., 1974; Ono and Smith, 1983; Robyt and Martin, 1983; McCabe, 1985; Walker and Scheurch, 1986). Primer dextran can accept growing dextransyl chains or single glucose residues from the GTF active site, just as fructose does. However, since dextran is such an efficient acceptor, the rate of autopolymerization decreases markedly in the presence of primer, and primer-dependent synthesis increases (Fukui et al., 1982; Ono and Smith, 1983).

In view of the exclusive presence of  $\alpha$ -1,3 and  $\alpha$ -1,6 glycosidic bonds in GTF products, glucosyl residues could theoretically be transferred either to the 6-hydroxyl or 3-hydroxyl groups of primer dextran. It has been shown by several different methods that the 3-hydroxyl groups are the sites of attachment. Robyt and Corrigan (1977) showed that

modification of the 6-hydroxyl groups of primer dextran with tripsyl chloride, a reagent that specifically reacts with primary hydroxyls, did not result in any loss of priming activity. Thus, the 6-hydroxyls could not be the sites of glucosyl attachment. Walker and Schuerch (1986) demonstrated that chemically synthesized dextrans containing various proportions of  $\alpha$ -1,3 branches showed less priming efficiency as the number of branches increased. Since the number of free 6-hydroxyl groups increases as the number of branches increases, the priming efficiency should have become better with increasing branching if 6-hydroxyls were the glucosyl acceptor sites. It has similarly been shown (McCabe, 1985; Walker and Schuerch, 1986) that highly branched dextrans synthesized by GTF-S are poorer primers than more nearly linear dextrans. Another piece of evidence was provided by Robyt and Martin (1983), who showed that the  $\alpha$ -1,6-endodextranase cleavage pattern of  $^{14}\text{C}$ -labeled primer dextran changed dramatically when the primer was modified by reaction with GTF and  $^3\text{H}$ -labeled sucrose. The pattern changed in such a way as to demonstrate that the 3-hydroxyls, not the 6-hydroxyls, were the sites of glucose attachment to the primer. Finally, Walker (1980) used paper chromatography to separate the products of the reaction between GTF,  $[\text{U-}^{14}\text{C}]\text{sucrose}$ , and  $\alpha$ -1,6-linked oligosaccharide primers of lengths 7, 8, and 9. The  $R_f$  values of the products and the cleavage patterns the products displayed

when treated with  $\alpha$ -1,6-endodextranase indicated that GTF had added glucosyl residues to the 3-hydroxyl groups of the short primers.

The sucrose-independent branching activity of GTF-S (McCabe and Hamelik, 1983; McCabe, 1985) has barely been studied, so that its mechanism is unknown. However, the slow transfer of glucosyl groups from dextran to acceptor molecules (Binder et al., 1983; Ditson et al., 1986), as discussed earlier, could account for this activity if dextran served as the acceptor. Thus, this branching reaction may be a specific example of a known, minor GTF side reaction.

Therefore, as the studies cited here demonstrate, all of the known GTF reactions can be accounted for based on the existence of the glucosyl-enzyme intermediate, which can transfer glucose, either as single residues or chains, to acceptor molecules.

#### F. Physical and Catalytic Properties of GTF's

GTF-S and GTF-I from all oral streptococcal species studied are large proteins, with most molecular weight estimates being in the range 140 - 180 thousand (Fukui et al., 1982; Shimamura et al., 1982; McCabe, 1985; Asem et al., 1986; Kumada et al., 1987). GTF-S and GTF-I both have catalytically active isozyme forms, and some of the smaller molecular weight forms have been shown to be proteolytic degradation fragments of larger forms (Grahame and Mayer,



1984; Asem et al., 1986). However, the origins of most isozyme forms observed have not been established. Although GTF-S and GTF-I have similar molecular weights and amino acid compositions (Ciardi et al., 1977; Shimamura et al., 1982; Furuta et al., 1985), recent genetic cloning experiments indicate that the enzymes represent different gene products (Pucci et al., 1987; Ueda, 1988). The GTF's are known to aggregate into high molecular weight forms consisting of ten or more enzyme molecules per aggregate (Germaine and Schachtele, 1976; Germaine et al., 1977; Luzio et al., 1982). Both dextran-dependent and dextran-independent aggregates have been observed, but only the dextran-independent aggregates are dissociable in 1.0 Molar KCl (Schachtele et al., 1976).

The GTF's are catalytically active in the acidic pH range, usually with an optimum around pH 5 - 6, although some preparations have been reported to have activity at pH values as high as 7.5 to 8.0. The  $K_m$  for sucrose in the presence of primer is generally in the range 1 - 10 mM, and half maximal primer dextran stimulation occurs at about 10 - 100 mg/l (1 - 10  $\mu$ M for a 10,000 M.W. primer). There are no known metal ion or cofactor requirements for the oral streptococcal GTF's.

The only naturally occurring substrate that will donate glucosyl residues to primer dextran is sucrose. However, chemically synthesized  $\alpha$ -D-glucopyranosyl fluoride,

which has a fluoride in place of the fructosyl moiety of sucrose, has been shown to serve as a substrate for primer dependent dextran synthesis, and to have a  $K_m$  similar to that of sucrose (Figures and Edwards, 1976). Also, the compounds 3-deoxysucrose and 3-deoxy-3-fluoro sucrose, in which the 3-hydroxyl group of the glucose moiety is replaced with hydrogen or fluorine, can donate single glucosyl residues to primer dextran, but cannot be used for synthesis of high molecular weight polymers. Also, the affinity of GTF for these compounds is an order of magnitude less than for sucrose (Binder and Robyt, 1986). This very limited number of known substrates indicates the high specificity of the sucrose-binding site for the natural substrate sucrose. In contrast, the acceptor binding site has a very broad specificity, and will bind a wide range of acceptor molecules, as discussed earlier, although the site does display a marked preference for high molecular weight dextran (Chludzinski et al., 1976; Fukui and Moriyama, 1983).

The binding of molecules in either binding site can inhibit GTF activity. Such sucrose analogs as  $\alpha$ -D-xylosyl- $\beta$ -D-fructofuranoside (Nisizawa et al., 1986),  $\alpha$ -D-glucopyranosyl fluoride in high concentrations (Figures and Edwards, 1976), fructose itself (Chludzinski et al., 1974), and the sucrose analogs modified at the 3-position with hydrogen or fluorine (Binder and Robyt, 1986), inhibit GTF by competing with sucrose for the sucrose-binding site. On

the other hand, inefficient acceptors, such as maltose and isomaltose, inhibit GTF by competing with dextran for the acceptor binding site. It has also been shown that  $\alpha$ -1,6-linked dextran that has been oxidized with periodic acid binds to the acceptor site irreversibly and inactivates GTF (Inoue and Smith, 1980). Most of these inhibitors, except for the oxidized dextran, display very weak inhibition, a reflection of the specificity of the binding sites for sucrose and high molecular weight dextran.

Although the GTF's from oral streptococci have many interspecies similarities, the enzymes from different species do differ enough to warrant a discussion of the species separately.

#### G. Characteristics of Oral Streptococcal GTF's from Different Species

The GTF's from the oral streptococcal species S. mutans, S. sobrinus (the species studied in this dissertation), and S. sanguis have been the most intensively studied streptococcal GTF's. Since the taxonomy of these organisms has changed recently, it is necessary to understand how the species are now classified.

The most highly cariogenic organisms were once grouped into the single species S. mutans, and strains were identified by serotype according to a scheme originally proposed by Bratthall (1970). However, this "species" has now been divided into five species, four of which are found in humans

(Coykendall, 1977). Two of these species--S. mutans, comprising serotypes c, e, and f, and S. sobrinus, comprising serotypes d and g--are isolated regularly from humans in about a 4 to 1 ratio of mutans to sobrinus (Loesche, 1986) and have relatively well-studied GTF's. The other species are isolated only rarely. Although many authors still use the serotype system, it has been argued that the four-species system is preferable for use in caries research studies, due to differences in cariogenic activity between species (Loesche, 1986).

Another common oral bacterium whose GTF has been relatively well-studied is S. sanguis.

#### 1. The GTF's of S. sobrinus

S. sobrinus produces both GTF-S and GTF-I enzymes. The GTF-S synthesizes, from sucrose alone, a dextran product consisting of about a 2 to 1 ratio of  $\alpha$ -1,6 to  $\alpha$ -1,3 bonds (Shimamura et al., 1982; Furuta et al., 1985). As discussed earlier, GTF-I enzymes from oral streptococci generally synthesize 100% linear  $\alpha$ -1,3-linked polymers from sucrose. Although the S. sobrinus GTF-I will not synthesize high molecular weight products in the absence of primer (Fukui et al., 1982; Hanada and Takehara, 1987), the enzyme has been reported to synthesize mainly  $\alpha$ -1,3 bonds when primer dextran is present (Fukui et al., 1982). Although both enzymes are highly primer dependent, no study designed to determine

the structures of the branches added to primer dextran has been reported in the literature for either enzyme.

GTF-S has been reported to have two isozymes of pI 3.9 and 4.1 on isoelectric focusing, while GTF-I has one band at pI 4.9 (Tsumori et al., 1983a). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) also shows two isozyme bands for GTF-S (Germaine et al., 1977; McCabe, 1985). On SDS PAGE, GTF-I has been reported to have 1 band (Fukui et al., 1982) and 3 bands (McCabe, 1985). Reports of single bands of GTF-S (Koga et al., 1983; Furuta et al., 1985) and GTF-I (Fukui et al., 1982) are probably due to poor resolution on gels or inability of the staining methods to detect minor bands. All the GTF forms have molecular weights in the range 140 - 180 thousand.

A second GTF-S has been reported (Shimamura et al., 1983; McCabe, 1985; Hanada et al., 1987), which is found in culture media only when the bacteria are grown in the presence of the nonionic detergent Tween 80. In contrast to most other GTF's reported, this enzyme is not stimulated by primer dextran. The product synthesized by this enzyme is a nearly linear dextran with very little branching, and is, therefore, a better primer of the other GTF's than the product synthesized de novo from sucrose by the primer-dependent GTF-S. This observation has led to the proposal that the primer-independent GTF-S may function in dental

plaque synthesis by providing efficient primers for the primer-dependent GTF's (McCabe, 1985).

The GTF-I and primer-stimulated GTF-S of S. sobrinus 6715-7 are the ones purified and studied in this dissertation. Therefore, in all subsequent discussions, unless otherwise stated, the term "S. sobrinus GTF-S" will be used to refer only to the primer-stimulated enzyme.

## 2. The GTF's of S. mutans

It is not entirely clear exactly how many forms of GTF are produced by S. mutans. Two major GTF-S forms have been described, with pI values of 7.5 (Kuramitsu and Wondrack, 1983; Asem et al., 1986; Baba et al., 1986) and 8.1 - 8.4 (Mukasa et al., 1982). The molecular weights are in the range 140 - 160 thousand, and both enzymes have isozyme forms. Both GTF-S enzymes synthesize a typical, highly branched soluble dextran product in dilute buffer. However, in the presence of protein aggregation agents, such as ammonium sulfate or polyethylene glycol, the pI 7.5 enzyme can increase the proportion of  $\alpha$ -1,3 bonds synthesized enough to make the product insoluble (Asem et al., 1986; Baba et al., 1986). However, this insoluble product contains only branched  $\alpha$ -1,3 linkages, with no linear  $\alpha$ -1,3 linkages characteristic of GTF-I products.

Two GTF-I forms from S. mutans have also been described. One has a pI of 4 - 5 and a molecular weight of 140 - 150 thousand (Kenny and Cole, 1983; Kuramitsu and

Wondrack, 1983). The other has a pI of 8.5 and a molecular weight of 99,000 (Mukasa et al., 1985). This latter enzyme may be a proteolytic degradation fragment of a larger form, and synthesizes from sucrose the typical linear,  $\alpha$ -1,3-linked mutan characteristic of GTF-I.

The enzymes are primer stimulated, but can also synthesize significant amounts of products in the absence of primer dextran. Because of the similarities in physical properties of many of the isozymes, the ability of various forms to aggregate, and the tendency of all the forms to undergo proteolytic degradation in culture, complete purification of the various forms has proved very difficult, and there is no general agreement about the number of S. mutans GTF's that exist or their physical properties.

### 3. The GTF's of S. sanguis

Compared to the complex GTF profile of S. mutans, that of S. sanguis is relatively simple. S. sanguis produces only GTF-S, and no GTF-I (Mayer, 1987). Two fully active isozymes are produced, one of which is a proteolytic degradation fragment of the other (Grahame and Mayer, 1984, 1985). Although the rate of dextran synthesis increases markedly in the presence of primer dextran, the enzyme can synthesize a dextran product from sucrose alone consisting of about a 2.5 to 1 ratio of  $\alpha$ -1,6 to  $\alpha$ -1,3 bonds (Huang et al., 1979; Grahame and Mayer, 1985), similar to the highly-branched GTF-S products of other oral streptococci.

#### 4. Other Oral Streptococcal GTF's

GTF-S and GTF-I enzymes have also been isolated from the species S. salivarius (Wittenberger et al., 1983) and S. cricetus (Tsumori et al., 1985a, 1985b). From the limited amount of work that has been done, it appears that the enzymes from these species are, in general, similar to those of the other oral streptococci with respect to catalytic properties and structures of the glucan products synthesized.

#### H. Purification of the GTF's

In order to study the physical and catalytic properties of the GTF's, many investigators have developed purification schemes for the enzymes. The large number of purifications attempted have been reviewed (Montville et al., 1978; Ciardi, 1983), and many other schemes have been reported since then. However, no completely satisfactory method has ever been reported, despite the use of many purification methods, such as ammonium sulfate precipitation (Chludzinski et al., 1974; Koga et al., 1983), ethanol precipitation (Shimamura et al., 1982; Mukasa et al., 1985), ultrafiltration (Ciardi et al., 1977; Tsumori et al., 1985a), gel filtration (Kuramitsu, 1975; Germaine et al., 1977), ion-exchange chromatography (Germaine et al., 1977; Baba et al., 1986; Kumada et al., 1987), chromatofocusing (Furuta et al., 1985; Kametaka et al., 1987b), hydroxyl-apatite chromatography (Chludzinski et al., 1974; Asem



et al., 1986), hydrophobic chromatography (McCabe and Smith, 1977; Furuta et al., 1985), and preparative isoelectric focusing (Chludzinski et al., 1974; Mukasa et al., 1985). The most successful schemes reported have involved affinity chromatography on Sephadex resins, which bind GTF's because the beads are made of cross-linked dextran (Russell, 1979; McCabe, 1985; Mooser et al., 1985). The reason for this large number of purification schemes is that none of them is entirely adequate. Due to this extensive literature, it is impractical to describe the strengths and weaknesses of each method that has been developed.

However, it can be stated that, in general, all the purification schemes developed thus far have one or more of the following weaknesses. Some of the purified GTF's simply contain unacceptable quantities of contaminating proteins and enzyme activities, or fail to separate the different forms of GTF from one another. Others require a large number of purification steps to obtain sufficiently purified enzymes, and generate very low yields. Some schemes recover only one form of GTF, and, therefore, comparisons between the different forms of GTF cannot be made. Another problem is that the purified enzyme preparations often contain dextran, which makes the enzymes difficult to separate due to dextran-dependent aggregation, and which complicates the interpretation of kinetic data, since dextran is a GTF substrate. Dextran contamination can be introduced in two

different ways. First, some common bacterial culture media that are used to grow oral streptococci contain traces of sucrose, which GTF can polymerize into dextran during bacterial growth. Second, dextran has been used as an eluting agent in affinity chromatography (McCabe and Smith, 1977; Figures and Edwards, 1979). Once dextran is present in a GTF preparation, it is almost impossible to remove, due to its high molecular weight and its GTF-binding capacity. Even the most successful affinity chromatography schemes have not proven to be completely acceptable, particularly because guanidine hydrochloride, which completely denatures proteins, has been used to remove GTF's from affinity columns. For enzymes as large as the GTF's (M.W. greater than 140,000), there is no guarantee that the native enzyme structure is completely regained when the guanidine hydrochloride is dialyzed away and the enzymes renature.

Thus, because of the lack of sufficiently purified GTF preparations, some important aspects of GTF enzymatic activity have not been adequately studied. It will be shown in the RESULTS and DISCUSSION sections how the purification scheme proposed in this dissertation for the S. sobrinus GTF's solves many of the problems associated with other schemes reported in the literature.

#### I. Experimental Determination of Dextran Structures

Because of the presence of streptococcal dextrans in dental plaque, several studies have been done to elucidate

the structures of these polysaccharides. Carbohydrate analysis techniques similar to those used to determine the structures of other large polysaccharides have been applied to the study of streptococcal dextrans. Five of the major parameters of interest in describing dextran structure are:

1. The identity of the polymerized sugar residues;
2. The anomeric configurations of glycosidic linkages;
3. The percentages of different interresidue linkage types present;
4. The distribution of branches along the polymer backbone; and
5. The lengths of the branches.

Studies of these parameters have revealed that dextrans synthesized by GTF-containing culture broths from Leuconostoc and Streptococcus have generally similar characteristics, except that the percentages of different linkage types present vary widely with the individual bacterial strain.

Early studies of dextran structure using complete or partial acid hydrolysis techniques, including a more specific hydrolysis technique known as acetolysis, demonstrated that the dextran synthesized by culture broths from Leuconostoc and Streptococcus strains consist exclusively of glucose residues, possibly with traces of fructose, and that the residues are all of the  $\alpha$  configuration (Suzuki and

Hehre, 1964; Newbrun, 1972). Furthermore, the  $\alpha$ -1,6-linkage was found to be predominant in the dextrans studied.

Information about the types of linkages present was obtained not only from the hydrolysis studies, but from early studies using periodate oxidation of dextrans (Rankin and Jeanes, 1954; Sidebotham et al., 1971). This technique is based on the fact that, when polysaccharides react with periodic acid, the oxidation products obtained depend upon the linkage types present in the molecule. Although periodate oxidation was convenient for screening large numbers of dextrans for their approximate content of different linkage types, the results obtained with this method are rarely unequivocal (Aspinall, 1982). Dextrans have also been analyzed by a related technique called Smith degradation (Goldstein et al., 1965), that is, reduction of the aldehydes in periodate-oxidized dextrans with sodium borohydride, followed by hydrolysis and analysis of the hydrolysis products (Guggenheim, 1970; Bourne et al., 1972). These techniques, as well acid hydrolysis data, indicated that dextrans could contain essentially any combination of  $\alpha$ -1,2,  $\alpha$ -1,3, and  $\alpha$ -1,4 linkages, along with the predominant  $\alpha$ -1,6. However, Smith degradation is an experimentally difficult technique which can involve unwanted side reactions that make the data difficult to interpret (Aspinall, 1982).

A more specific technique for the determination of linkage types is methylation analysis (Hakomori, 1964; Lindberg, 1972). This technique involves methylation of free hydroxyl groups in polysaccharides with methyl iodide or methyl sulfate, followed by complete (or, more rarely, partial) depolymerization and quantitative analysis of the resulting methylated sugar derivatives, which are now most often determined by gas-liquid chromatography and mass spectrometry (GLC-MS). Methylation analysis reveals which hydroxyl groups of the sugar residues are involved in glycosidic linkages, since these groups are not methylated. Although methylation cannot distinguish between  $\alpha$  and  $\beta$  anomeric configurations, the exact percentages of each linkage type in the original polymer can theoretically be determined. Methylation analysis confirmed that Leuconostoc dextrans contain various combinations of all possible linkage types (Sidebotham, 1974; Jeanes and Seymour, 1979). However, all streptococcal dextrans studied by methylation were shown to contain exclusively  $\alpha$ -1,3 and  $\alpha$ -1,6 glycosidic linkages (Baird and Ellwood, 1972; Ceska, 1972; Trautner, 1982).

These studies demonstrated that the  $\alpha$ -1,6-linked backbone of dextran contains branches of various linkage types. Investigations designed to determine the lengths of the branches have been performed by a variety of techniques, such as analysis of physical properties of dextran

solutions, immunochemical reactions of dextrans, and analysis of acid hydrolysis and acetolysis products of native dextrans. Also, dextran modification techniques have been developed in which the glycosidic bonds of sugar residues are stabilized to acid hydrolysis by converting the carbon-6  $\text{CH}_2\text{OH}$  groups to carboxyl groups or p-toluenesulfonate esters (Abbot et al., 1966; Rees et al., 1969). Since the modified 6-carbon groups lie at the non-reducing termini of branches, analysis of acid degradation products of these modified dextrans gives an indication of branch length. Such analyses have indicated that most dextran branches consist of a single glucosyl residue, especially in highly branched dextrans (Sidebotham, 1974; Kenne and Lindberg, 1982), although longer branches have been shown to be present in some more nearly linear dextrans (Larm et al., 1971; Taylor et al., 1985). Effective techniques for studying the distribution of the branches along the backbone remain to be developed, so that such distribution patterns have not been conclusively determined (Kenne and Lindberg, 1982).

One potentially informative technique for determining polysaccharide structures is enzyme-catalyzed degradation, followed by analysis of the resulting oligosaccharide products. Such techniques have been used with great success in analyzing the structures of such molecules as amylose and glycogen (Whelan, 1971). However, not nearly as much information has been obtained by this technique for dextrans, due

to the limited number of known dextran-degrading enzymes and the limited ability of these enzymes to hydrolyze highly branched dextrans. Enzymatic techniques for the  $\alpha$ -1,6,  $\alpha$ -1,3 system, such as that of streptococcal dextrans, are still in the developmental stage (Taylor et al., 1985).

A very powerful technique for dextran analysis is NMR spectroscopy. The characteristics of proton NMR spectra of dextrans have been described (Pasika and Cragg, 1963; Meyer et al., 1978). The chemical shifts of anomeric protons are downfield of those for the rest of the molecule, and the shifts observed are dependent upon the type of linkage in which the anomeric carbon is bound. Proton NMR has not, however, been extensively used in dextran analysis because the linkage-dependent chemical shifts in the anomeric region of the spectrum are not very widely separated, and the HOD peak can cause interference. Two-dimensional NMR could improve the resolution achievable, but analyses using this technique have been limited, thus far, to oligosaccharides up to about eight residues in length (Bax et al., 1984).

Carbon-13 NMR spectra of dextrans have been much more thoroughly analyzed because the chemical shift differences observed are much larger, and linkage dependent shifts are observed both for the anomeric carbon and for the nonanomeric carbons involved in glycosidic linkages. The details of C-13 NMR spectra of dextrans will be discussed thoroughly later. In general, C-13 NMR spectra have confirmed earlier

methylation and other data regarding the structures of dextrans synthesized by Leuconstoc and Streptococcus culture broths.

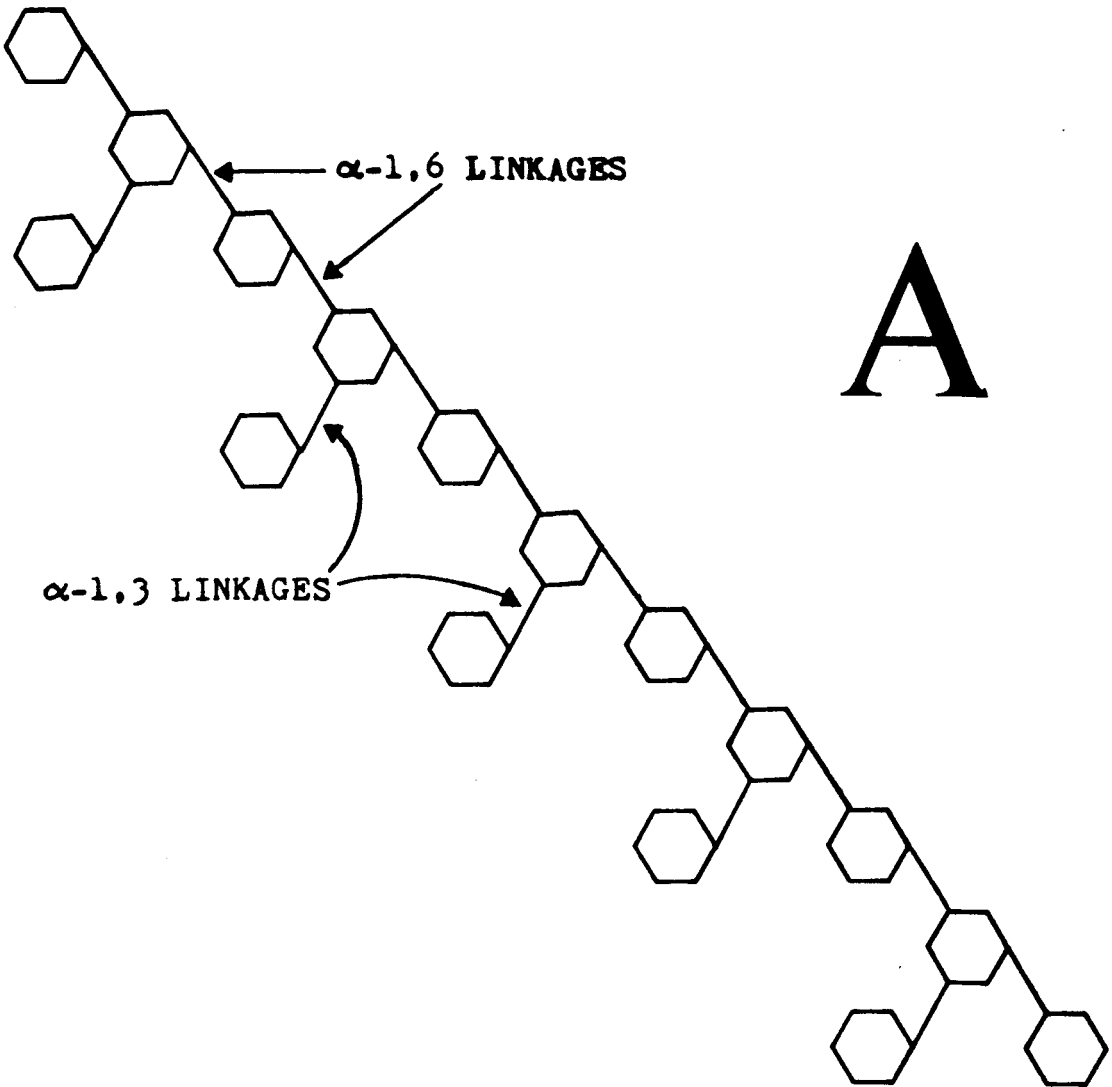
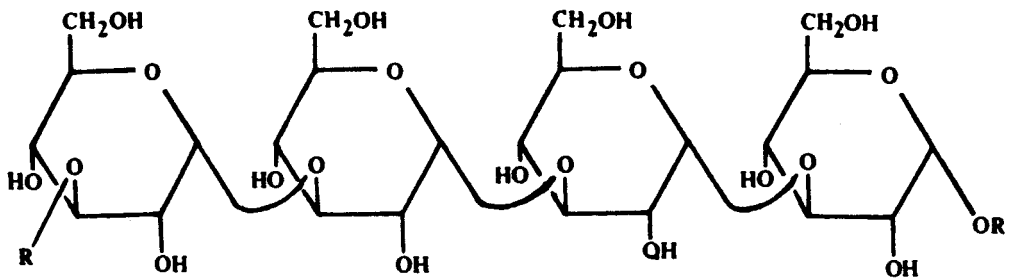
The techniques described have established many important features of dextran structure. In summary, dextrans contain stretches of  $\alpha$ -1,6-linked glucose homopolymer, which can occasionally be interspersed with linear  $\alpha$ -1,3 residues. The  $\alpha$ -1,6 backbone invariably contains branches, most of which are simply a single glucosyl residue. The structures are, therefore, probably "comb"-like, with the single glucosyl branches representing the "teeth" of the comb (Kenne and Lindberg, 1982). Depending on the dextran being studied, there can be many branches that are close together, or fewer branches that are farther apart. Figure 4A shows the proposed "comb" structure of the de novo product synthesized by S. sobrinus GTF-S in the absence of primer dextran (Robyt, 1983). This particular structure contains a branch on every other glucose residue in the backbone.

J. Dextran Structures Synthesized by GTF-I and GTF-S of S. sobrinus

All of the dextrans analyzed in the studies referred to above were synthesized from sucrose by the crude culture broths in which the bacterial cells had grown. However, especially for the streptococci, the concerted action of more than one enzyme is responsible for the final dextran structures synthesized. Therefore, attempts have been made



Figure 4. Proposed structures of glucan products synthesized by GTF-S and GTF-I de novo from sucrose. A: "Comb" dextran structure (Robyt, 1983) synthesized by S. sobrinus 6715 GTF-S in the absence of primer dextran. The hexagons represent glucose residues, and a branch is attached to every other glucose residue in the backbone. The  $\alpha$ -1,3 branch residues represent the "teeth" of the comb. B: Structure of the linear,  $\alpha$ -1,3-linked "mutan" synthesized by the GTF-I enzymes of the oral streptococci in the absence of primer dextran. The symbol "R" represents the rest of the chain, which extends in both directions.

**B**

to study the structures of the products synthesized by the individual enzymes. Although the lack of sufficiently purified enzyme preparations has hampered these efforts, enough information has been obtained to draw some conclusions.

As discussed earlier, GTF-I enzymes from streptococci have been reported to synthesize from sucrose a 100% linear,  $\alpha$ -1,3-linked "mutan" structure (Figure 4B). However, since GTF-I from S. sobrinus will not synthesize large polymers in the absence of primer dextran, reaction mixtures from which GTF-I products are obtained for structural studies must contain primer dextran. Although the S. sobrinus GTF-I enzyme synthesizes mainly  $\alpha$ -1,3 bonds under these conditions (Fukui et al., 1982), it has not been quantitatively demonstrated in the literature that only  $\alpha$ -1,3 bonds are synthesized.

The S. sobrinus GTF-S product synthesized de novo from sucrose has been studied by methylation analysis (Shimamura et al., 1982; Furuta et al., 1985). These studies demonstrate that the product contains approximately a 2 to 1 ratio of  $\alpha$ -1,6 to  $\alpha$ -1,3 linkages, and that all the  $\alpha$ -1,3 bonds are contained in branched 1,3,6-linked glucose residues. A structure consistent with this data has been proposed by Robyt (1983) (Figure 4A). As indicated in the figure, the product probably has a comb dextran structure, like most other known dextrans, with a single  $\alpha$ -1,3-linked

glucosyl group on approximately every other residue in the backbone. Although this enzyme is known to be stimulated by primer dextran, no study designed to determine the structure of the branches added to the primer has been reported in the literature. Also, no study designed to assess the contribution of the sucrose-independent branching activity to the overall formation of  $\alpha$ -1,3 bonds by GTF-S has been reported.

Most of the analyses of the GTF-synthesized product structures have been done using methylation analysis. However, methylation is a time-consuming and labor-intensive process that is not well suited to analyzing large numbers of samples. On the other hand, C-13 NMR analysis of dextran structure is a relatively rapid technique, which has the potential to yield information similar to that of methylation analysis. This technique could, therefore, be used to study the modification of primer dextran by the GTF enzymes, especially with regard to the structures of the branches added to the primer.

#### K. The Analysis of Dextran Structure by C-13 NMR

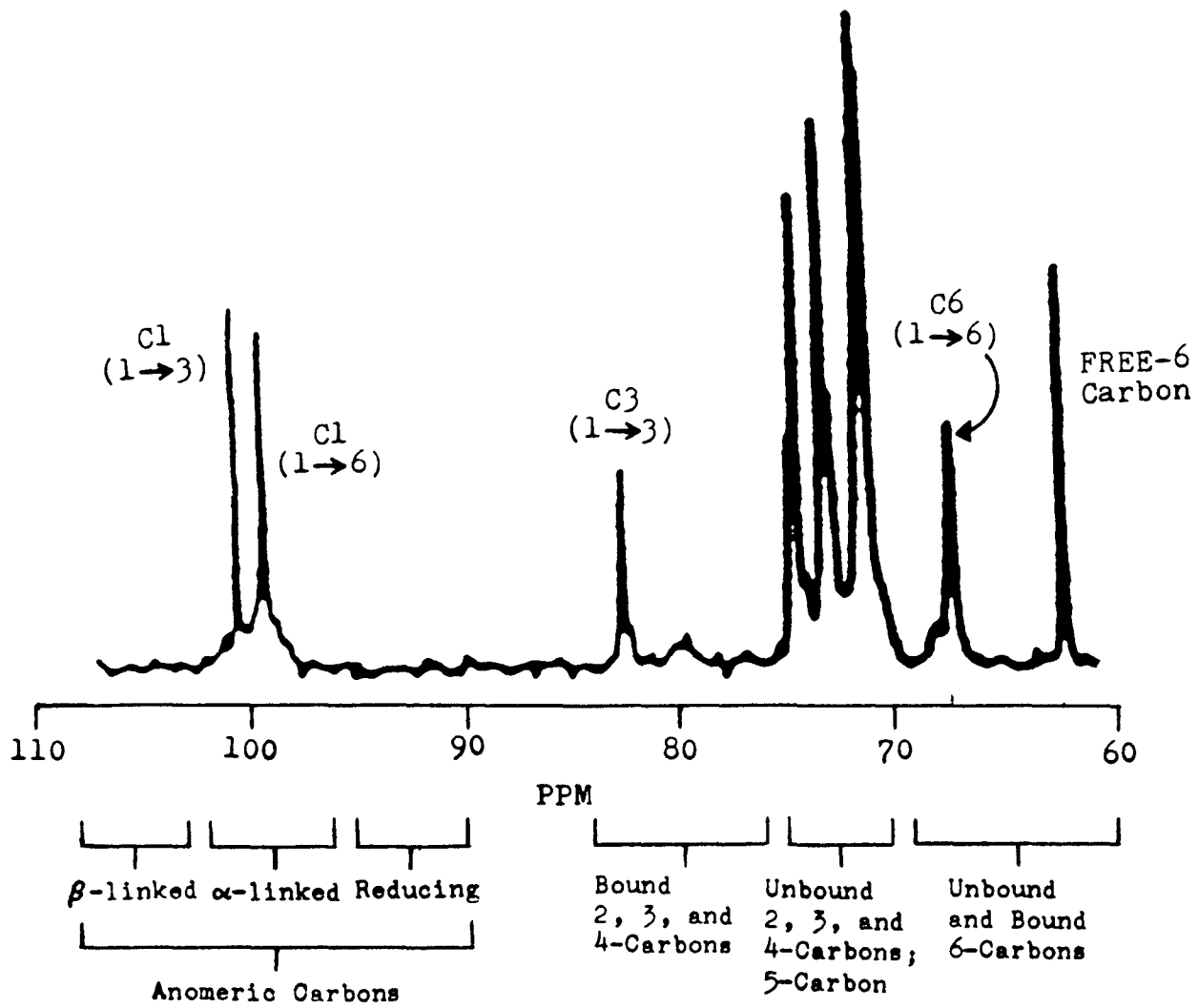
By far, the most extensive study of the C-13 NMR spectra of dextrans has been performed by F.R. Seymour and associates using dextrans from the species Leuconostoc mesenteroides (Seymour et al., 1976; Seymour, 1979; Seymour et al., 1979b, 1979c). By comparing the spectra of several dextrans that had already been studied by methylation analysis, these investigators showed that the chemical shifts of

carbon species in dextrans were similar to the shifts of the corresponding carbons in simple sugar analogs, which had been analyzed previously (Perlin et al., 1970; Dorman and Roberts, 1971).

The spectral region containing the dextran resonances is shown in Figure 5, along with the spectrum (from Seymour et al., 1979b) of an *L. mesenteroides* dextran containing both  $\alpha$ -1,3 and  $\alpha$ -1,6 linkages. The peaks due to anomeric carbons (90 - 110 ppm) are shifted more than 10 ppm downfield of the peaks from the other carbons. Peaks due to  $\beta$ -linked,  $\alpha$ -linked, and reducing anomeric carbons can be distinguished, although only  $\alpha$ -linked peaks are observed in dextran spectra. The very small number of reducing ends in dextran cannot be detected above the spectral noise. The bound 2-, 3-, and 4-carbon peaks at 76 - 84 ppm are also shifted downfield of the unbound ring carbon peaks (70 - 75 ppm), although not as dramatically as the anomeric region. The bound and unbound 6-carbon peaks (60 - 69 ppm) are upfield of the unbound ring carbon peaks.

The carbon atoms involved in different types of linkages in dextran show peaks at characteristic ppm values within each of these regions. The chemical shifts associated with various linkage types were found to be very similar ( $\pm$  about 0.2 ppm) in all the dextrans studied. Characteristic chemical shifts at 70°C for various carbon species in dextran, as calculated from the data of the

Figure 5. C-13 NMR spectrum, at 90°C, of a dextran fraction synthesized by L. mesenteroides B-742 culture supernatant. The spectrum is from Seymour, et al., 1979b. This dextran contains exclusively  $\alpha$ -1,3 and  $\alpha$ -1,6 glycosidic linkages. The regions of C-13 NMR spectra into which the chemical shifts of various carbon species in dextrans fall are indicated at the bottom of the figure.



Seymour group, are tabulated in Table 1. The temperature 70°C is specified for three reasons: the chemical shifts of all the peaks show a temperature dependence; the resolution and sensitivity increase markedly at higher temperatures (Seymour et al., 1979b); and most of the spectra reported in this dissertation were, therefore, run at 70°C. As shown in the table,  $\alpha$ -1,2,  $\alpha$ -1,3, and  $\alpha$ -1,4 linkages can be easily distinguished by their characteristic chemical shifts in the 76 - 84 ppm and 96 - 102 ppm regions. Furthermore, peaks due to bound and free 6-carbons, in the 60 - 69 ppm region, are well-separated. Peaks in the 70 - 75 ppm region are closely spaced, and do not provide particularly useful information about the types of linkages present.

The spectrum of the L. mesenteroides dextran shown in Figure 5 was run at 90°C, so that its chemical shifts are similar to those recorded in Table 1. The spectrum shows  $\alpha$ -1,3 and  $\alpha$ -1,6-linked anomeric carbon peaks at 100.9 and 99.6 ppm respectively. An  $\alpha$ -1,3-linked 3-carbon peak is visible at 82.9 ppm, and the  $\alpha$ -1,6-linked 6-carbon peak appears at 67.7 ppm. The free-6-carbon peak is at 62.5 ppm. Therefore, this dextran can be characterized as containing both  $\alpha$ -1,6 and  $\alpha$ -1,3 linkages.

Although the peak areas in C-13 NMR spectra are, in general, not proportional to the number of carbon atoms giving rise to the signal, it has been determined in C-13 NMR studies of dextrans (Usui et al., 1975; Colson et al.,



TABLE 1. Positions of Peaks in C-13 NMR Spectra of Dextrans

Carbon Species	Chemical Shift <sup>b</sup> at 70°C
β-Anomeric Carbons	103 - 108
α-Anomeric Carbons	
1,6-linked	99.4
1,3-linked	100.8
1,2-linked	97.0 and 97.9
1,4-linked	101.5
6-Carbon α-1,6	67.3
3-Carbon α-1,3	82.6
2-Carbon α-1,2	77.2
4-Carbon α-1,4	80.0
Free-6-Carbons	62.1

<sup>a</sup>Characteristic values calculated from the data of Seymour et al., 1976, 1979c. Values for β-Anomeric Carbons are from Dorman and Roberts, 1971 and Seymour, 1979.

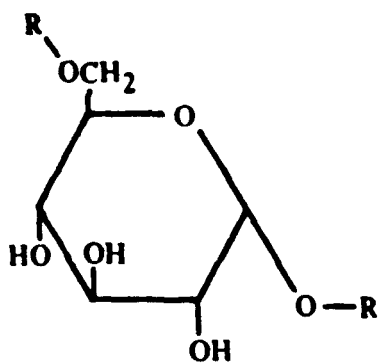
<sup>b</sup>PPM values relative to tetramethylsilane (TMS).

1979; Seymour et al., 1979c) that the proportions of linkage types calculated from peak areas in dextran spectra are, in general, very close to those determined by methylation analysis. This finding is understandable, since the carbon atoms in dextrans relax very efficiently and have very short spin-lattice ( $T_1$ ) relaxation times, on the order of 0.1 - 0.25 sec (Seymour et al., 1979a). The validity of using C-13 NMR peak areas to estimate the proportions of carbon species present in dextrans will be more thoroughly discussed in later sections. Thus, C-13 NMR spectra can determine not only the linkage types present in dextrans, but also the proportions of these linkage types present.

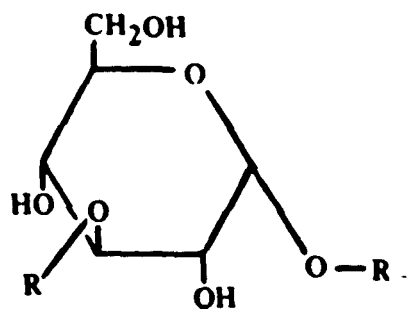
For the oral streptococcal,  $\alpha$ -1,6 and  $\alpha$ -1,3-linked dextrans, which are of interest in the present research, there are four main types of glucose residues possible in the polymers (Figure 6). These residue types are present in the glucan structures synthesized by GTF-I and GTF-S (Figure 4). Each of the residues shown in Figure 6 has its own characteristics in C-13 NMR spectra, as follows.

Linear  $\alpha$ -1,6 residues (Figure 6A). These are the backbone residues of dextrans. The anomeric carbon is bound to the 6-hydroxyl group of the next residue, and the 6-carbon hydroxyl is bound to the anomeric carbon of the preceding residue. Linear 1,6 groups, therefore, give a C1(1 $\rightarrow$ 6) peak and a C6(1 $\rightarrow$ 6) peak in C-13 NMR spectra.

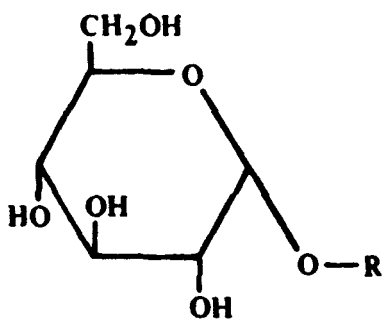
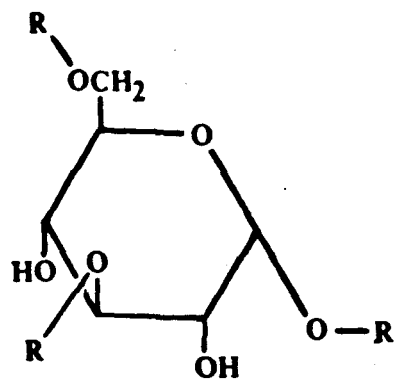
Figure 6. The four major types of glucose residues that can exist in oral streptococcal dextrans. Each of these residue types gives rise to characteristic peaks in C-13 NMR spectra, as discussed in the text.



A. LINEAR 1,6



B. LINEAR 1,3

C. NON-REDUCING  
ENDD. 1,3 BRANCH  
POINT

Linear  $\alpha$ -1,3 residues (Figure 6B). These are the residues characteristic of the insoluble mutan synthesized by GTF-I. The anomeric carbon is bound to the 3-hydroxyl group of the next residue, and the 3-carbon hydroxyl is bound to the anomeric carbon of the preceding residue. Linear 1,3 groups, therefore, give a C1(1 $\rightarrow$ 3) peak and a C3(1 $\rightarrow$ 3) peak in spectra.

Non-reducing end, 1,6-linked (Figure 6C). These groups represent the non-reducing ends of sections of dextran backbone, but since dextran is such a large molecule, very few of these residues exist. They show a C1(1 $\rightarrow$ 6) peak and a free-6-carbon peak in spectra.

Non-reducing end, 1,3-linked (Figure 6C). These non-reducing ends are much more numerous, and represent the branches consisting of single glucose residues in the comb dextran structure. They show a C1(1 $\rightarrow$ 3) peak and a free-6-carbon peak in spectra.

Branched 1,3 residues (Figure 6D). These residues are dextran backbone residues to which a branch is attached in the comb dextran structure. These residues display a C1(1 $\rightarrow$ 6) peak, a C3(1 $\rightarrow$ 3) peak, and a C6(1 $\rightarrow$ 6) peak in spectra.

These considerations lead directly to predictions about the spectral characteristics that should be observed in spectra of the GTF-I mutan and GTF-S comb dextran. The linear 1,3 residues in the mutan should give prominent

C1(1→3) and C3(1→3) peaks. Furthermore, since each of these residues has a free-6-carbon, the ratio of the area of the free-6-carbon peak to that of either the C1(1→3) or C3(1→3) peak should be 1. The observation of a ratio of 1 would provide a good check on the validity of using the peak areas for quantitative purposes.

For the GTF-S comb dextran, the ratio of the peak areas of the  $\alpha$ -1,6 to  $\alpha$ -1,3 peaks should be 2 to 1. Furthermore, since each branched  $\alpha$ -1,3 residue contains a free-6-carbon, the area ratio of free-6 to  $\alpha$ -1,3 peaks should again be 1.

Thus, the data of the Seymour group, using other dextrans, provides a promising method for analyzing the structure of products synthesized by oral streptococcal GTF's. Particularly, the method can be used to study a major question about GTF product structure alluded to earlier, namely, how the GTF's modify primer dextran.

## CHAPTER III

### MATERIALS AND METHODS

#### A. Materials

##### 1. Growth of Bacteria

S. sobrinus 6715-7 (catalog number 27351) and S. mutans NCTC 10449 (catalog number 25175), which was grown as a source of fructosyltransferase, were obtained as lyophilized cultures from the American Type Culture Collection (ATCC). Mitis-Salivarius agar and Tryptose were from Difco, and Trypticase Peptone and yeast extract were from BBL. The glucose used in culture media was Mallinkrodt Analytical Reagent (AR) grade. Anaerobic gas mixture (85% N<sub>2</sub>, 10% CO<sub>2</sub>, 5% H<sub>2</sub>) was obtained from Union Carbide/Linde Division.

##### 2. Enzyme Assays and Liquid Scintillation

[U-<sup>14</sup>C]Sucrose was obtained from New England Nuclear (673 mCi/mmol) or Pathfinder Labs (Sigma, 522 mCi/mmol), and [1'-<sup>3</sup>H]fructose-sucrose was a product of New England Nuclear. Methanol used for washing in assays was Tissue-Tek grade, 99.8%, from American Scientific Products. PPO (2,5-diphenyloxazole) and POPOP (p-Bis[2-(5-phenyloxazolyl)]-Benzene) were from New England Nuclear. Toluene was Mallinkrodt ScintillAR grade. D-raffinose (O- $\alpha$ -D-galactopyranosyl-[1->6]- $\alpha$ -D-glucopyranosyl- $\beta$ -D-fructofuranoside) was obtained from Aldrich.

### 3. Chromatography Resins

DEAE-Sephacel and coarse Sephadex G-25 were products of Pharmacia. Bio-Gel A 0.5m (100 - 200 mesh) and Bio-Gel A 1.5m (200 - 400 mesh) were from Bio-Rad.

### 4. Gel Electrophoresis, Isoelectric Focusing, and Staining Reagents

The gels and buffer strips used in SDS PAGE, isoelectric focusing, and native PAGE were obtained from Pharmacia for use with the PhastSystem electrophoresis instrument. Coomassie blue R 350 was supplied by Pharmacia as PhastGel Blue R tablets. Bromphenol blue and basic fuschin were "Baker Analyzed" reagents from J. T. Baker Chemical Company. Glutaraldehyde was Sigma grade II, supplied as a 25% aqueous solution. Analytical grade sodium dodecyl sulfate (SDS) and research grade 2-mercaptoethanol were Ephortec electrophoresis chemicals from Haake-Buchler Instruments. In general, other electrophoresis and gel staining chemicals were obtained from commercial chemical suppliers and were reagent grade or better.

### 5. NMR Study

Ethanol (200 proof) for precipitation of dextran products was obtained from U.S. Industrial Chemicals Company.  $D_2O$  (99.8 atom% D) and NaOD (30 wt.% in  $D_2O$ , 99+ atom% D) were products of Sigma. NMR tubes, 10 mm diameter for high-field NMR instruments, were obtained from Wilmad Glass Company.



## 6. Miscellaneous Reagents

Sucrose and ammonium sulfate were Mallinckrodt AR grade. Dextran T fractions were obtained from Pharmacia. D-(+)-maltose monohydrate was a product of J. T. Baker Chemical Company. All water used in this study was filtered through a Millipore Milli-Q, 4-cartridge water purification system. Dialysis tubing (Spectra Por 2) was obtained from Spectrum Medical Industries, Inc., and PM-10 ultrafiltration membranes (10,000 M.W. cutoff) were from Amicon. All chemicals not specifically mentioned were reagent grade or better and were obtained from commercial sources.

### B. Methods

#### 1. Absorbance and pH Measurements

Absorbance measurements were performed on a Perkin-Elmer Model 320 spectrophotometer with spectrophotometer cells that had a 1 cm path length. Measurements of pH were performed on a Beckman Model 71 pH meter equipped with either a Broadley-James or an Orion Ross combination pH electrode.

#### 2. Growth of Bacteria

All bacterial culturing was done at 37°C in anaerobic jars (BBL) that had been evacuated and refilled three times with an anaerobic gas mixture (85% N<sub>2</sub>, 10% CO<sub>2</sub>, 5% H<sub>2</sub>). New cultures were started from the lyophilized ATCC bacterial stock each time a culture was needed. The liquid culture medium used was the TTY broth of Hamada and Torii (1978),

which consists of 15 g Trypticase Peptone, 4 g Tryptose, 4 g yeast extract, 2 g  $K_2HPO_4$ , 5 g  $KH_2PO_4$ , 2 g  $Na_2CO_3$ , 2 g NaCl, and 10 g glucose per liter of water. Glucose was autoclaved separately from the other components as a 5% solution. All autoclaving was at 18 lbs/in<sup>2</sup> pressure and 121°C for 20 minutes.

In the standard procedure for an enzyme purification, lyophilized S. sobrinus 6715-7 stock was streaked directly on Mitis-Salivarius agar, and the plates were allowed to grow for 3 days. S. sobrinus forms on this agar characteristic dark blue, hard colonies with rough edges. Colonies were picked and grown in 15 ml TTY broth for 24 hr. Liter flasks of TTY broth were inoculated with these 15 ml cultures (1.5% inoculum) and grown for 18 hr. After growth, all cultures were checked for identity and purity by gram-staining and by growth on Mitis-Salivarius agar. S. sobrinus cells are gram-positive cocci that grow in the "chains" characteristic of streptococci. They also display their characteristic colony morphology when grown on Mitis-Salivarius agar. Bacteria were removed from the TTY broth by centrifugation at 10,000xg and 4°C for 30 minutes, and the supernatant was used as the source of crude GTF's for the purification.

### 3. GTF Assay

GTF was assayed by the method of Germaine et al. (1974b). This assay is based on the incorporation of

[U-<sup>14</sup>C]glucose into methanol insoluble polysaccharide. At desired time points during the reaction, 10 or 20  $\mu$ l of the reaction mixture, which contained sucrose, dextran, and [U-<sup>14</sup>C]sucrose, was spotted on Whatman 3MM filter paper disks. These disks were washed 3 times in methanol for at least 15 minutes each time, using at least 10 ml of methanol per disk. The methanol washing removed fructose and unreacted sucrose, but left labeled polysaccharide on the disks. The disks were dried under a heat lamp and placed in scintillation vials for counting. Blanks containing the same reaction mixture without enzyme were spotted, washed, and counted, and their value was subtracted from that of the enzyme-containing samples. Disks that were spotted but not washed were counted to determine the total radioactivity in reaction mixtures.

This assay was used as described (Germaine et al., 1977) to determine what percentage of total polysaccharide synthesis was soluble or insoluble. After the reaction, aliquots were removed and spotted to determine total dextran synthesis. The reaction mixtures were centrifuged at 3700xg in a clinical centrifuge to pellet insoluble product. The supernatant was then spotted and counted to determine the percent soluble synthesis. The difference between the total synthesis and soluble synthesis yielded the percent insoluble synthesis.

Reaction mixtures used in our work consisted of buffer containing a GTF enzyme, dextran, sucrose, and enough [ $U-^{14}C$ ]sucrose to give 70,000 to 95,000 total CPM per 10  $\mu$ l of reaction mixture. The sucrose specific activity varied, but was always known, and was used to calculate the amount of GTF activity from the number of CPM observed in scintillation counting.

The reaction mixture to determine yields in the purification contained 50 mM sodium acetate buffer, pH 5.5, 20 mM sucrose, 10  $\mu$ M dextran T10, and 1.55 M ammonium sulfate, which was found to linearize enzyme activity. The usual reaction mixtures for kinetic measurements contained [ $U-^{14}C$ ]sucrose, varying concentrations of sucrose, dextran, and inhibitor, and 100 mM sodium acetate buffer, pH 5.5, for GTF-S, or 100 mM potassium phosphate buffer, pH 6.2, for GTF-I.

In order to standardize the amount of GTF enzyme added to reactions mixtures in this work, one unit of GTF activity was defined as the amount of enzyme that would incorporate 1  $\mu$ mole of glucose per ml reaction mixture into methanol insoluble glucan per minute. For GTF-I, the reaction mixture used to determine the unit activity was arbitrarily defined as 100 mM potassium phosphate buffer, pH 6.2, 10 mM sucrose, and 0.714  $\mu$ M dextran T70. For GTF-S, it was 100 mM sodium acetate buffer, pH 5.5, 20 mM sucrose, and 0.714  $\mu$ M dextran T70.

#### 4. Scintillation Counting

Scintillation counting was performed in an LKB 1211 MiniBeta liquid scintillation counter. The spotted, dried filter paper disks were placed in 20 ml glass scintillation vials (Kimble) filled with 10 ml of a scintillation solution that contained 4 g PPO and 0.1 g POPOP per liter of toluene. Since only ratios of CPM values were used for calculating GTF activity, no conversion to DPM was performed. For the same reason, no quench correction was applied, since the small amount of quenching observed was determined by the external standard ratio method to be identical for all samples. Samples were counted with the wide open C-14 window recommended by the manufacturer.

#### 5. Column Chromatography

For ion-exchange and affinity columns, to which large samples were applied, 2.6 cm diameter Pharmacia columns with flow adaptors were used. For gel filtration procedures with smaller sample sizes, 1.0 cm diameter Bio-Rad or Whatman columns were used. All chromatography steps were performed at room temperature except for the ion-exchange column, which was run at 4°C. Affinity columns were run at room temperature with fractions collected at 4°C.

Flow rate and fraction size were precisely controlled using a calibrated LKB 2132 Microperpex Peristaltic pump attached to an LKB 2112 RediRac fraction collector. Protein elution was detected with an LKB 2138 Uvicord S UV monitor

attached to an LKB 2210 2-channel recorder, or by measuring individual fractions for absorbance at 280 nm. GTF activity was detected in column fractions by the filter paper spotting assay.

DEAE-Sephacel was supplied preswollen. It was prepared for use by several cycles of mixing with 1.0 M sodium acetate buffer, pH 5.5, and decanting. The last time, it was poured into a column and equilibrated with 10 mM sodium acetate buffer by passing several column volumes over the resin. The salt gradient was formed with a home-made assembly consisting of two bottles connected at the bottom with tubing. The bottle from which solution was directly pumped contained 10 mM sodium acetate buffer, pH 5.5, and the other bottle contained the same buffer with 0.35 M KCl added. The concentration of KCl in column fractions was measured with an American Optical T/C Hand-Held Refractometer, Model 10431. After an ion-exchange run, the column was regenerated by successively passing over the column the 1.0 M and 10 mM sodium acetate buffers.

Bio-Gel A 0.5m and A 1.5m resins were supplied preswollen, and were prepared for use by mixing with the eluting buffer, pouring the beads into a column, and passing several column volumes of buffer over the column. Blue dextran (Sigma) was used to determine the void volume,  $V_0$ , of gel filtration columns.

## 6. Protein and Carbohydrate Determination

Protein concentrations were determined by the method of Lowry et al. (1951) with Bovine serum albumin as the standard.

Reducing sugar was determined by the method of Nelson (1944). Briefly, sample was mixed with an alkaline copper sulfate reagent and placed in a boiling water bath for 20 minutes. An arsenomolybdate reagent was added, the mixture was diluted, and absorbance was read at 500 nm.

Carbohydrates that could not be readily detected by the reducing sugar assay were determined by the phenol-sulfuric acid method, which detects most carbohydrates (DuBois et al., 1956). Phenol solution was added to the sample, followed by concentrated sulfuric acid. The absorbance of the resulting solution was measured at 490 nm.

## 7. Gel Electrophoresis

### a. Apparatus

All gels were run on the PhastSystem horizontal electrophoresis system produced by Pharmacia. All functions were microprocessor controlled by a program entered by the operator.

The separation unit consisted of a gel compartment with removable electrodes. Gels were precast on a plastic backing and had dimensions of 43 x 50 mm with varying thicknesses. Gel temperature was automatically controlled by heating and cooling units in the gel support bed. Buffer

compartments consisted of agarose buffer strips impregnated with various salts, and were in contact with both the electrodes and the gel during a run. Samples were drawn up into the controlled volume capillary tubes of a plastic sample applicator, and were applied near the cathode (for SDS and native PAGE) or at the center of the gel (for isoelectric focusing).

The development unit stained gels at a controlled temperature for each step. The chamber volume was 70 ml, and solutions were pumped in and out at desired times, according to the program entered by the operator. Stained gels were dried by leaving them in the open air overnight. Measurements of  $R_f$  values were performed on the screen of a slide viewer into which the dried gel had been inserted. Since wet gels photograph better, gels were rehydrated for photography.

All electrophoresis and staining was performed according to the manufacturer's instructions. These procedures were all PhastSystem adaptations of standard techniques.

b. SDS PAGE

SDS PAGE was performed by a modification of the method of Laemmli (1970) using a gradient separating gel. Protein solutions were prepared by heating at 100°C for 5 minutes in 10 mM Tris/HCl, pH 8.0, 1 mM EDTA, and 2.5% SDS, with or without 5.0% 2-mercaptoethanol. Bromphenol blue, 0.01%, was



added as a tracking dye. Gels contained a continuous 10 - 15% polyacrylamide gradient with 2% crosslinking, and a 4.5% polyacrylamide stacking gel region. Gel thickness was 0.45 mm. The gel buffer system was 0.112 M acetate and 0.112 M Tris, pH 6.4. The buffer strips contained 0.20 M tricine, 0.20 M Tris, and 0.55% SDS, pH 8.5. Gels were run at 250 V and 10.0 mA at 15°C until the tracking dye reached the anode.

c. Gel Isoelectric Focusing

Isoelectric focusing (IEF) was performed in pH 3-9 ampholyte-containing gels of 0.35 mm thickness. The gels were homogenous 5% polyacrylamide with 3% cross-linking. No buffer strips are required for polyacrylamide gel isoelectric focusing on the PhastSystem since the buffering capacity of the ampholytes in the gel is sufficient. Also, no special sample preparation is required, provided the sample contains only small quantities of salts or buffers. The gel was prefocused at 2000 V and 2.5 mA to develop the pH gradient, the sample was applied to the center of the gel, and focusing was continued at 2000 V and 2.5 mA at 15°C.

d. Native PAGE

Native PAGE was performed by a modification of the method of Davis (1964). No special sample preparation was required, but 0.01% bromphenol blue was added as a tracking dye. The 0.45 mm thick gel contained a continuous 8 - 25%

polyacrylamide gradient with 2% cross-linking, and a 4.5% polyacrylamide stacking gel region. The gel buffer system was 0.112 M acetate and 0.112 M Tris, pH 6.4. The buffer strips contained 0.88 M L-Alanine and 0.25 M Tris, pH 8.8. Gels were run at 400 V and 10.0 mA at 15°C until the tracking dye reached the anode.

e. Coomasie Blue Staining

IEF gels were fixed before staining with 20% trichloroacetic acid (TCA) at 20°C. All gel types were stained at 50°C with 0.02% Coomassie blue R 350 in 30% methanol/10% acetic acid, and destained in the same solution without dye. SDS and native PAGE gels were exposed before drying to a preserving solution containing 5% glycerol.

f. Silver Staining

Silver staining was performed by a modification of the method of Heukeshoven and Dernick (1985). IEF gels were first fixed with 20% TCA at 20°C. Gels were washed with ethanol/acetic acid/H<sub>2</sub>O and exposed to 8.3% glutaraldehyde at 50°C. After washes with ethanol/acetic acid/H<sub>2</sub>O and plain H<sub>2</sub>O, gels were exposed to silver nitrate, 0.25% for SDS PAGE and 0.5% for IEF, at 40°C. After washes with water, gels were developed with 0.04% formaldehyde in 2.5% sodium carbonate at 30°C. Development was stopped with 5% acetic acid at 50°C. SDS PAGE gels were soaked in 5% glycerol-containing preserving solution before drying.

g. Periodic Acid-Schiff (PAS) Stain for GTF Activity

Unstained SDS PAGE gels that had been washed with buffer and exposed to the GTF substrates sucrose and dextran T10 were PAS stained for regions of polysaccharide synthesis, which indicates GTF activity, by a modification of the method of Zacharius et al. (1969). Gels were exposed to 0.7% periodic acid in 5% acetic acid, washed with water, and soaked in an acidified 0.5% basic fuchsin solution containing 0.5% sodium metabisulfite. Destaining was with 50% methanol/30% acetic acid.

8. Assays for Contaminating Enzymes

Dextranase was assayed by the method of Hamelik and McCabe (1982). Enzyme sample was incubated at 37°C in 100 mM sodium acetate buffer, pH 5.0, containing 14 mg/ml dextran T2000. A blank containing no enzyme was treated similarly. At desired times, aliquots were removed and subjected to the Nelson (1944) reducing sugar assay, which yields a product in the presence of reducing sugar that absorbs at 500 nm. The commercial enzyme preparation used as a standard was Calbiochem Grade B bacterial dextranase.

Invertase was assayed by the method of McCabe and Smith (1983). Enzyme sample was incubated at 37°C in 30 mM sodium phosphate buffer, pH 6.8, containing 13.5 mM raffinose. A blank containing no enzyme was treated similarly. At desired times, aliquots were removed and subjected to the Nelson (1944) reducing sugar assay. The commercial enzyme

preparation used as a standard was Sigma Grade VII yeast invertase.

Fructosyltransferase, an enzyme that polymerizes the fructose moiety of sucrose into a polyfructose polymer, was assayed by the method of McCabe (1985). Enzyme sample was incubated at 37°C in 125 mM sodium phosphate buffer, pH 6.0, containing 24.3 mM sucrose and [1'-<sup>3</sup>H]fructose-sucrose. Activity was measured as the amount of [1-<sup>3</sup>H]fructose incorporated into methanol insoluble polysaccharide by the filter paper spotting assay. A blank containing no enzyme was treated similarly. Since no commercial enzyme preparation was available, an ammonium sulfate precipitate fraction from the culture supernatant of *S. mutans* NCTC 10449 was used as a standard.

#### 9. Synthesis of Products and Recording of Spectra for C-13 NMR

Dextran products for NMR spectra were synthesized in large-volume, GTF-containing reaction mixtures similar to the smaller mixtures used for determination of kinetic constants. Procedures for the synthesis of various dextran samples and preparation of the samples for spectral recording are described in the RESULTS section.

Natural abundance C-13 NMR spectra were recorded in Fourier Transform mode on a Bruker Aspect 3000, 300 MHz (75 MHz C-13) NMR instrument in the Chemistry Division of Argonne National Laboratory. Samples were dissolved in D<sub>2</sub>O

(or, for water-insoluble samples, D<sub>2</sub>O adjusted to pD 14 with NaOD) and placed in 10 mm diameter, high-field NMR tubes for recording of spectra. Routine C-13 NMR spectra were run with proton-noise decoupling, a 90° pulse width, and a five second pulse delay, using a computer program supplied by Bruker. Tubes containing sample solutions were spun at 20 Hz during spectral recording. Spectra were run using a deuterium lock signal, and the ppm values relative to tetramethylsilane (TMS) were calculated automatically using an external chloroform standard.

Spin-lattice (T<sub>1</sub>) relaxation times were measured by the inversion-recovery method using a Bruker computer program designed for this purpose. The procedure for measuring T<sub>1</sub> times is described in the RESULTS.

## CHAPTER IV

### RESULTS

#### A. Growth of Bacteria

Before the purification of the GTF enzymes could be attempted, it was necessary to choose a suitable culture medium for S. sobrinus, which is a fastidious organism that must be grown in a rich medium. The TTY broth of Hamada and Torii (1978), which contains Trypticase Peptone and Tryptose (protein sources), yeast extract (a source of vitamins, cofactors, and nucleotide bases), and glucose (a carbon source) was chosen for growing the bacteria. Of several media tested, TTY broth provided the most consistent bacterial growth and enzyme yield.

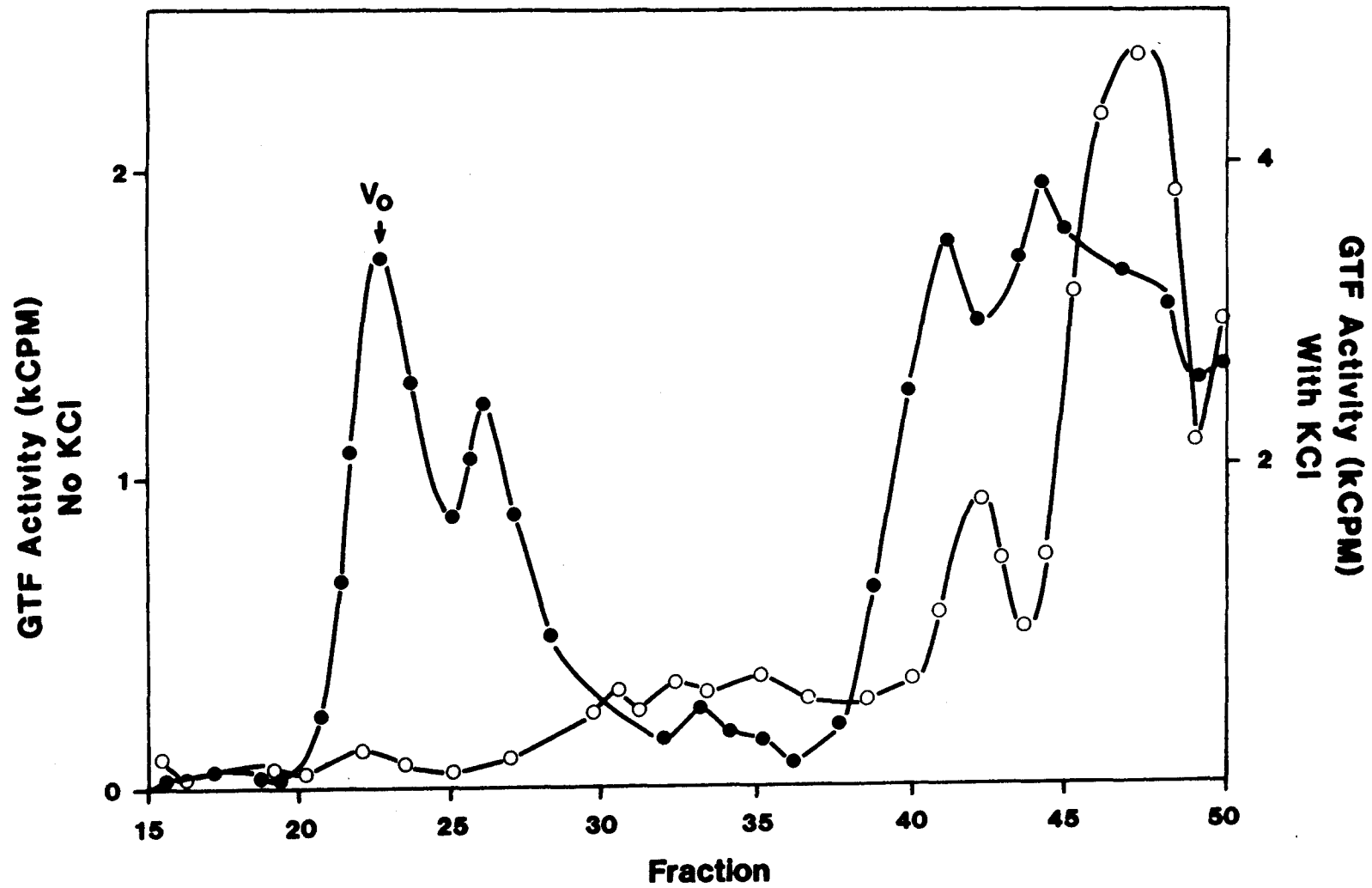
After growth of S. sobrinus in this medium for 18 hr. and removal of the cells by centrifugation at 4°C and 10,000xg for 30 minutes, the culture supernatant served as the source of crude enzyme. Because the pH of the culture medium decreased to about 4.7 during bacterial growth, due to accumulation of lactic acid in the culture medium, the pH was adjusted to 6.0 before any further purification steps. Also, due to the richness of the culture medium, 0.02% sodium azide ( $\text{NaN}_3$ ), which was determined in test experiments to have no effect on GTF activity, was added as a bacteriostatic agent.

Since obtaining a dextran-free GTF preparation was so critical, the GTF-containing culture supernatant was tested for the presence of dextran after bacterial growth. The most sensitive method of testing for dextran in GTF preparations is that of Schachtele et al. (1976). These investigators showed that when dextran is present in the preparation, GTF aggregates are not dissociable with 1.0 M KCl, but emerge in the void volume of gel filtration columns having a molecular weight cutoff of  $1.5 \times 10^6$ . When dextran is absent from the preparation, GTF aggregates are dissociable in the presence of 1.0 M KCl, and the GTF elutes after the void volume as low molecular weight material.

The results of gel filtration of a concentrated TTY culture supernatant in the presence and absence of 1.0 M KCl are shown in Figure 7. A Bio-Gel A 1.5m column (1 x 31 cm, 24.5 ml, M.W. cutoff  $1.5 \times 10^6$ ) was equilibrated with 10 mM potassium phosphate buffer, pH 6.0, containing 0.02%  $\text{NaN}_3$ . A sample (0.5 ml) of culture supernatant, which had been concentrated 18-fold through an Amicon PM-10 membrane, was applied and eluted with the same buffer at 4.0 ml/hr (5.1 cm/hr). Fractions of 0.5 ml were collected. When no KCl was present, about half the GTF activity eluted as high molecular weight aggregates in the void volume. When 1.0 M KCl was added to the concentrated culture supernatant and the buffer, essentially all the GTF dissociated and eluted as low molecular weight material.

Figure 7. Gel filtration of 18-fold concentrated culture supernatant on a Bio-Gel A 1.5m column. The column was 1 x 31 cm, 24.5 ml. The supernatant sample (0.5 ml) was eluted with 10 mM potassium phosphate buffer, pH 6.0, containing 0.02%  $\text{NaN}_3$ . The flow rate was 4.0 ml/hr (5.1 cm/hr), and 0.5 ml fractions were collected. (● - ●) GTF activity with no KCl; (○ - ○) GTF activity with 1.0 M KCl added to supernatant sample and buffer.





Therefore, since TTY broth produced good bacterial growth, consistent enzyme yield, and a dextran-free GTF preparation, as shown by the gel filtration result, this broth was used for all bacterial culturing.

#### B. GTF Assay

Another requirement for any successful enzyme purification is that there be available a rapid and sensitive assay that is linear with increasing enzyme concentration, so that yields from each of the purification steps may be accurately determined. The filter paper spotting GTF assay method of Germaine et al. (1974b), which is based on the incorporation of [U-<sup>14</sup>C]glucose from [U-<sup>14</sup>C]sucrose into methanol-insoluble polysaccharide, as described in MATERIALS AND METHODS, was found to be effective except for two problems.

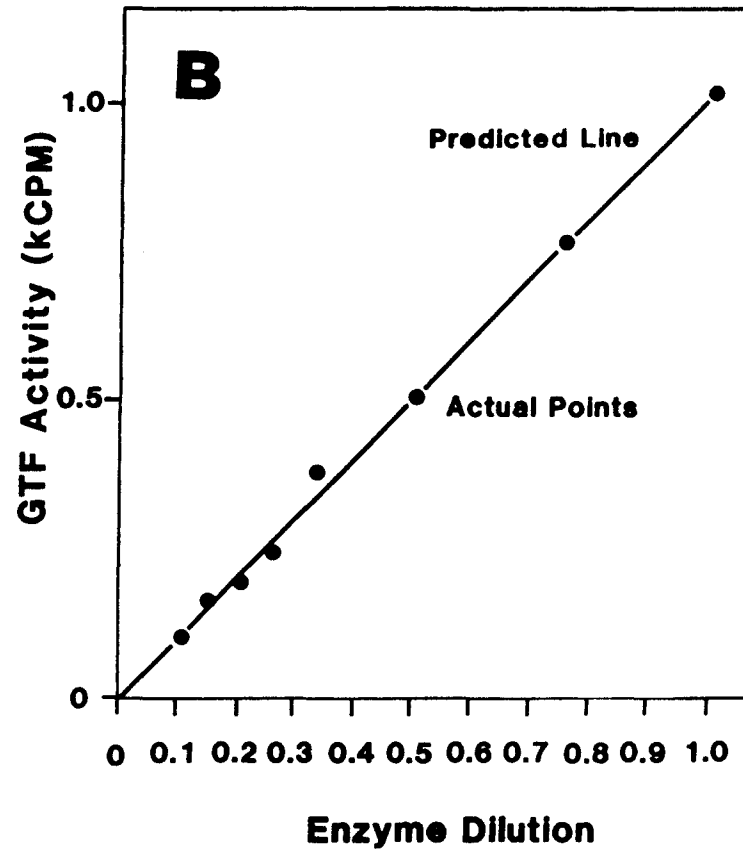
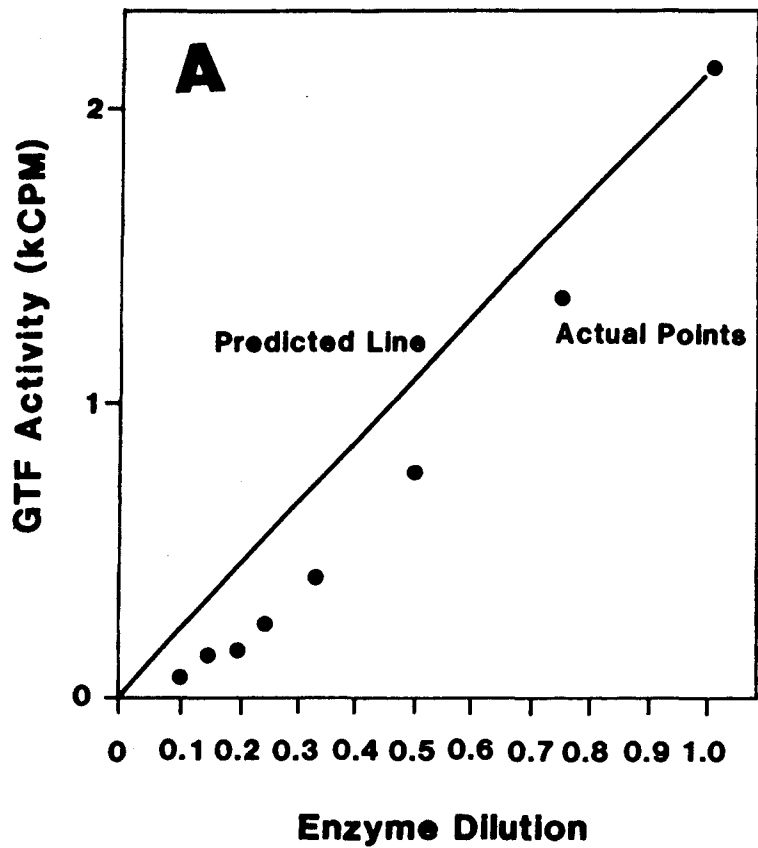
First, when GTF-I was present, the insoluble nature of the product produced a sampling error which could cause the results of duplicate spottings from the same reaction mixture to vary by 100% or more. This error could be eliminated by assaying the mixture for very short periods of time so that the amount of glucose added to the soluble dextran primer remained small enough (5% conversion of sucrose or less) to keep the product soluble. If an experimental procedure required the formation of insoluble product, such as in the NMR product synthesis experiments, the sampling error could be greatly reduced by sonicating the reaction

mixtures in the water bath of an ultrasonic cleaner before spotting. Purified GTF-S assays did not present this problem since the products remained soluble no matter how much glucose was added to the primer.

Another problem was that, with crude enzyme preparations, the measured initial rate of GTF activity was not linear with increasing enzyme concentration in the standard assay buffer of 50 mM sodium acetate (Figure 8A). The activity of concentrated culture supernatant showed an upward curvature with increasing enzyme concentration, rather than the desired linear behavior, shown by the predicted line drawn in the figure from the highest activity point through zero. The addition of 1.55 M ammonium sulfate to the assay mixture linearized the activity (Figure 8B), although the activity at high enzyme concentrations was inhibited about 50%. This assay was repeated several times, using different enzyme concentration ranges, always with a similar result. It will be shown later that this behavior is most likely due to the aggregation of GTF-I. Therefore, when enzyme preparations from purification steps were assayed to determine percent yields, 1.55 M ammonium sulfate was included in the assay mixture. It was not, however, included for kinetic experiments, such as determination of kinetic constants, or for NMR product synthesis experiments.

For typical reaction mixtures, the incubation times to determine initial rates in quantitative assays, such as

Figure 8. GTF activity with varying crude enzyme concentration using an initial rate assay. Culture supernatant, concentrated 18-fold by positive pressure through an Amicon PM-10 membrane, was used as the source of enzyme. The assay buffer was 50 mM sodium acetate, pH 5.5, containing 20 mM sucrose and 10  $\mu$ M dextran T10. The points represent the average of duplicate spottings and show the measured activity in the absence (A) and presence (B) of 1.55 M ammonium sulfate. The predicted lines are drawn from the highest activity point through zero.



determination of kinetic constants or yields from purification steps, were five minutes for crude enzyme or GTF-I preparations and ten minutes for GTF-S preparations. When column fractions were assayed to determine the position of elution of GTF activity, and not for quantitative purposes, reaction mixtures were often incubated overnight or longer, to be certain of detecting GTF activity.

### C. Purification of the GTF's

Due to the inadequacies of previously reported purification schemes for the GTF's, it was determined that a new purification method would be required. The goal of the purification was to obtain highly purified enzyme preparations that were free of dextran and contaminating enzymes, and that could be obtained with a reasonable number of steps in sufficient yield for further kinetic and product structure experiments. The scheme which was developed involved three steps--ammonium sulfate precipitation, anion-exchange chromatography, and affinity chromatography.

#### 1. Ammonium Sulfate Precipitation

Since large amounts of culture medium were needed to obtain sufficient quantities of enzyme, the activity was first concentrated 40-fold from the pH 6.0 culture supernatant by ammonium sulfate precipitation at 4°C. Solid ammonium sulfate was slowly added to 55% of saturation, the mixture was allowed to stand at 4°C for 18 hours to complete precipitation, and the precipitate was collected by

centrifugation at 10,000xg. The precipitate was dissolved in 10 mM sodium acetate buffer, pH 5.5, centrifuged again at 10,000xg to remove a small, black precipitate of caramelized culture medium components, and dialyzed against the same buffer.

This procedure recovered the GTF activity quantitatively and resulted in a purification greater than 100-fold. This unusually high degree of purification was due to the presence in the culture medium of Trypticase Peptone and Tryptose, which contained small peptides that were Lowry-positive, but were not precipitated by ammonium sulfate. When assayed for 1 hr. or longer, the dialyzed ammonium sulfate fraction produced visible turbidity in reaction tubes because of the insoluble dextrans produced by this enzyme fraction.

## 2. Anion-Exchange Chromatography

Since, as will be discussed later, the affinity chromatography step did not separate GTF-S and GTF-I, the enzymes had to be separated by an independent technique at some point in the purification. However, gel filtration of the ammonium sulfate fraction on Bio-Gel A 0.5m (M.W. cutoff  $5 \times 10^5$ ), in the presence of 2.0 M KCl to keep the enzymes dissociated, did not result in any separation. All GTF activity eluted as a single peak after the void volume. Therefore, the enzymes were separated by a modification of the anion-exchange technique of Germaine et al. (1977).

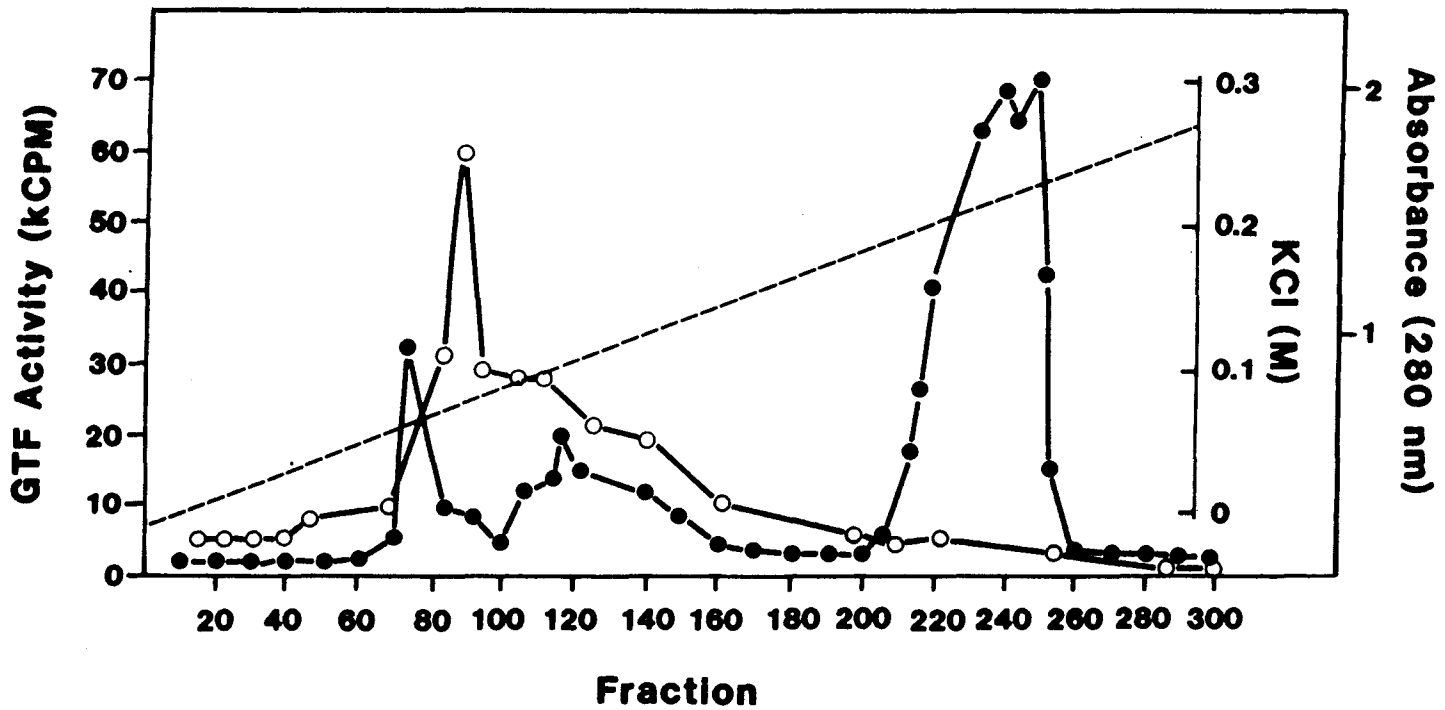
Since GTF-S has a pI of about 4.2 and GTF-I about 4.9, these enzymes were separated by a salt gradient on a DEAE resin at pH 5.5.

All ion-exchange steps were performed at 4°C. The dialyzed ammonium sulfate preparation (300 ml) was pumped at 50 ml/hr onto a DEAE-Sephacel column (2.6 x 23 cm, 122 ml) equilibrated with 10 mM sodium acetate buffer, pH 5.5, and the column was washed overnight at 20 ml/hr with the same buffer. The enzymes were eluted by a KCl gradient (0 - 0.28 M) in the acetate buffer at 20 ml/hr, with 4 ml fractions collected.

The elution profile for a typical ion-exchange run is shown in Figure 9. Although the GTF activity eluted in three peaks, the peak at fractions 70 - 90 was not obtained reproducibly and, when present, accounted for less than 1% of the total GTF activity. This peak may have comprised a small amount of the primer-independent GTF-S that leached into the culture medium during bacterial growth. This activity was not further purified because of the small quantity present. The peak at fractions 100 - 160, eluting at about 0.1 M KCl, was GTF-I, as evidenced by the visible turbidity formed in assay tubes from this peak. Although this peak represented 81.3% of the eluted GTF activity when an initial rate assay was used, the peak was smaller than the other two GTF peaks because, in the overnight assay performed on the column fractions, the insoluble product



Figure 9. Anion-exchange chromatography of a solubilized ammonium sulfate precipitate on a DEAE-Sephacel column. The column was 2.6 x 23 cm, 122 ml. The sample (300 ml) was applied in 10 mM sodium acetate buffer, pH 5.5, the column was washed overnight, and protein was eluted at 20 ml/hr with a 0 - 0.28 M KCl gradient in the same buffer. Fractions of 4.0 ml were collected. The sizes of the GTF peaks are not proportional to the amounts of enzyme present because an overnight assay, not an initial rate assay, was used. (● - ●) GTF activity; (○ - ○) absorbance at 280 nm; (-----) KCl gradient.



formed by GTF-I appeared to inhibit the enzyme activity. The peak at fractions 200 - 260, eluting at about 0.2 M KCl and representing 18.7% of the eluted GTF activity, was GTF-S, as evidenced by the lack of turbidity in assay tubes from this peak. That GTF-S elutes after GTF-I can be predicted based on their relative pI values. The peak of protein elution, around fraction 90, did not correspond to any of the GTF activity peaks.

The ion-exchange step successfully separated GTF-I and GTF-S, purified the enzymes a further 4 to 6-fold, and resulted in recovery of over 90% of the applied GTF activity. For the affinity step, the GTF-I and GTF-S peak fractions from ion exchange were pooled, and the two preparations were applied to and eluted from the affinity resin separately.

### 3. Affinity Chromatography

Because of the objections to eluting GTF's from affinity columns with soluble dextran or strong denaturants, a milder elution technique was sought. It was discovered that high concentrations of the disaccharide maltose could elute GTF's from Sephadex beads. Furthermore, the elution was very effective since, after maltose elution of GTF's from a test Sephadex column, no further GTF activity could be eluted even with dextran T10, an agent which normally eluted GTF's very efficiently. Thus, an affinity technique was developed incorporating maltose as the eluting agent.

The capacity of Sephadex G-25 beads to bind GTF's was measured by assaying concentrated culture supernatant before and after overnight mixing with a known quantity of the preswollen beads. Since the resin bound 1.4 units of GTF per ml of wet beads, the ratio when mixing the ion-exchange fractions with Sephadex was kept at 0.5 GTF units per ml of beads or less, to ensure maximum binding.

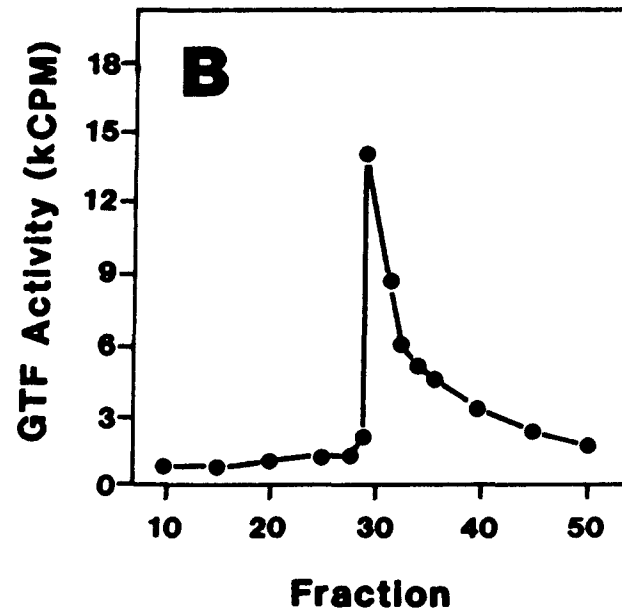
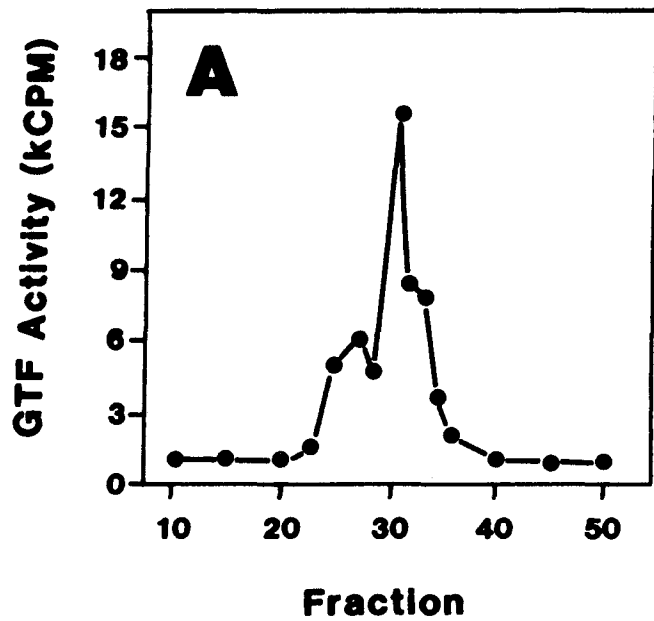
For the affinity procedure, coarse Sephadex G-25 beads were swollen at room temperature overnight in 50 mM potassium phosphate buffer, pH 6.0, containing 1.0 M KCl and 0.02%  $\text{NaN}_3$  (S buffer). Enough solid KCl, 1 M potassium phosphate buffer, and  $\text{NaN}_3$  were added to a pooled ion-exchange fraction (GTF-S or GTF-I) to make the salt concentrations the same as in S buffer, and the solution was adjusted to pH 6.0. This enzyme solution (about 200 ml) and the swollen Sephadex beads (150 - 175 ml) were stirred together in a batch process with a paddle stirrer at 4°C for 18 hr. The beads were poured into a column (2.6 cm diameter) and washed by upward flow at 50 ml/hr until the effluent absorbance at 280 nm was zero (about 2 column volumes). GTF was eluted at room temperature with 33.3% maltose (w/v, about 1 M) in S buffer by upward flow at 5 ml/hr (0.94 cm/hr). Upward flow elution was used because the maltose solution was dense and sank in the column when downward flow was attempted. Fractions of 4.0 ml were collected at 4°C.

Affinity column elution profiles of GTF activity are shown in Figure 10. Again, visible turbidity was observed in assays from the GTF-I peak, but not from the GTF-S peak.

Pooled fractions from the affinity columns were dialyzed at 4°C against 0.02%  $\text{NaN}_3$  and then concentrated to about 0.75 ml, at 4°C, in a Pierce Micro-ProDiCon protein concentrator. In this device, the dialyzing solution (0.02%  $\text{NaN}_3$ ) was placed under vacuum, and the protein solution was concentrated by negative pressure dialysis through cellulose dialysis membrane. The membrane was fitted with a fixed-volume plastic cup to prevent the solution from dialyzing to dryness. These concentrated solutions were the final enzyme preparations from the purification, and were used for all subsequent experiments.

When the concentrated GTF-I preparation was assayed, it produced visible turbidity in reaction tubes, while GTF-S did not. The filter paper spotting assay was used as described (Germaine et al., 1977) to quantitate this effect by determining the percentage of soluble and insoluble product in the GTF assay mixtures, as described in MATERIALS AND METHODS. Briefly, aliquots from a reaction mixture were spotted, the reaction tube was centrifuged to pellet insoluble product, and the supernatant was spotted again. The difference between the spottings before and after centrifugation yielded the percent insoluble synthesis.

Figure 10. Affinity column elution profiles. Ion-exchange fractions were stirred overnight with coarse Sephadex G-25 beads at 4°C. The beads were poured into a column (2.6 cm diameter), washed with S buffer, and eluted with 33.3% w/v maltose in S buffer at 5 ml/hr (0.94 cm/hr) by upward flow at room temperature. Fractions of 4.0 ml were collected at 4°C. The points show the eluted GTF activity profile for GTF-I (A) and GTF-S (B) incubated overnight in the reaction mixtures.



This technique was used to demonstrate that the purified GTF-S affinity chromatography preparation synthesized a product which was, within experimental error, 100% soluble after incubation for 1 hr. or 19 hr. in an optimal GTF-S reaction mixture containing 100 mM sodium acetate buffer, pH 5.5, 20 mM sucrose, and 50  $\mu$ M dextran T10. On the other hand, GTF-I synthesized a product that was 92% insoluble after a 19 hr. incubation in an optimal GTF-I reaction mixture containing 100 mM potassium phosphate buffer, pH 6.2, 20 mM sucrose, and 1  $\mu$ M dextran T10. Because GTF-I needed time to add enough  $\alpha$ -1,3 bonds to primer dextran to make the product insoluble, GTF-I product was initially soluble, but always became insoluble on long incubation. GTF-S could not be made to synthesize insoluble product in the presence of 1.55 M ammonium sulfate, as has been reported for the pI 7.5 GTF-S from *S. mutans* (Asem et al., 1986; Baba et al., 1986).

The dialyzed and concentrated enzymes (0.2 - 0.5 mg protein/ml) from the affinity columns were stable for over a year at 4°C. Concentrated enzyme solutions could be frozen at -20°C or -70°C and thawed without activity loss, but they lost 50 - 70% of their activity when lyophilized and reconstituted. Therefore, the concentrated enzymes were routinely stored at 4°C, with 0.02% NaN<sub>3</sub> from the dialyzing solution included as a bacteriostat.



Thus, the affinity technique as developed yielded highly concentrated, stable enzyme preparations, provided a further 8 - 12 fold purification, and gave a 5.5% yield of GTF activity over the preceding ion-exchange step.

#### 4. Loss of GTF Activity on Reversing the Ion-Exchange and Affinity Steps

Although it was possible to perform the affinity step first, by directly mixing either concentrated or unconcentrated culture supernatant with Sephadex beads and eluting the GTF's together, the enzymes became so highly purified in this procedure that they lost almost all activity upon dilution in the subsequent ion-exchange step. In the procedure actually adopted, ion exchange, which gave only a 5-fold purification, was performed first, so that other proteinaceous material remained in the GTF preparations and stabilized their activity. Then, since the subsequent affinity step involved less enzyme dilution and was done in the presence of high concentrations of the inhibitor maltose, the GTF activity was preserved. Thus, the order in which these two purification steps were performed was critical.

Incidentally, when the GTF's were bound to Sephadex directly from the culture supernatant, elution with a maltose concentration gradient did not separate GTF-S and GTF-I. Thus, the ion-exchange step was necessary to separate the enzymes.

## 5. Purification of the GTF's: Summary

Table 2 summarizes the purification scheme for GTF-I and GTF-S. These enzymes were purified from the bacterial culture supernatant 3970 and 6870-fold respectively, with a 5.1% overall yield.

Although the ammonium sulfate precipitation and ion exchange gave nearly quantitative yields of GTF activity, the major loss of activity occurred at the affinity step. However, this loss was not equivalent for the two enzymes. For GTF-S, 23% of the applied activity was recovered from the affinity column, but only 1.5% of the GTF-I. The reason for this difference in recovery is not known. In any case, the quantities of enzymes obtained from 7 liters of culture supernatant were sufficient for numerous experiments involving electrophoresis, kinetics, and product structure analysis.

### D. Assessment of the Purity of the GTF Preparations

Since the bacterial culture broth from which the GTF's were purified contained a great deal of nutrient protein from the Trypticase Peptone and Tryptose, the calculated value of the fold purification is not particularly meaningful as an index of the success of the purification. Therefore, the purity of the preparations was tested both by electrophoresis and by assays for contaminating enzymes.

TABLE 2. Purification Table for S. sobrinus GTF's

Fraction	Volume (ml)	mg Protein	GTF Units	Specific <sup>a</sup> Activity	Fold Purification	Percent Yield	Overall Yield
Supernatant	6800	61400	110.2	0.00179	1	100.0	-----
Ammonium Sulfate	300	574	110.1	0.192	107	99.9	-----
Ion Exchange							
GTF-I	196	101.0	82.9	0.821	457	75.2	92.5%
GTF-S	232	17.5	19.0	1.090	607	17.3	
Affinity							
GTF-I	0.85	0.171	1.22	7.13	3970	1.1	5.1%
GTF-S	0.75	0.353	4.36	12.30	6870	4.0	

<sup>a</sup>Expressed as GTF units/mg protein.

## 1. Polyacrylamide Gel Electrophoresis

One of the most common techniques used for assessing protein purity and determining molecular weights is sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS PAGE), which separates proteins according to size (Weber and Osborn, 1969; Wyckoff et al., 1977). The purified GTF enzymes were analyzed by SDS PAGE using the PhastSystem electrophoresis and gel staining apparatus produced by Pharmacia. This instrument gives results analogous to those obtained on larger gels in other systems, but requires less sample and less time to run and stain gels. Proteins are usually detected after SDS PAGE by Coomassie blue staining. However, since silver staining is about 20 times as sensitive, and thus provides a stricter test of protein purity, both staining methods were used.

Figure 11 shows the results of SDS PAGE of two gels run under identical, non-reducing conditions, with Coomassie blue staining (Gel A) and silver staining (Gel B). Lane 1 on each gel contained molecular weight standard proteins, lane 2 was GTF-S, and lane 3 was GTF-I. Each enzyme can be seen to have two isozyme bands and to be very pure, since no other bands are visible, even on the silver-stained gel, which was overloaded, according to the manufacturer's product literature. A gel run under identical conditions was stained for GTF activity by washing the unstained gel after the run to remove SDS, incubating the gel at 37°C in three

Figure 11. SDS PAGE of purified GTF's under non-reducing conditions. Samples (1.0  $\mu$ l each), including 0.01% bromophenol blue as a tracking dye, were applied to a precast 10 - 15% gradient PhastSystem polyacrylamide gel with a stacking gel region. Two gels run under identical conditions were stained with Coomassie blue (Gel A) or silver stain (Gel B). Lanes 1A, 1B: Molecular weight marker proteins at 238 ng per band. Lanes 2A, 2B: Purified GTF-S, 359 ng. Lanes 3A, 3B: Purified GTF-I, 153 ng.

**A**

**B**

Separating  
Gel Boundary -

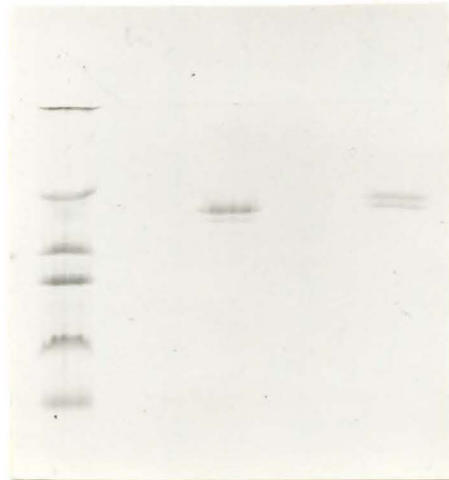
205K -

116K -

97K -

66K -

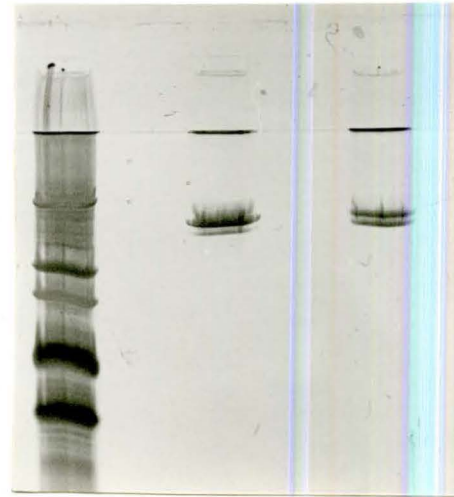
45K -



**1**

**2**

**3**



**1**

**2**

**3**

changes of 50 mM sodium acetate buffer, pH 5.5, containing 20 mM sucrose and 5  $\mu$ M dextran T10, and using periodic acid-Schiff (PAS) staining, a carbohydrate stain, to detect regions of polysaccharide synthesis. Both of the GTF-S bands showed enzyme activity using this stain, and correspond to the two GTF-S activities observed in SDS PAGE experiments in other studies (Chludzinski et al., 1974; McCabe, 1985). No GTF-I activity bands were visualized because the linear,  $\alpha$ -1,3-linked product of GTF-I does not contain adjacent hydroxyl groups and is, therefore, not detected by this periodic acid stain. It is also possible that GTF-I did not regain activity after removal of SDS. Multiple GTF-I forms in SDS PAGE experiments have also been reported elsewhere (McCabe, 1985).

Figure 12 shows the results of SDS PAGE under reducing conditions, that is in the presence of 5% 2-mercaptoethanol, with Coomassie blue staining (Gel A) and silver staining (Gel B). Again, lane 1 on each gel contained molecular weight markers, lane 2 was GTF-S, and lane 3 was GTF-I. The sensitivity of the silver staining technique was demonstrated by the distinct bands observed on the silver stained gel using amounts of protein that were barely detectable by Coomassie blue staining. Each enzyme again shows two isozymes, apparently at the same molecular weights as under non-reducing conditions, with no contaminating bands visible.

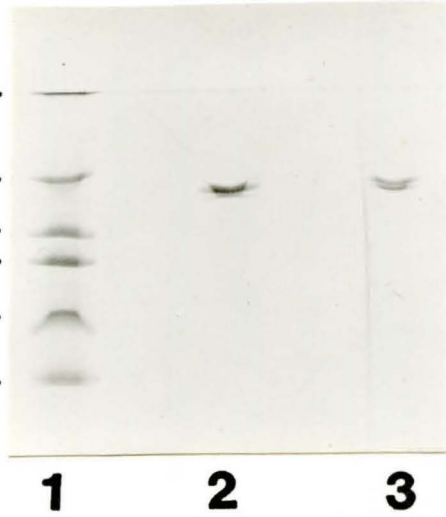
Figure 12. SDS PAGE of purified GTF's under reducing conditions, that is, in the presence of 5% 2-mercaptoethanol. Samples (1.0  $\mu$ l each), including 0.01% bromphenol blue as a tracking dye, were applied to a precast 10 - 15% gradient PhastSystem polyacrylamide gel with a stacking region. Two gels run under identical conditions were stained with Coomassie blue (Gel A) or silver stain (Gel B). Lane 1A: Molecular weight marker proteins at 238 ng per band. Lane 1B: Molecular weight marker proteins at 20 ng per band. Lane 2A: Purified GTF-S, 336 ng. Lane 2B: Purified GTF-S, 50 ng. Lane 3A: Purified GTF-I, 144 ng. Lane 3B: Purified GTF-I, 50 ng.



# A

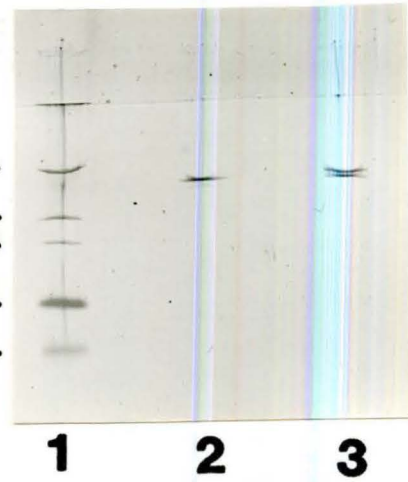
Separating  
Gel Boundary -

205K -  
116K -  
97K -  
66K -  
45K -



# B

205K -  
116K -  
97K -  
66K -  
45K -

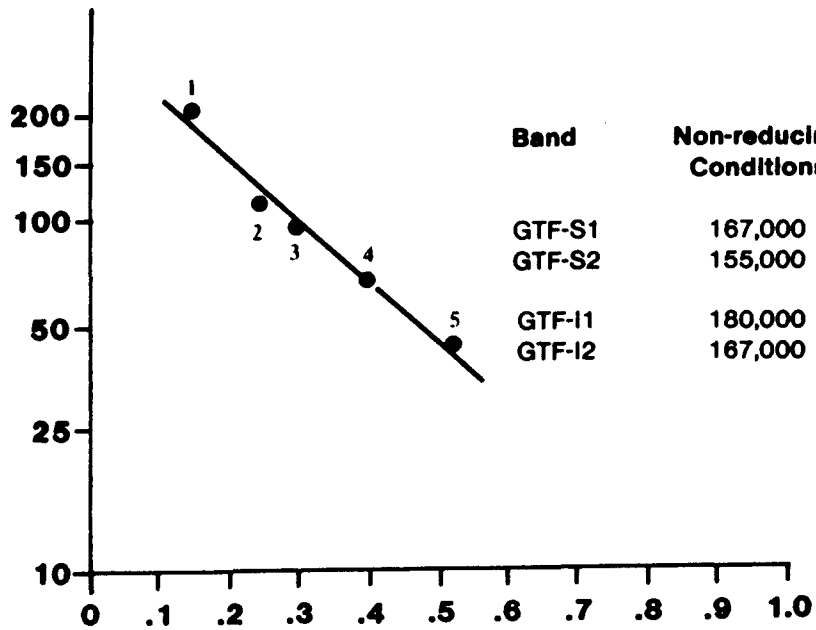


The molecular weights of the GTF bands were calculated using the graph shown in Figure 13, constructed by plotting the logs of the molecular weights of the standard proteins vs. their  $R_f$  values, that is, the ratio of the migration distance of the protein to that of the tracking dye. The  $R_f$  values of the GTF bands under reducing and non-reducing conditions were then used to determine the GTF molecular weights, which are tabulated in the figure. Because of the small size of the gels, measurements were taken on the screen of a slide viewer into which the dried gel had been inserted. Since the plastic backing prevented the gels from expanding or shrinking during staining or drying, no correction for changes in gel size needed to be applied, as is necessary for larger gels (Weber and Osborn, 1969).

As discussed earlier, the molecular weights of S. sobrinus GTF's in several other studies (Fukui et al., 1982; Shimamura et al., 1982; McCabe, 1985) have been reported to be similar to those determined here, in the range 150,000 to 180,000, although a comparison of gels run under reducing and non-reducing conditions with the same enzyme preparation has not been previously reported for either enzyme. Since the molecular weights were similar under reducing and non-reducing conditions, the isozymes of GTF-S and GTF-I are apparently single polypeptide chains, with no interchain disulfide bonds.

Figure 13. SDS PAGE molecular weight calibration curve and calculated molecular weights of the GTF's. The marker proteins were the following, with molecular weights (in thousands) in parentheses. 1. Myosin (205); 2.  $\beta$ -Galactosidase (116); 3. Phosphorylase b (97.4); 4. Bovine Albumin (66); 5. Egg Albumin (45).

**Molecular Weight (in thousands)  
(log scale)**



<b>Band</b>	<b>Non-reducing Conditions</b>	<b>Reducing Conditions</b>
GTF-S1	167,000	169,000
GTF-S2	155,000	162,000
GTF-I1	180,000	177,000
GTF-I2	167,000	169,000

## 2. Polyacrylamide Gel Isoelectric Focusing

Isoelectric focusing (IEF) in precast PhastSystem polyacrylamide gels was done according to the manufacturer's instructions to assess the purity and determine the pI values of the GTF's. Gel A in Figure 14 was Coomassie blue stained and contained pI markers (Lane 1A) and GTF-S (Lane 2A). GTF-S focused as two bands with no contaminating bands visible, but GTF-I was barely visible with Coomassie staining. Thus, gel B, with pI markers (Lane 1B) and GTF-I (Lane 2B), was run under identical conditions and silver stained. GTF-I can be seen to be smeared from the point of application in the center of the gel toward the acidic pH range, with one focused band visible just to the basic side of where the GTF-S bands focused. It will be shown later that this smearing was due to the aggregation behavior of GTF-I.

The pI's of the proteins were determined from the standard curve in Figure 15, which was constructed using measurements of the focused positions of the pI markers. The GTF-S bands focused at pI 4.18 and 4.33, whereas the position of the farthest advancing GTF-I protein was at pI 4.91. These values are similar to those obtained by others for S. sobrinus 6715 GTF's using partially purified enzyme preparations (Chludzinski et al., 1974; Tsumori et al., 1983a).

Figure 14. Polyacrylamide gel isoelectric focusing of purified GTF's. Samples (1.0  $\mu$ l each) were applied to the center of a precast and prefocused PhastSystem pH 3-9 polyacrylamide isoelectric focusing gel, and focusing was continued at 2000 V and 2.5 mA at 15°C. Gel A: Coomassie blue stained gel. Lane 1A: pI marker proteins, 400 ng per band. Lane 2A: Purified GTF-S, 471 ng. Gel B: Silver-stained gel. Lane 1B: pI marker proteins, 40 ng per band. Lane 2B: Purified GTF-I, 201 ng.

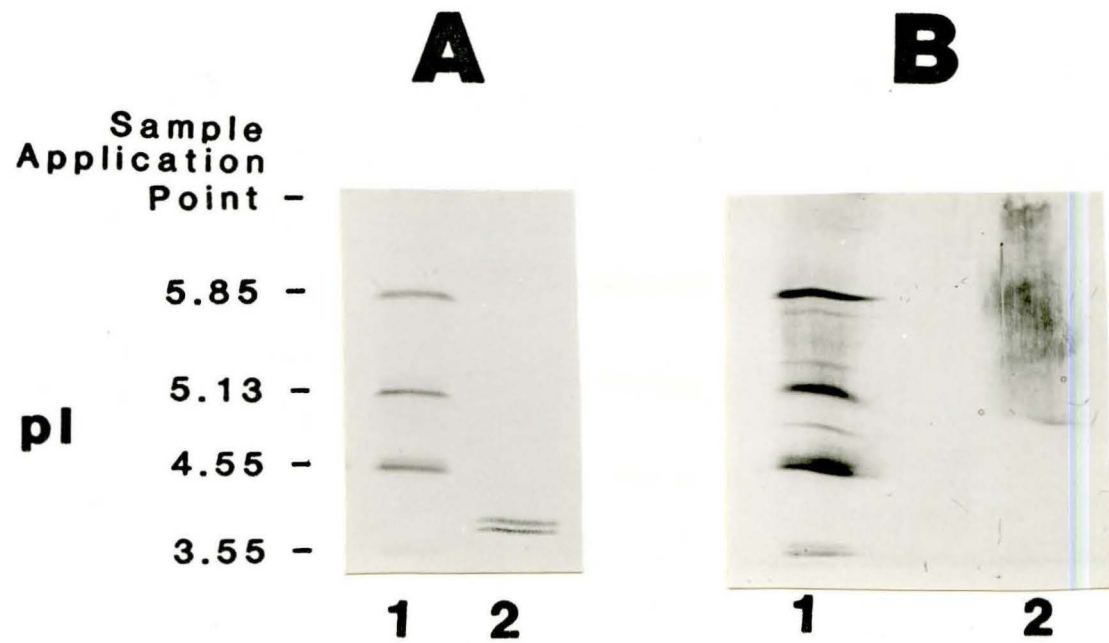
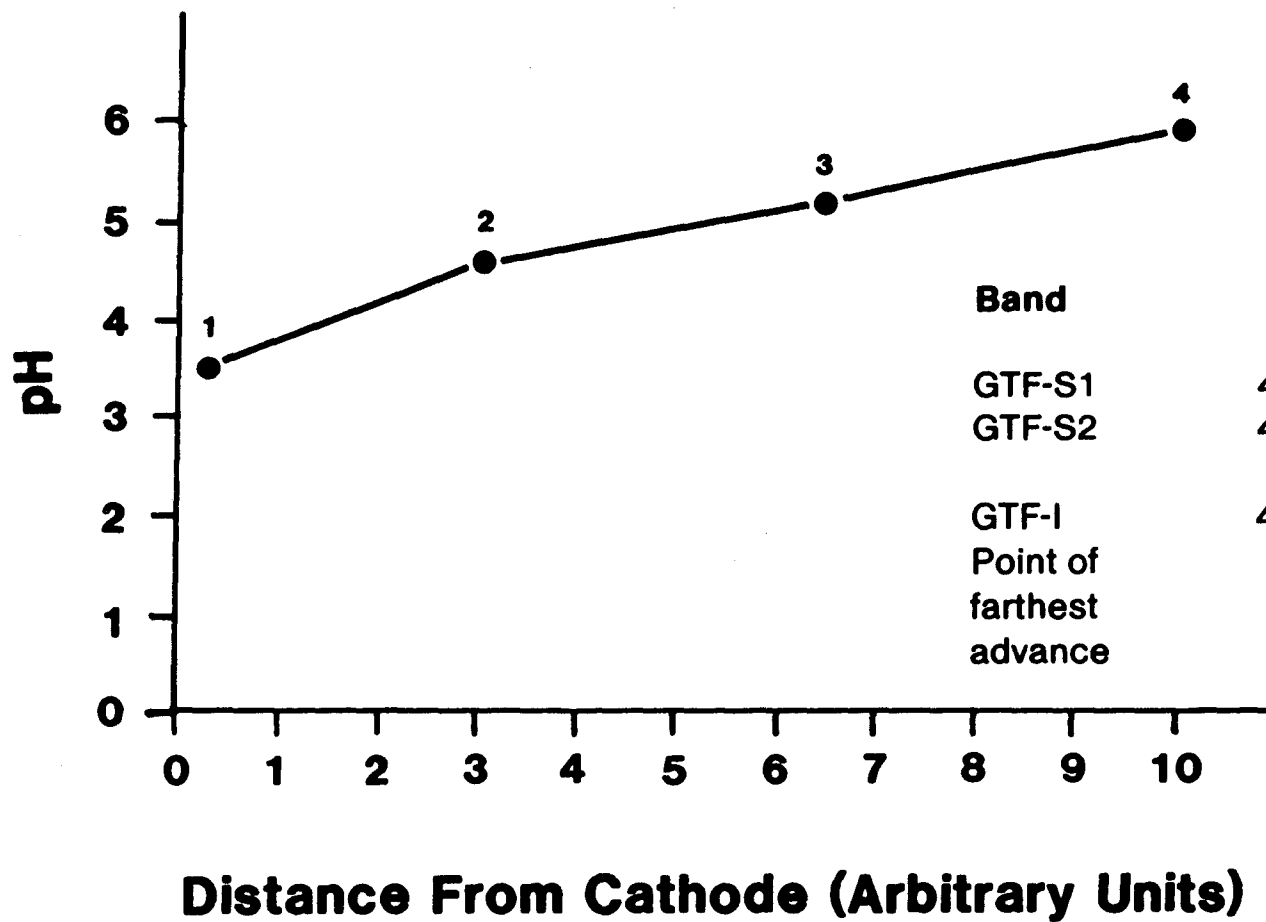


Figure 15. Gel isoelectric focusing pI calibration curve and calculated pI's of the GTF's. The marker proteins were the following, with pI's in parentheses. 1. Amyloglucosidase (3.55); 2. Trypsin soybean inhibitor (4.55); 3.  $\beta$ -Lactoglobulin A (5.13); 4. Carbonic Anhydrase B (5.85).





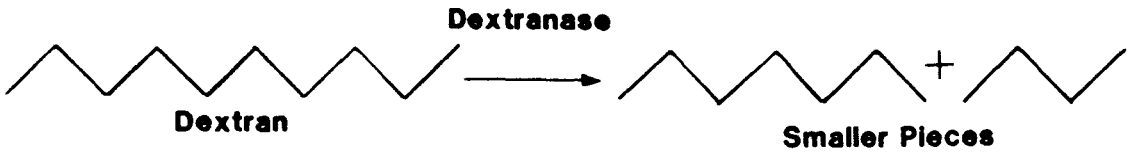
### 3. Assays for Contaminating Enzymes

Although the electrophoresis experiments indicated that the GTF preparations were highly purified, sucrose metabolizing enzymes could have been present in quantities below the level of detection by staining. Three such enzymes are potential contaminants produced by oral streptococci, and their absence from the purified GTF preparations was assured by direct assay.

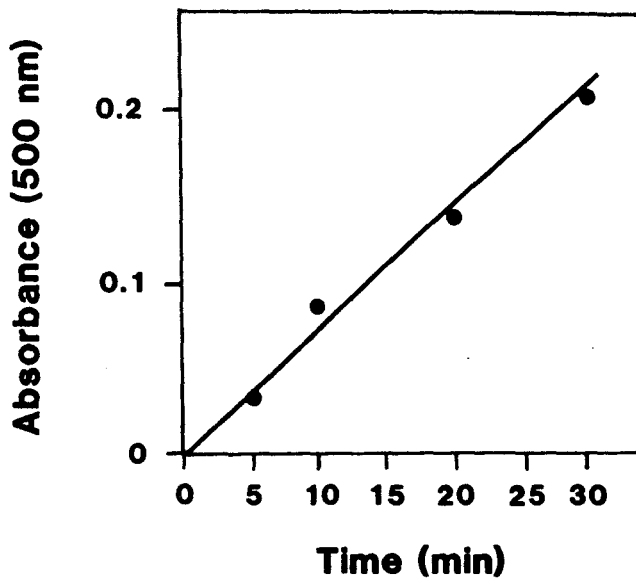
Dextranase cleaves  $\alpha$ -1,6-linked dextran into smaller fragments, as shown at the top of Figure 16. Since each cleavage releases a reducing terminus, the enzyme was assayed by measuring the release of reducing sugar (Nelson, 1944) from dextran T2000 (Hamelik and McCabe, 1982). Commercial dextranase at 0.12 unit/ml showed easily detectable activity in 5 minutes (Figure 16, middle), whereas 0.12 unit/ml of GTF-S and 0.18 unit/ml of GTF-I showed no dextranase activity even after overnight (17 hr.) incubation (Figure 16, bottom). As shown in the figure, the dextranase activity that was present in the ammonium sulfate fraction was removed during the purification.

Invertase cleaves sucrose to glucose and fructose, as shown at the top of Figure 17. Since sucrose is not a reducing sugar, but glucose and fructose are, this enzyme was also assayed by release of reducing sugar. However, since GTF's have invertase activity themselves, the substrate raffinose, which is cleaved by invertase but not by

Figure 16. Assay of purified GTF's to demonstrate the absence of dextranase. Top. Diagram of the dextranase reaction. Middle. Assay, with dextran T2000 substrate, of 0.12 units/ml of a commercial dextranase preparation (Calbiochem grade B) using the Nelson (1944) reducing sugar assay, which produces a blue species that absorbs at 500 nm. The points represent the average of triplicate determinations. Activity was easily detected in 5 minutes. Bottom. The same assay incubated overnight (17 hr.) using 0.12 unit/ml of GTF-S and 0.18 unit/ml of GTF-I. Dextranase was present in the ammonium sulfate fraction, but not in the purified enzymes.

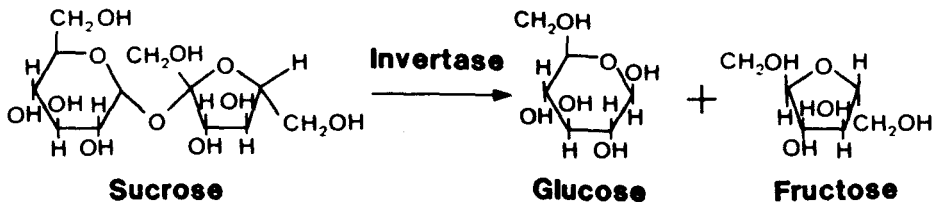


### COMMERCIAL DEXTRANASE

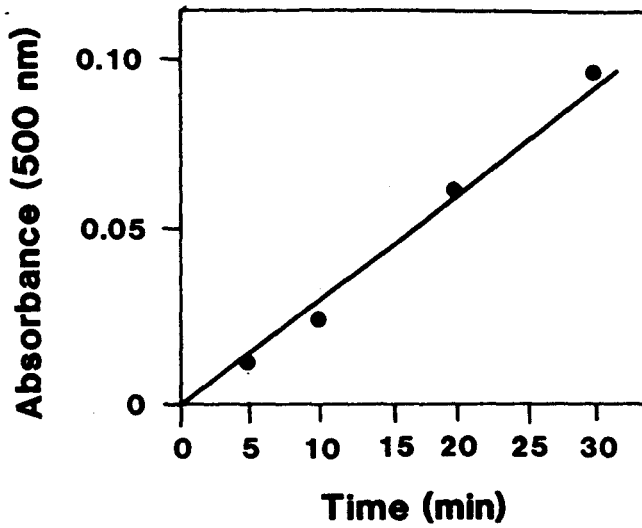


Enzyme Prep	Absorbance at 500 nm (n = 3)
GTF-I	0.003
GTF-S	0.005
Ammonium Sulfate Fraction	0.709

Figure 17. Assay of purified GTF's to demonstrate the absence of invertase. Top. Diagram of the invertase reaction. Middle. Assay, with raffinose substrate, of 0.12 unit/ml of a commercial invertase preparation (Sigma Grade VII) using the Nelson (1944) reducing sugar assay. The points represent the average of triplicate determinations. Activity was easily detected in 5 minutes. Bottom. The same assay incubated overnight (17 hr.) using 0.12 unit/ml of GTF-S and 0.18 unit/ml of GTF-I. Invertase was present in the ammonium sulfate fraction, but not in the purified enzymes.



### COMMERCIAL INVERTASE



Enzyme Prep	Absorbance at 500 nm (n = 3)
GTF-I	0.000
GTF-S	0.005
Ammonium Sulfate Fraction	0.535

GTF, was used for the test (McCabe and Smith, 1973). Raffinose contains a sucrose moiety that has an  $\alpha$ -linked galactose bound to the 6-hydroxyl of the glucose unit. Again, 0.12 unit/ml of commercial invertase gave detectable activity in 5 minutes (Figure 17, middle), whereas 0.12 unit/ml of GTF-S and 0.18 unit/ml of GTF-I showed no activity in a 17 hr. incubation (Figure 17, bottom). All the invertase activity that was present in the ammonium sulfate fraction was removed during the purification.

Fructosyltransferase (FTF), or levansucrase, polymerizes the fructose moiety of sucrose into a polyfructose polymer, with the release of free glucose. The enzyme was assayed by measuring the incorporation of [ $1-^3\text{H}$ ]fructose from [ $1'-^3\text{H}$ ]fructose-sucrose into methanol insoluble product by the filter paper spotting technique (McCabe, 1985). No commercial FTF preparation was available, but FTF activity was readily detected in an ammonium sulfate precipitate fraction from the culture supernatant of an S. mutans strain (NCTC 10449). This strain was chosen since S. mutans is known to produce large quantities of FTF (Russell, 1978). However, in accordance with other data (Schachtele et al., 1972; Kametaka et al., 1987a), which show that S. sobrinus produces only traces of this enzyme, FTF activity could not be detected in the S. sobrinus ammonium sulfate fraction or in the purified enzyme preparations. It was detected in small quantities only in unpurified culture supernatant that

had been concentrated 18-fold through an Amicon PM-10 membrane.

Thus, the final GTF-S and GTF-I preparations were extremely pure, based on results from both electrophoresis and contaminating enzyme assays.

#### E. Kinetic Characterization of the Enzymes

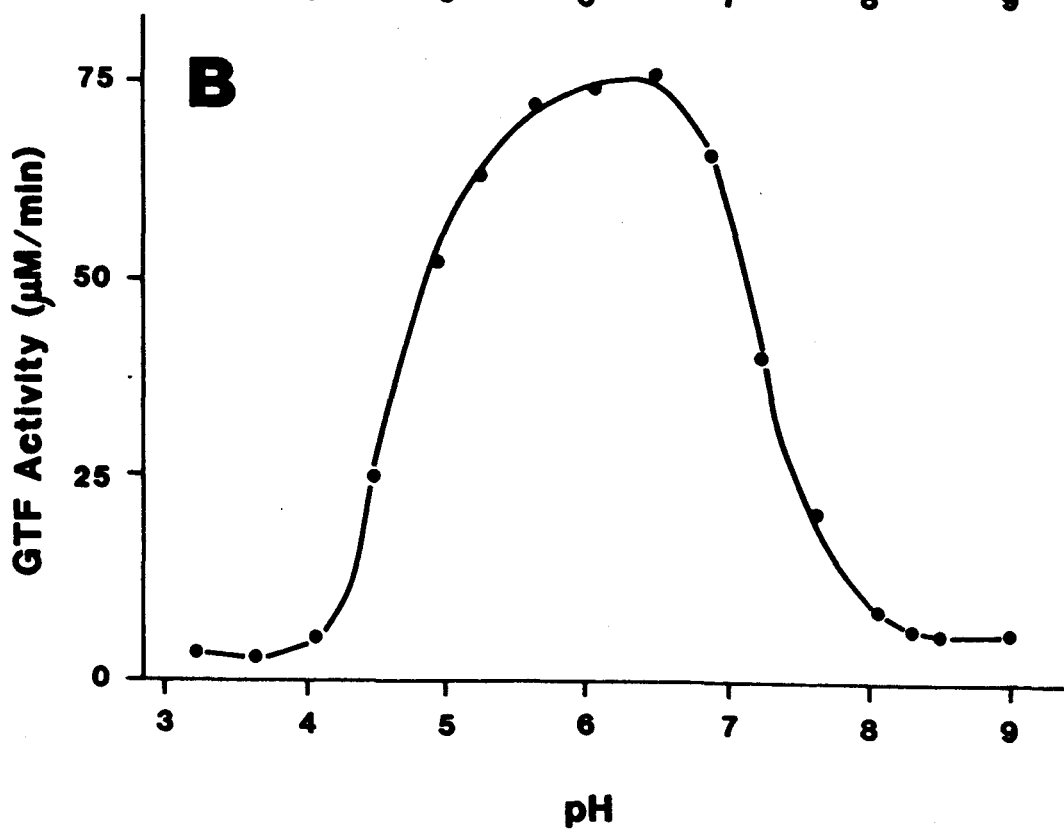
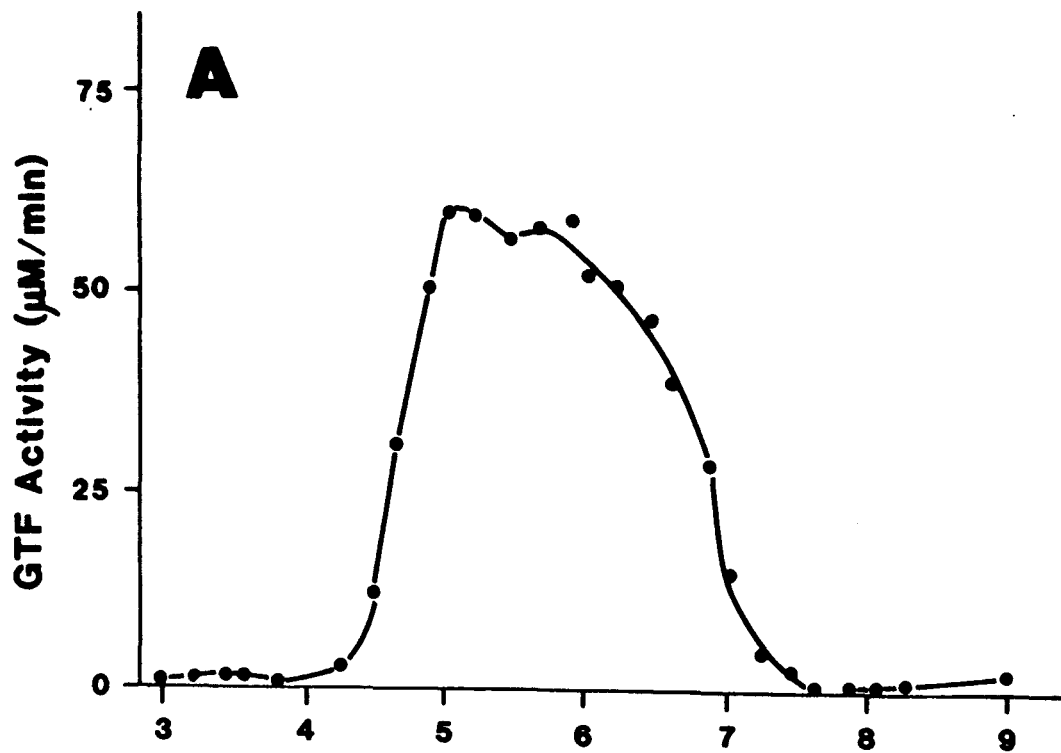
Now that highly purified GTF-S and GTF-I preparations were available, it was possible to do kinetic studies to further characterize the enzymes and to determine appropriate conditions for synthesizing the products to be used in the NMR study.

The kinetic constants  $K_m$  and  $V_{max}$  were determined using direct linear plots (Eisenthal and Cornish-Bowden, 1974; Cornish-Bowden and Eisenthal, 1974, 1978). This graphical method, including statistical error analysis by the method of Porter and Trager (1977), is described in APPENDIX A. A BASIC computer program was written to calculate the required intersections and medians, and the statistical errors were determined by hand from tabulated values of Kendall's K statistic (Kendall, 1970).

Since no buffer tested inhibited GTF activity, a citrate/phosphate/glycine buffer, which was 100 mM in each component and effectively covered the pH interval 3 to 9, was used to determine the pH range of activity of the purified enzymes. Figure 18 shows initial rate vs. pH graphs for GTF-S (A) and GTF-I (B) at 20 mM sucrose and 20  $\mu$ M



Figure 18. Initial rate vs. pH profiles for GTF-S (Graph A) and GTF-I (Graph B). The points represent the average of duplicate spottings. The reaction mixtures contained citrate/phosphate/glycine buffer, 100 mM in each component, 20 mM sucrose, 20  $\mu$ M dextran T10, and GTF-S (0.058 unit/ml) or GTF-I (0.115 unit/ml).



dextran T10. Although these graphs are not highly significant for quantitative kinetics, they do give a qualitative feel for the activity range of the GTF's at typical sucrose and dextran concentrations. Both enzymes showed single peaks of activity in the acidic pH range, with the GTF-S optimum region at pH 5 to 6 and the GTF-I region at pH 5.5 to 6.5. Thus, GTF-S kinetic assays were routinely performed in pH 5.5 sodium acetate buffer, and GTF-I assays in pH 6.2 potassium phosphate buffer. It will be shown later that these pH's were also near the maximum regions of enzyme specificity, that is, near the maximum points of the  $\log(V_{\max}/K_m)$  vs. pH graphs (see Figures 24 and 25). Thus, these pH's were ideal ones at which to perform the assays.

#### 1. Behavior of the GTF's Toward the Substrate Dextran

The effect of the substrate dextran on the two enzymes was different. Figures 19 and 20 show the initial rate of GTF activity, with varying dextran T10 concentrations and 20 mM sucrose, for GTF-S and GTF-I respectively. The most striking difference was that dextran gave significant substrate inhibition for GTF-I, but did not inhibit GTF-S, even at a concentration of 1 mM. The dextran inhibition of GTF-I could not be overcome with high concentrations of sucrose. Substrate inhibition with one substrate in a two-substrate reaction is relatively common and is consistent with some well-known types of kinetic mechanisms (Cornish-Bowden, 1979).

Figure 19. Initial rate vs. dextran T10 concentration for GTF-S. The points represent the average of duplicate spottings. Reaction mixtures contained 100 mM sodium acetate buffer, pH 5.5, dextran T10, 20 mM sucrose, and GTF-S (0.058 unit/ml).

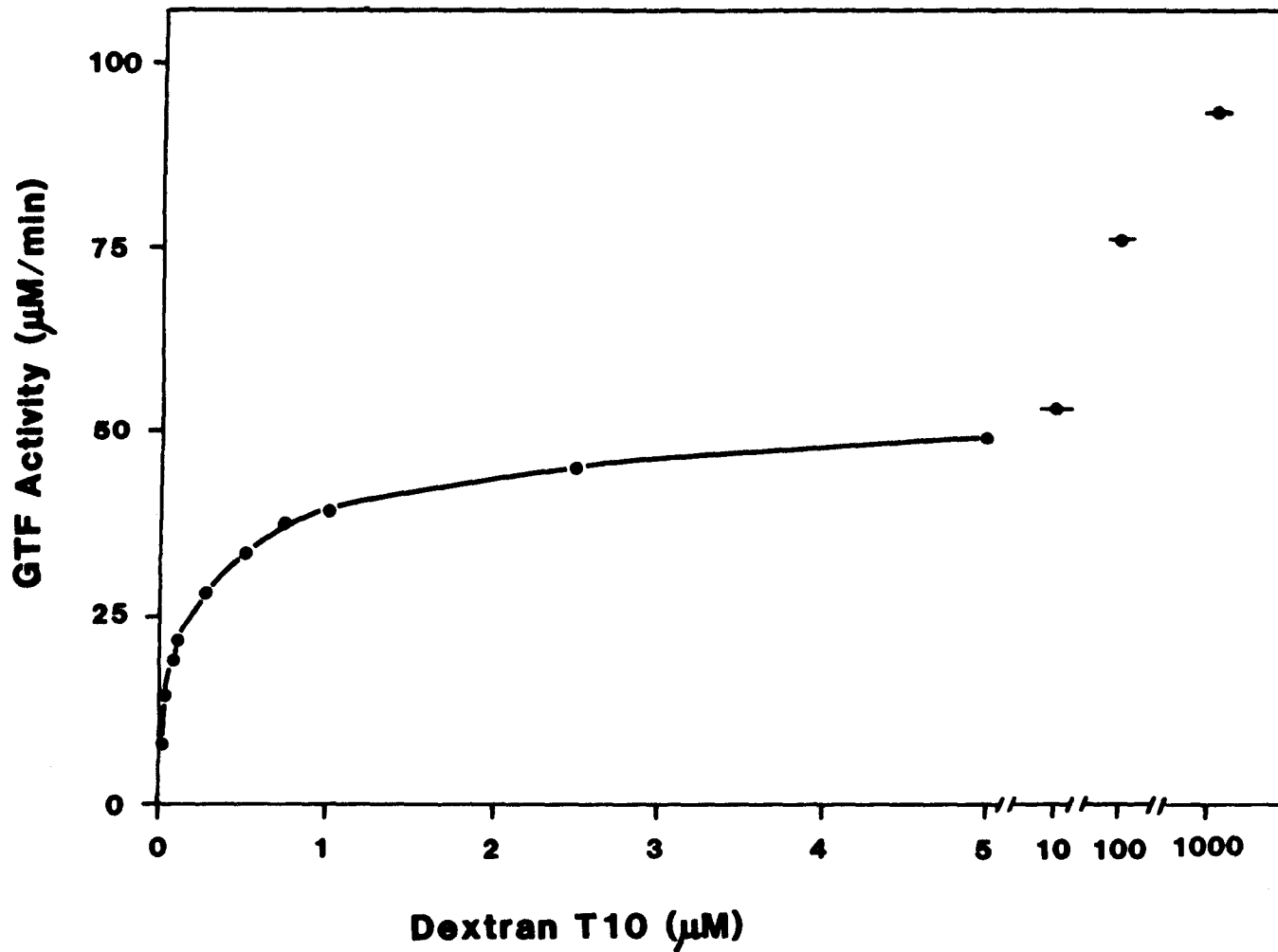
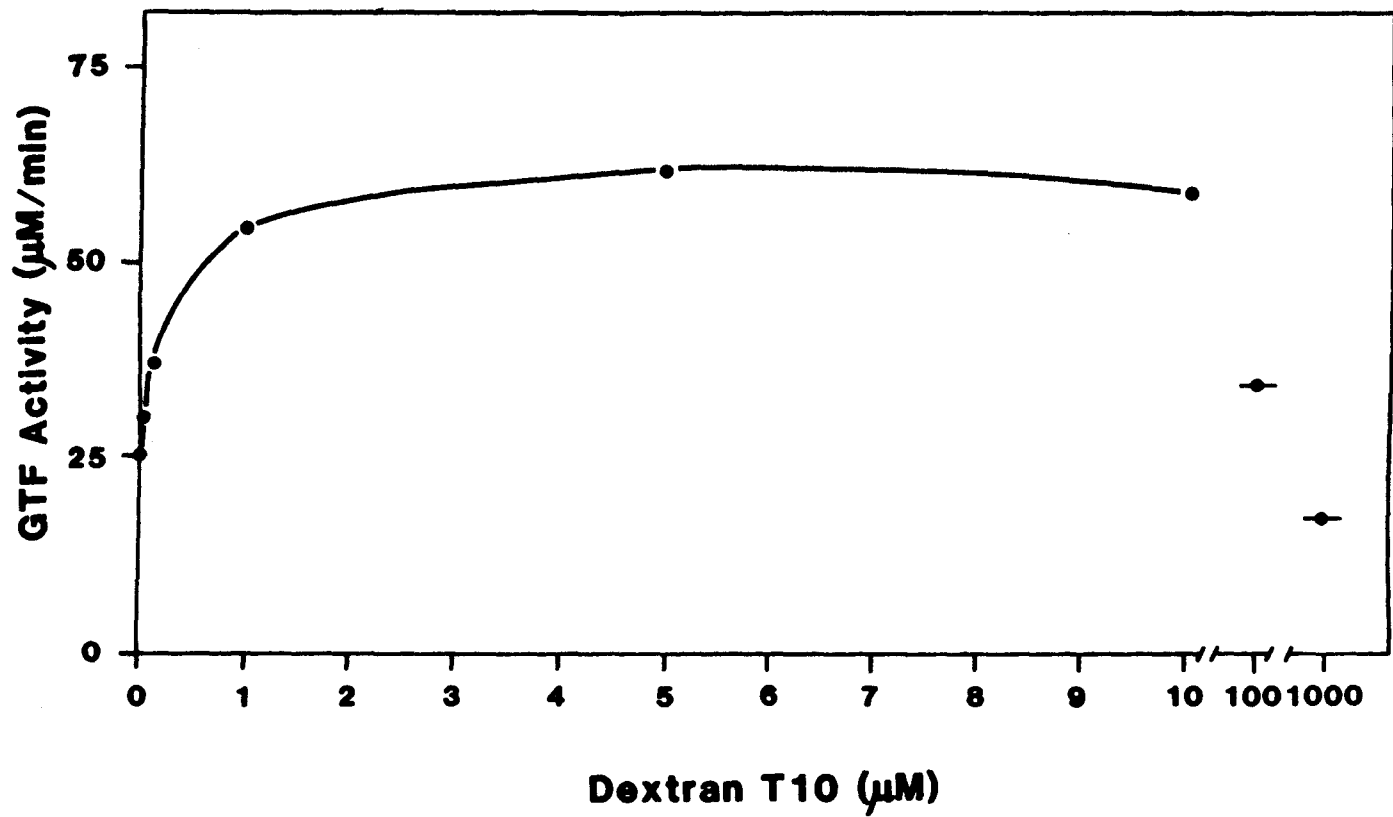


Figure 20. Initial rate vs. dextran T10 concentration for GTF-I. The points represent the average of duplicate spottings. Reaction mixtures contained 100 mM potassium phosphate buffer, pH 6.2, dextran T10, 20 mM sucrose, and GTF-I (0.092 unit/ml).

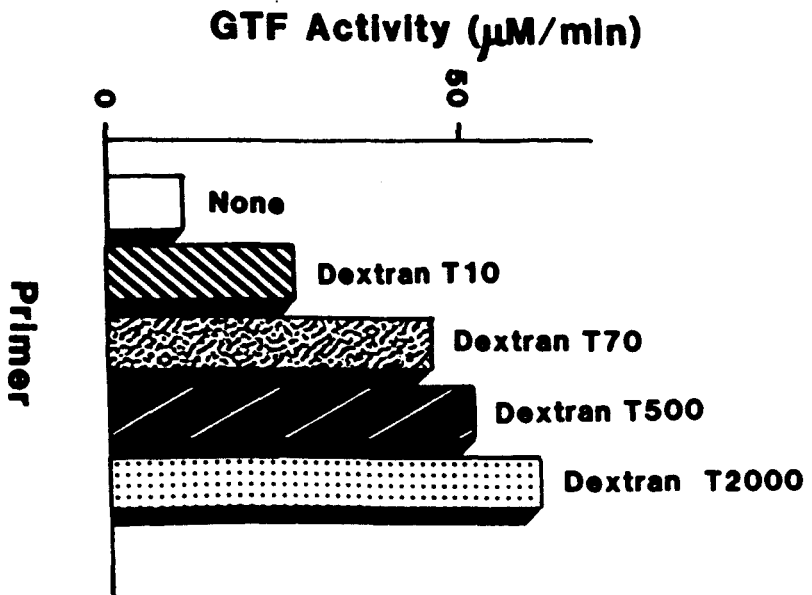
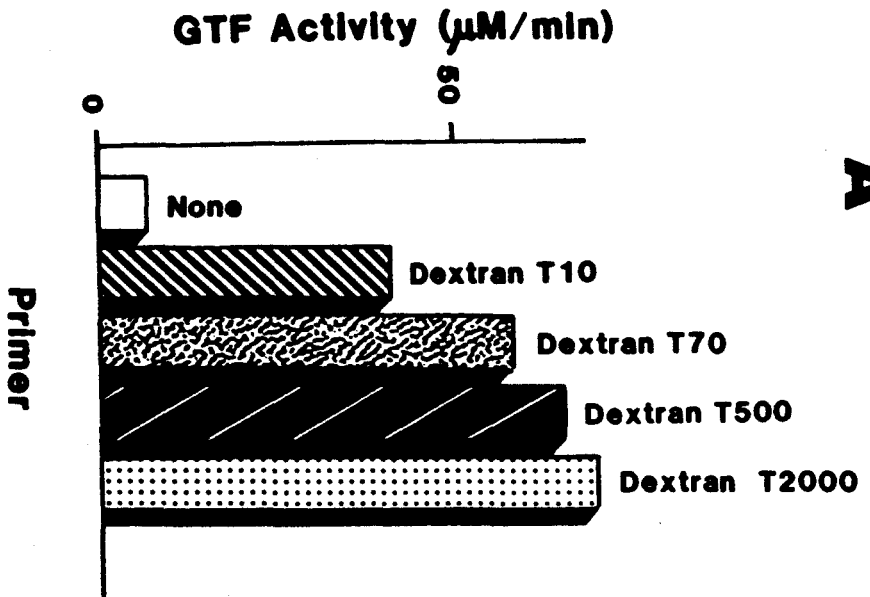


As the data indicate, both enzymes showed some activity in the absence of dextran. However, dextran stimulated GTF-S activity about 10-fold at saturating dextran concentrations, and GTF-I activity by about 2.5-fold at the optimal dextran concentration of 5  $\mu$ M. On long incubation, GTF-S was capable of synthesizing large amounts of dextran product from sucrose alone, although at a much slower rate than in the presence of dextran. However, GTF-I activity in the presence of sucrose alone declined very quickly, and no appreciable amount of product could be synthesized in the absence of dextran in 24 hours. The S. sobrinus GTF-I has also been reported in other studies (Fukui et al., 1982; Hanada and Takehara, 1987) to exhibit little ability to synthesize insoluble product in the absence of primer dextran. Thus, primer dextran was an absolute requirement for GTF-I to synthesize large, insoluble products.

Dextran molecules other than T10 were tested for their ability to stimulate GTF activity, as summarized for GTF-S (Figure 21A) and GTF-I (Figure 21B). The dextran T fractions are all partial degradation products, of varying molecular weights, prepared from the high molecular weight dextran synthesized from sucrose by Leuconstoc mesenteroides B512 dextransucrase. Thus, these dextrans all have a similar chemical structure (nearly 100% linear  $\alpha$ -1,6-linked dextran), but vary in molecular weight. The number following the "T" is the average molecular weight in thousands.



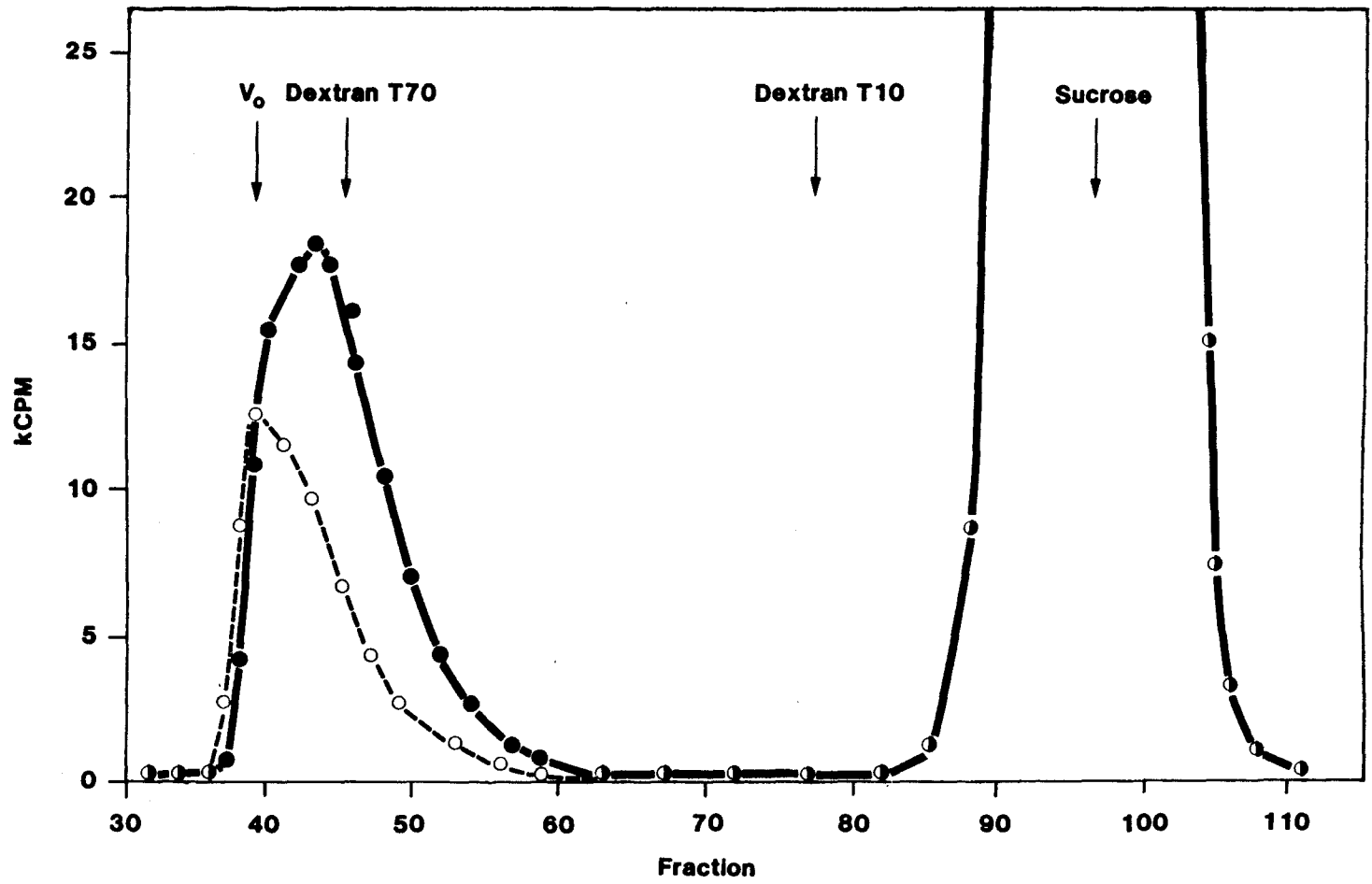
Figure 21. The effect of various dextran primers on the activity of GTF-S (Graph A) and GTF-I (Graph B). Reaction mixtures contained the following. GTF-S: 100 mM sodium acetate buffer, pH 5.5, 20 mM sucrose, 0.058 unit/ml GTF-S, and 50  $\mu$ g/ml primer. GTF-I: 100 mM potassium phosphate buffer, pH 6.2, 10 mM sucrose, 0.046 unit/ml GTF-I, and 50  $\mu$ g/ml primer. Each bar represents the average of duplicate determinations. Dextran T10, T70, T500, T2000: commercial  $\alpha$ -1,6-linked dextrans with the average molecular weight designated in thousands. The bar labeled None indicates that sucrose alone was present in the reaction mixture with no primer added.



As indicated in the figure, some autopolymerization activity was detected in the absence of primer, as expected. Dextran T10 showed characteristic priming activity, but the higher molecular weight dextrans T70, T500, and T2000 were better primers, although the enhanced priming activity was not proportional to molecular weight. These results are consistent with other data (Germaine et al., 1976) suggesting that once  $\alpha$ -1,6-linked dextran reaches a large enough size, the priming activity increases little with molecular weight.

Although many different kinds of data, as described in the REVIEW OF THE RELATED LITERATURE, indicate that primer dextran works by accepting glucose residues from sucrose, an experiment was performed to demonstrate this fact using the highly purified enzymes. When dextran T70 was incubated with [ $U$ - $^{14}C$ ]sucrose and GTF-S or GTF-I for only 10 minutes, the T70 became extensively labeled, as shown by gel filtration of the reaction mixtures on a Bio-Gel A 0.5m column (Figure 22). The elution positions of dextran T70, dextran T10, and sucrose are shown by the arrows in the figure, and were determined in separate gel filtration runs with these standard compounds detected in column fractions using the phenol-sulfuric acid assay, which can detect non-reducing carbohydrates (Dubois et al., 1956). When the pulse-labeled GTF reaction mixtures (0.20 ml) were run on this column, radioactivity eluted in the positions shown for GTF-I (open circles) and GTF-S (closed circles). Both reaction mixtures

Figure 22. Gel filtration of pulse-labeled dextran T70 on a Bio-Gel A 0.5m column. The column was 1 x 55 cm, 43.2 ml. Dextran T70 was incubated for 10 minutes in a reaction mixture containing 100 mM potassium phosphate buffer, [U-<sup>14</sup>C]sucrose (45  $\mu$ Ci/ml), and GTF-S (0.058 unit/ml) at pH 5.7, or GTF-I (0.038 unit/ml) at pH 6.2. The reactions were stopped by placing the tubes in boiling water for 5 minutes, and the reaction mixtures (0.20 ml) were eluted from the column with 100 mM potassium phosphate buffer at 3.1 ml/hr (4 cm/hr). Fractions of 0.5 ml were collected, and their radioactivity was measured by liquid scintillation after drying of aliquots from the fractions on Whatman 3MM filter paper. (● - ●) GTF-S reaction mixture; (○ - ○) GTF-I reaction mixture. The positions of elution of blue dextran ( $V_0$ ), dextran T70, dextran T10, and sucrose are indicated with arrows.



showed a large peak of unreacted sucrose as well as a higher molecular weight peak which eluted near the elution position of dextran T70. Since the pulse labeling added some molecular weight to the primer dextran, the high molecular weight peaks from the reaction mixtures eluted at slightly higher molecular weights than cold dextran T70. This effect was more pronounced for GTF-I, since a smaller concentration of primer ( $1.42 \mu\text{M}$  T70) had to be used than for GTF-S ( $14.2 \mu\text{M}$  T70) due to dextran substrate inhibition of GTF-I. However, the column results show that dextran T70 primer rapidly accepted glucose units from sucrose and became labeled, with no intermediate molecular weight dextrans being formed. The filter paper spotting assay was performed on the reaction mixtures before applying them to the column, and quantitative calculations showed that, within experimental error, 100% of the radioactivity incorporated into methanol-insoluble polymer eluted from the gel filtration column in the high molecular weight dextran T70 peaks. These results show that dextran T70 primer indeed acted by accepting glucose units from sucrose.

## 2. Behavior of the GTF's Toward the Substrate Sucrose

In the presence of primer dextran and varying concentrations of the substrate sucrose, both enzymes gave linear Hanes plots, as Figures 23 and 24 show for some typical kinetic data for GTF-S and GTF-I respectively. Thus, both enzymes obeyed typical Michaelis-Menton kinetics

Figure 23. Hanes plot for typical GTF-S kinetic data obtained by varying the sucrose concentration. The reaction mixture contained 100 mM sodium acetate buffer, pH 5.5, sucrose, 1 mM dextran T10, and GTF-S (0.058 unit/ml).

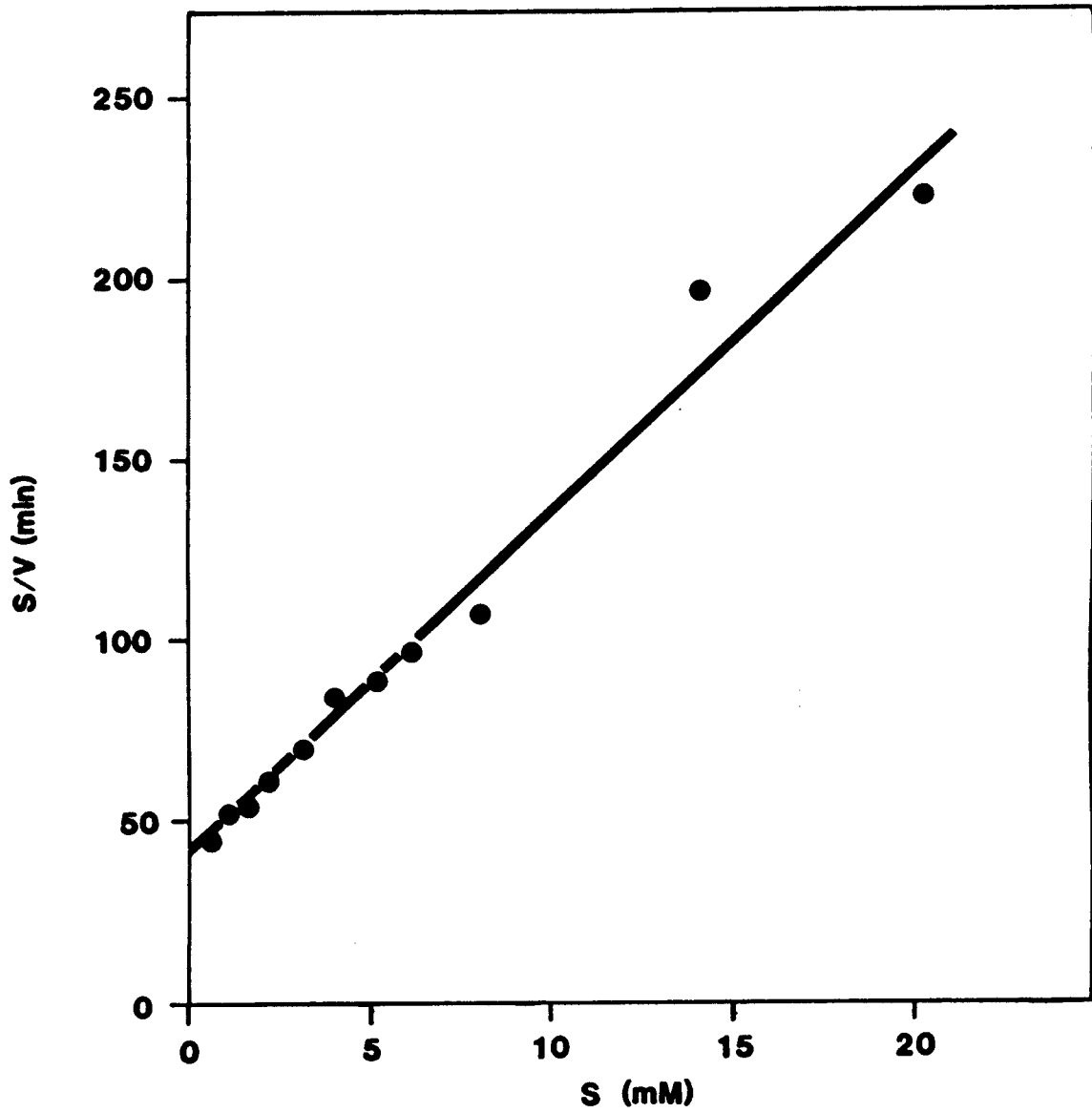
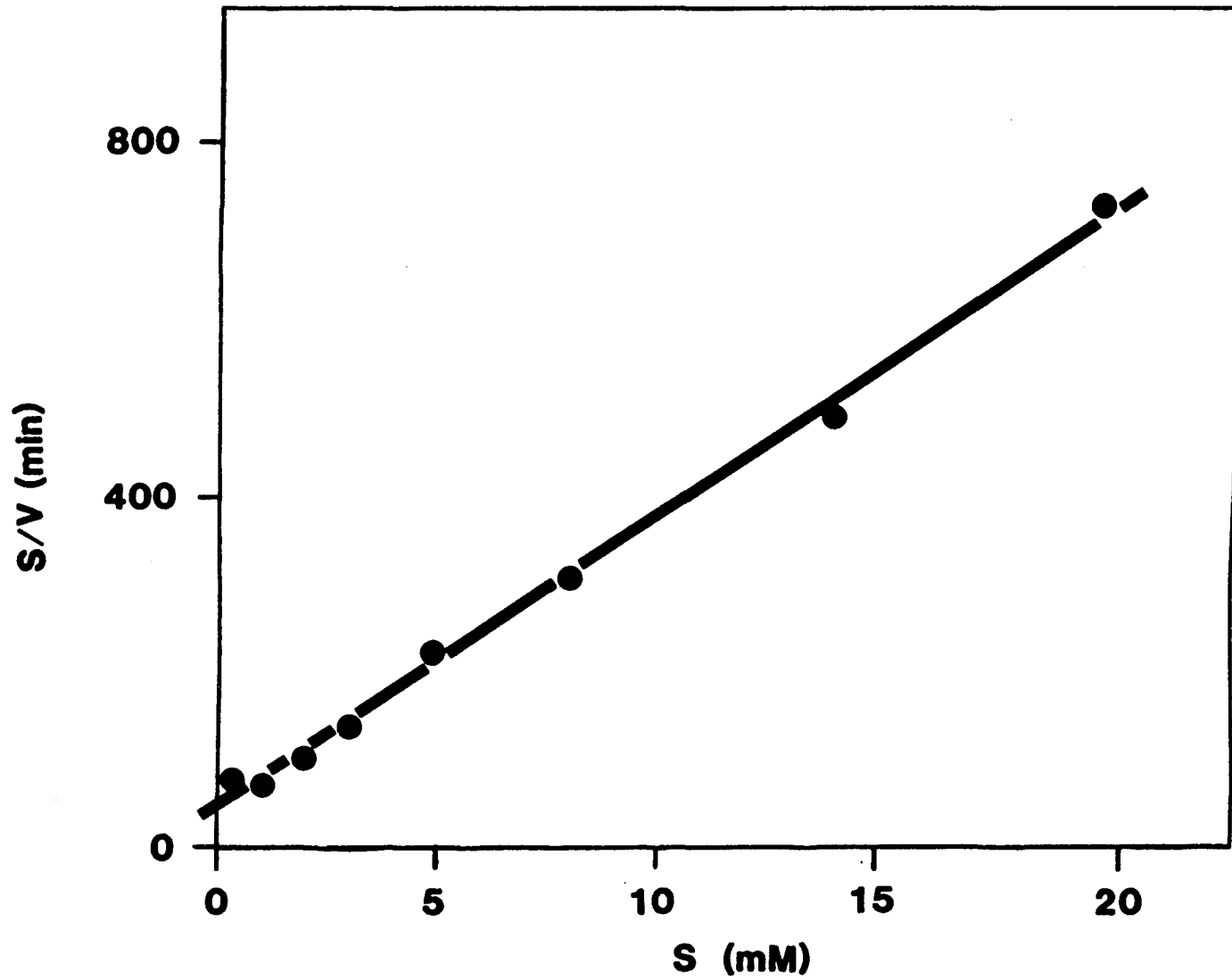




Figure 24. Hanes plot for typical GTF-I kinetic data obtained by varying the sucrose concentration. The reaction mixture contained 100 mM potassium phosphate buffer, pH 6.2, sucrose, 5  $\mu$ M dextran T10, and GTF-I (0.046 unit/ml).



with the substrate sucrose in the absence of added inhibitors. No sucrose substrate inhibition was observed for either enzyme at sucrose concentrations as high as 250 mM.

The  $K_m$ 's determined from such data with Cornish-Bowden direct linear plots were different for the two enzymes, but did not vary appreciably with enzyme concentration (Table 3).

### 3. Variation of Kinetic Constants with pH

For many enzymes, it is possible to obtain  $pK_a$  values for the ionizable groups in the active site by measuring the variation of the kinetic constants  $V_{max}$  and  $K_m$  with pH. The value of  $V_{max}/K_m$ , the apparent first-order rate constant for the enzyme reaction at low substrate concentrations, reflects the ability of the enzyme to form a complex with the substrate (Cleland, 1970). This complex is the first covalent intermediate in enzymes such as GTF, in which such an intermediate is formed. If two ionizable groups on the enzyme are involved in this complex formation, the ratio  $V_{max}/K_m$  will follow the equation:

$$(V_{max}/K_m) = \frac{(V_{max}/K_m)_{lim}}{1 + (H/K_{a1}) + (K_{a2}/H)} \quad (2)$$

where  $(V_{max}/K_m)_{lim}$  is the value of the ratio at the optimal hydrogen ion concentration,  $K_{a1}$  and  $K_{a2}$  are the acid dissociation constants for the ionizable groups, and  $H$  is the hydrogen ion concentration. The possibility of substrate ionization can be ignored in the case of GTF since the

TABLE 3.  $K_m$  Values for the GTF's at Varying Enzyme Concentrations

GTF-S

<u>RELATIVE ENZYME CONCENTRATIONS</u>	<u><math>K_m</math> (mM)</u>	<u>CONFIDENCE INTERVAL</u>
1.00 <sup>a</sup>	5.22 + 0.83 <sup>b</sup> - 0.13	72.4%
0.75	5.54 + 0.73 - 0.87	72.4%
0.50	4.60 + 0.40 - 0.38	69.4%

GTF-I

<u>RELATIVE ENZYME CONCENTRATIONS</u>	<u><math>K_m</math> (mM)</u>	<u>CONFIDENCE INTERVAL</u>
1.00 <sup>a</sup>	1.58 + 0.05 - 0.11	72.4%
0.75	1.60 + 0.28 - 0.24	74.0%
0.50	1.56 + 0.09 - 0.06	72.4%

<sup>a</sup>1.00 equals 0.177 unit/ml for GTF-S and 0.0613 unit/ml for GTF-I.

<sup>b</sup>Confidence intervals were calculated by the method of Porter and Trager (1977) using data generated from Eisenthal and Cornish-Bowden (1974) direct linear plots as described in APPENDIX A.

substrate sucrose is not ionizable. Equation (2) means that the value of  $V_{\max}/K_m$  at any pH equals its limiting value multiplied by the fraction of the enzyme that exists in the proper ionic state for complex formation. It should be pointed out that equation (2) represents a minimal mechanism in the sense that there is the possibility of more than two ionizable groups controlling the activity. However, the assumption of a minimum of two ionizable active site groups serves as a useful basis for discussion.

The values of  $V_{\max}$  and  $K_m$  at various pH's were measured for the GTF's at fixed dextran concentrations. Figures 25 and 26 show  $\log(V_{\max}/K_m)$  vs. pH plots for GTF-I and GTF-S respectively. A BASIC computer program was written which independently varied the values of  $(V_{\max}/K_m)_{\text{lim}}$ ,  $K_{a1}$ , and  $K_{a2}$  in equation (2) to obtain a best-fit curve which minimized the sum of the squares of the perpendicular distances from the experimental points to the curve. The best-fit curves are shown by the solid lines in Figures 25 and 26. Since the points below pH 4.8 for GTF-S show denaturation of the enzyme, these points were not used in the calculation of the predicted curve.

The GTF-I curve gives  $\text{pK}_a$  values of 4.65 and 6.59. The GTF-S curve gives values of 4.07 and 6.72. These values indicate that two ionizable groups, which may be the same in both enzymes, control the association of enzyme and sucrose.

Figure 25. Graph of  $\log(V_{\max}/K_m)$  vs. pH for GTF-I. The solid line is the best-fit curve in the form of equation (2) (see text). Error bars represent 69 - 74% non-parametric confidence intervals as calculated by the method of Porter and Trager (1977), using data from the direct linear plots. Calculation of these confidence intervals is described in APPENDIX A. Assays were run in sodium acetate/potassium phosphate buffer, 100 mM in each component, with sucrose and 5  $\mu$ M dextran T10. Values for the  $pK_a$ 's determined from the best-fit curve were 4.65 and 6.59.

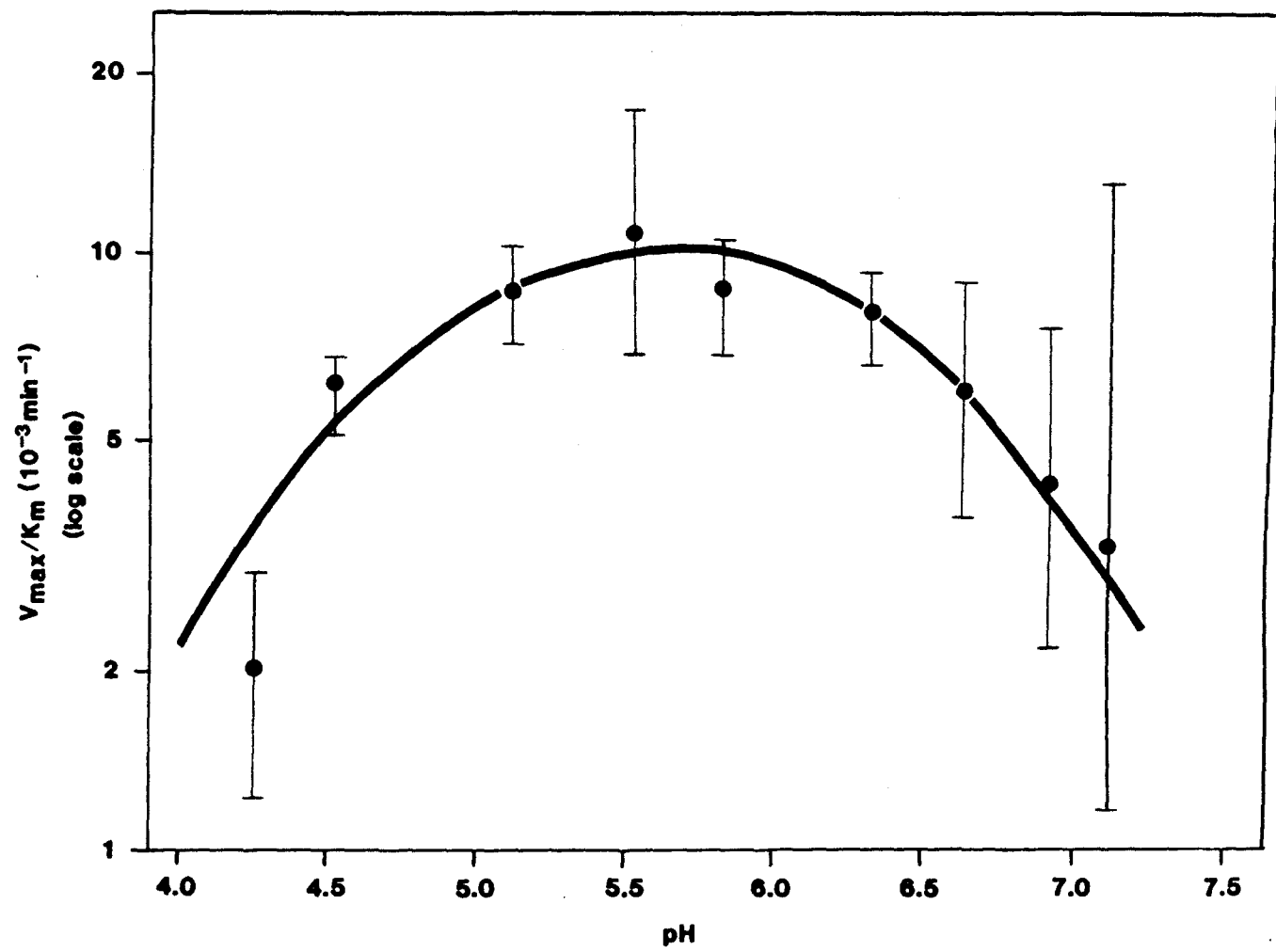
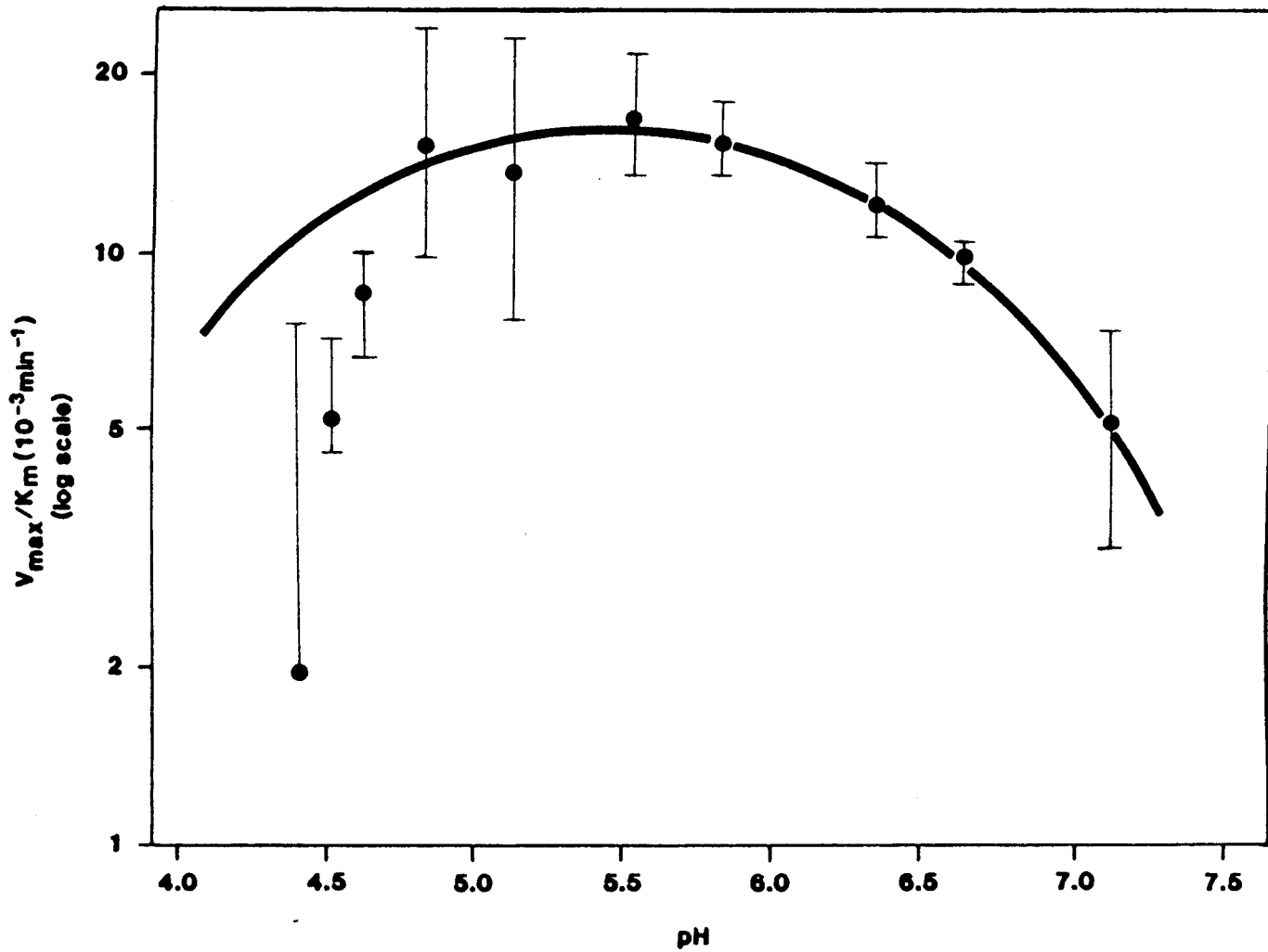


Figure 26. Graph of  $\log(V_{\max}/K_m)$  vs. pH for GTF-S. The solid line is the best-fit curve in the form of equation (2) (see text) with the three most acidic points not used in the calculation. Error bars represent 69 - 74% non-parametric confidence intervals (Porter and Trager, 1977), as described in Figure 25. Assays were run in sodium acetate/potassium phosphate buffer, 100 mM in each component, with sucrose and 1 mM dextran T10. Values for the  $pK_a$ 's determined from the best-fit curve were 4.07 and 6.72.





The variation of  $V_{\max}$  with pH reflects, if certain simplifying assumptions are true, the ionization of groups on the enzyme that are involved in the rate-limiting step (Cleland, 1970). If a single ionizable group that must be unprotonated for activity is involved in the rate-limiting step,  $V_{\max}$  follows the equation:

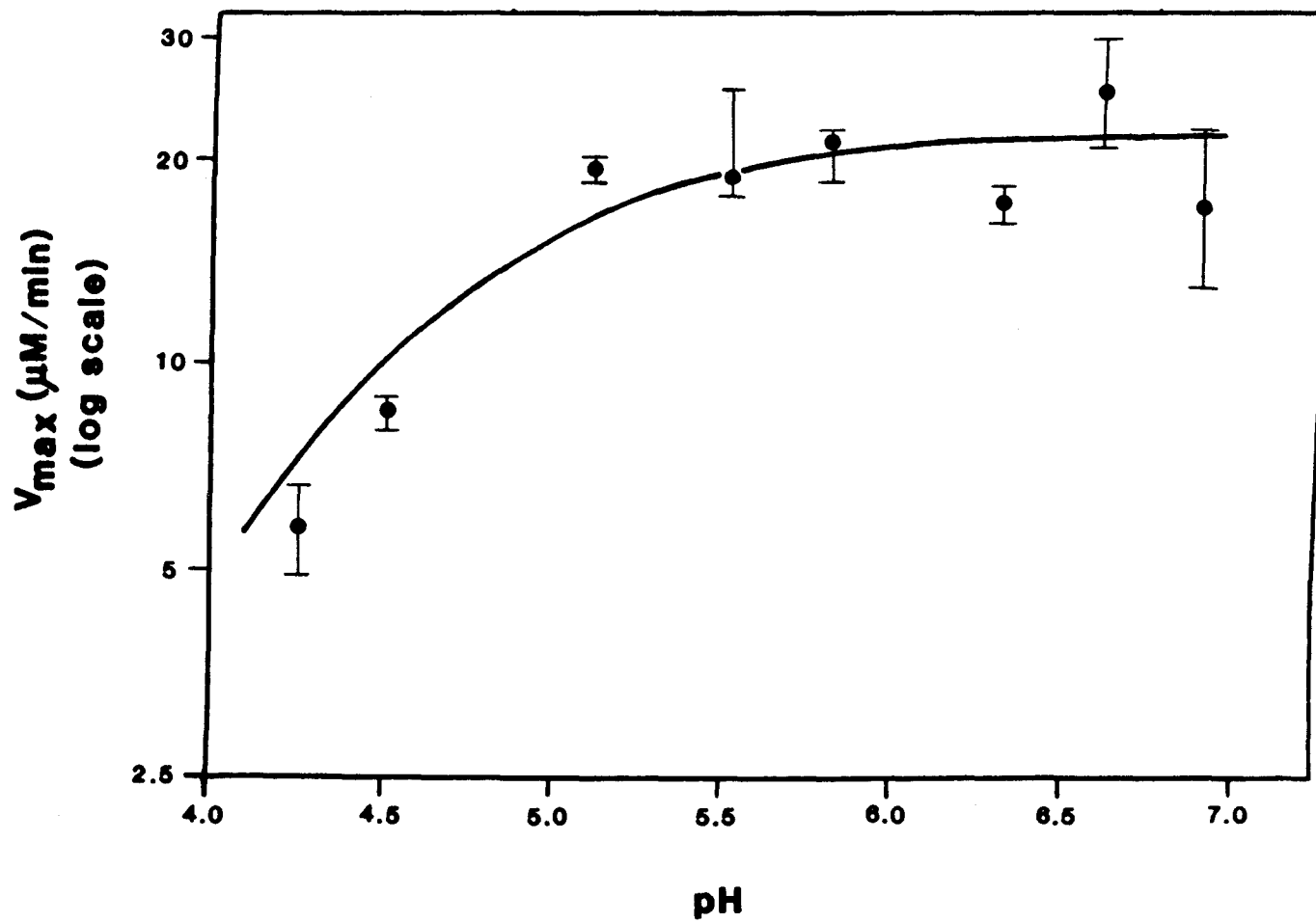
$$(V_{\max}) = \frac{(V_{\max})_{\lim}}{1 + (H/K_a)} \quad (3)$$

where  $(V_{\max})_{\lim}$  is the  $V_{\max}$  at high pH,  $K_a$  is the acid dissociation constant of the dissociable group, and H is the hydrogen ion concentration. Figure 27 shows a  $\log(V_{\max})$  vs. pH graph for GTF-I, including the best-fit curve in the form of equation (3). This curve gives a  $pK_a$  value of 4.55. Thus, a group with a  $pK_a$  of 4.55 appears to be involved in the catalytic reaction of GTF-I, and this group must be unprotonated for activity. For GTF-S, the  $V_{\max}$  values in the pH range 4.8 to 6.9 varied by only about 50% over the whole range. This range probably corresponds, for GTF-S, to the plateau region in Figure 27 for GTF-I. However, below pH 4.8, GTF-S denatured, so that the  $V_{\max}$  values obtained no longer reflected acid-base dissociation. Thus, a similar  $\log(V_{\max})$  vs. pH graph was not constructed for GTF-S.

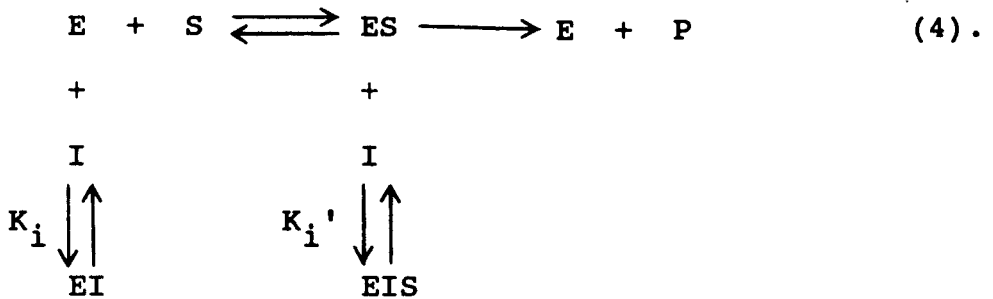
#### 4. Behavior of the GTF's Toward the Inhibitor Maltose

Since maltose was used to elute the GTF's from the affinity column, it was of interest to determine the type of inhibition it displays. A simple kinetic scheme for enzyme

Figure 27. Graph of  $\log(V_{\max})$  vs. pH for GTF-I. The solid line is the best-fit curve in the form of equation (3) (see text). The points represent the same  $V_{\max}$  data used to construct Figure 25. Error bars represent 69 - 74% non-parametric confidence intervals (Porter and Trager, 1977), as described in Figure 25. The  $pK_a$  determined from the best-fit curve was 4.55.



inhibition in general can be written as follows (Cornish-Bowden, 1979):



A so-called "mixed" inhibitor can bind either to the free enzyme (E), with a dissociation constant  $K_i$ , or to the enzyme-substrate complex (ES), with a dissociation constant  $K_i'$ . The type of inhibition displayed by a particular inhibitor can be determined by constructing linear regression plots of the Michaelis-Menton equation from kinetic data obtained in the absence and presence of inhibitor.

For GTF-S, Hanes plots, obtained by varying the sucrose concentration, were linear in the presence of maltose. Thus, in order to determine the form of inhibition, direct linear plots were first used to determine the values of  $K_m$  and  $V_{max}$  in the absence of maltose, as well as  $(K_m)_{app}$  and  $(V_{max})_{app}$  values at two different concentrations of maltose. The reciprocals of these values were then plotted on the axes of a Lineweaver-Burke type plot as shown in Figure 28. Since mixed inhibition is evident, the  $K_i$  and  $K_i'$  values were determined from the x-intercepts of the standard plots shown in Figure 29 (Cornish-Bowden, 1979). Because the  $K_i$  and  $K_i'$  values of 0.058 M and 0.227 M, respectively, are so

Figure 28. Lineweaver-Burke type plot of maltose inhibition data for GTF-S. Values of  $V_{\max}$  and  $K_m$  in the absence of maltose, and values of  $(V_{\max})_{\text{app}}$  and  $(K_m)_{\text{app}}$  in the presence of maltose were determined using Cornish-Bowden direct linear plots, and the reciprocals were used as the intercepts on the x- and y-axes. Mixed inhibition is indicated. Reaction mixtures contained 100 mM sodium acetate buffer, pH 5.5, sucrose, 1.0 mM dextran T10, and GTF-S (0.058 unit/ml).

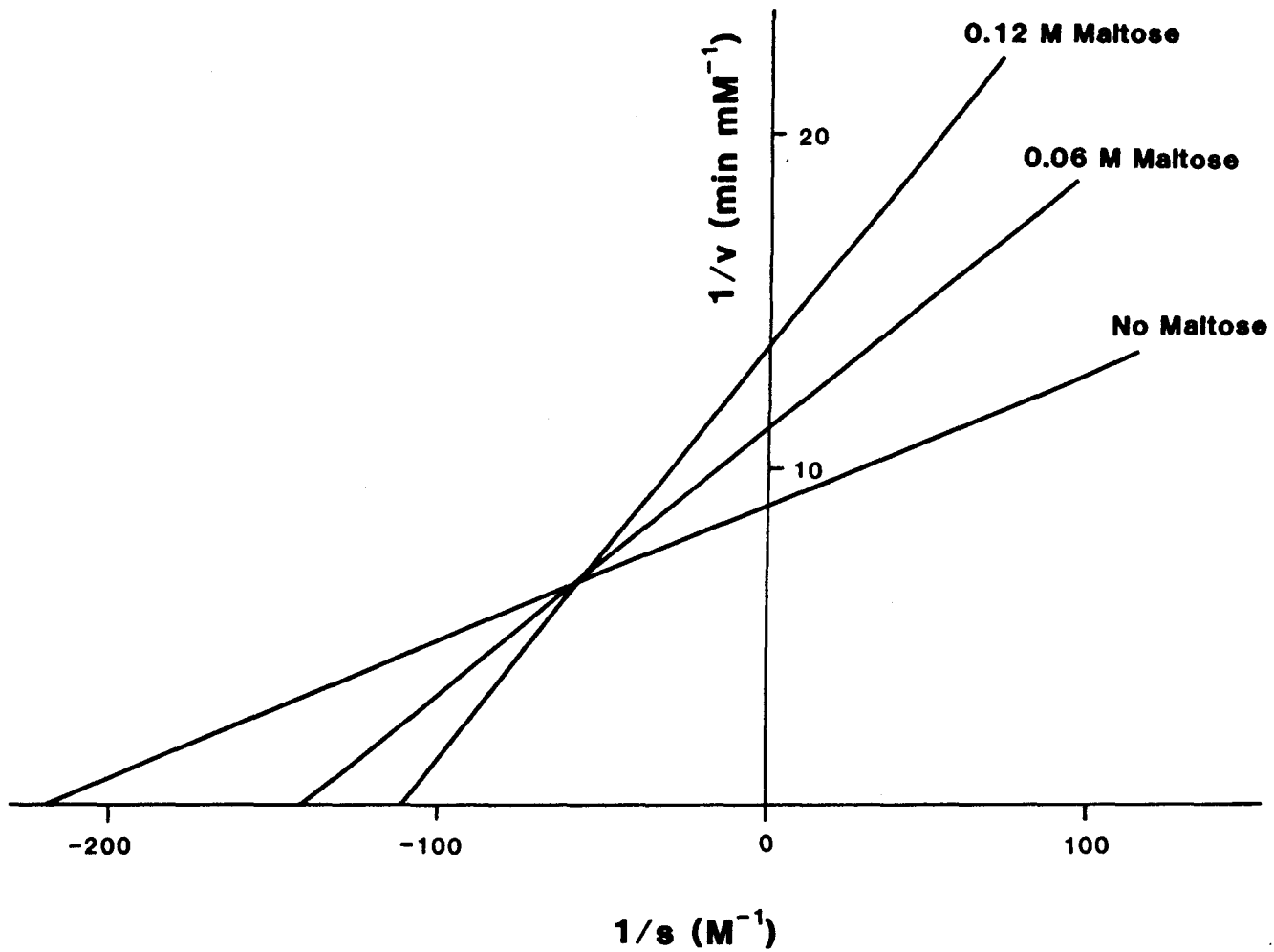
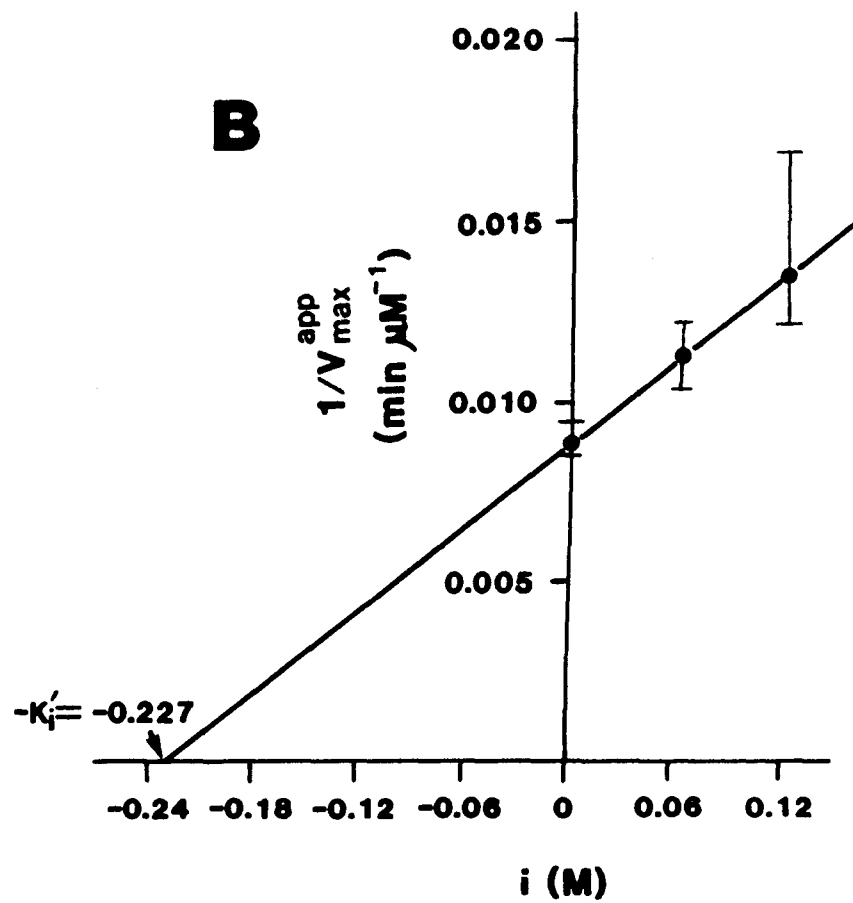
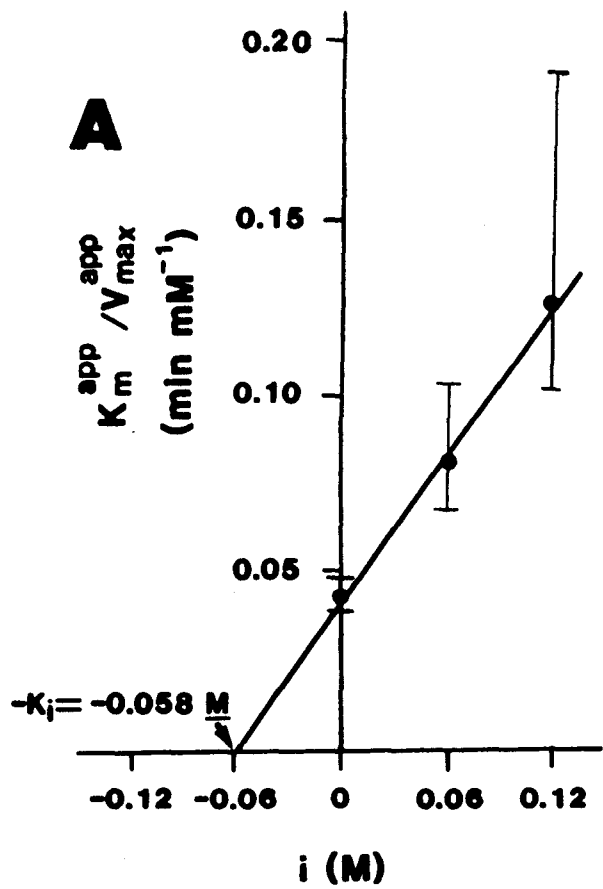


Figure 29. Graphs to determine  $K_i$  (Graph A) and  $K_i'$  (Graph B) for the inhibition of GTF-S by maltose. The data used were the same as for Figure 28. The x-intercept of a plot of  $(K_m)_{app}/(V_{max})_{app}$  vs.  $i$  is  $-K_i$  (Graph A), and the x-intercept of a plot of  $1/(V_{max})_{app}$  is  $-K_i'$  (Graph B). Error bars represent 69 - 74% non-parametric confidence intervals as calculated by the method of Porter and Trager (1977), using data from the direct linear plots. Calculations of these confidence intervals is described in APPENDIX A.





high and indicate such a low affinity of the enzyme for the inhibitor, high concentrations of maltose were required to elute the enzyme from Sephadex affinity columns.

Although maltose also inhibited GTF-I, the Hanes plots for this enzyme in the presence of maltose were not linear, but were concave upward, as is especially apparent near the y-axis (Figure 30). Such behavior is indicative of sucrose substrate cooperativity, which results in sigmoidal, rather than hyperbolic,  $v$  vs.  $s$  plots (Cornish-Bowden, 1979). As discussed later, this cooperativity is probably due to the aggregation behavior of GTF-I. Since the presence of maltose induced substrate cooperativity, the form of maltose inhibition of GTF-I could not be determined.

##### 5. Aggregation of GTF-I and Its Effect on Enzyme Activity

Several types of data gathered in this work demonstrate that GTF-I aggregates, and that this aggregation affects its catalytic activity.

The gel filtration column in Figure 7 indicates that some fraction of GTF activity aggregated in the absence of KCl and eluted in the void volume. This component was GTF-I, as demonstrated by the Coomassie blue-stained native PAGE gel shown in Figure 31. Lane 1 was GTF-S, which readily entered the gel matrix during electrophoresis. However, GTF-I, which was in lane 2, aggregated and failed to enter the gel matrix, forming a sharp band at the interface

Figure 30. Hanes plot for the inhibition of GTF-I by maltose. In the absence of maltose, the graph was linear, but in the presence of maltose, substrate cooperativity appeared, as evidenced by the upward concavity. The reaction mixtures contained 100 mM potassium phosphate buffer, pH 6.2, sucrose, 5.0  $\mu$ M dextran T10, and GTF-I (0.046 unit/ml).

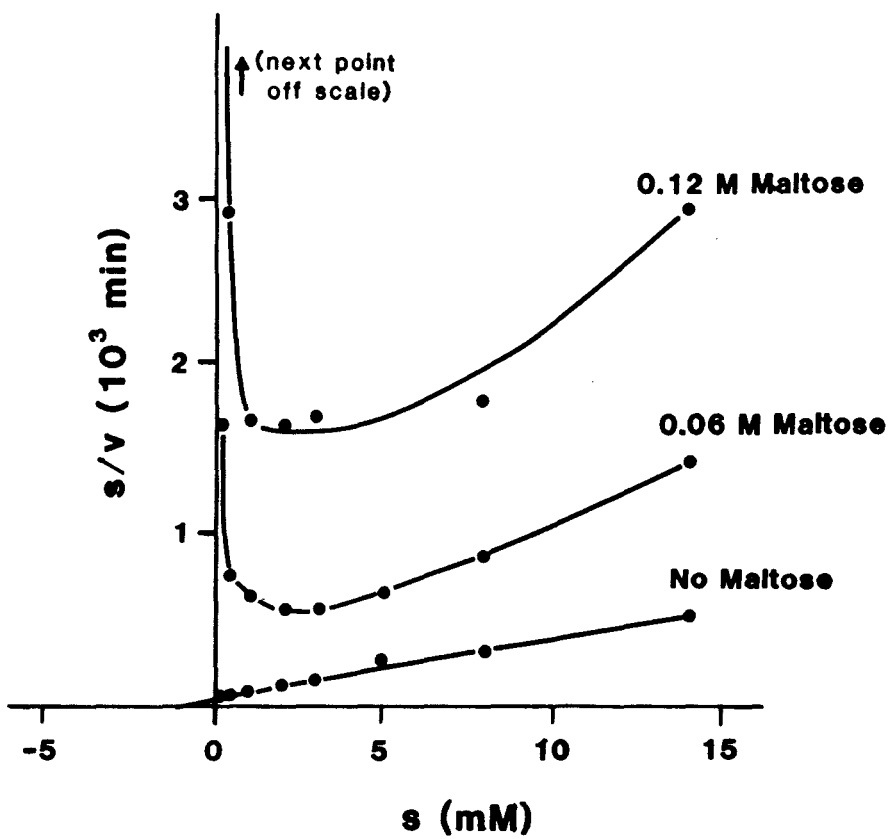


Figure 31. Coomassie blue stained native PAGE gel of purified GTF's. Samples (1.0  $\mu$ l each), including 0.01% bromophenol blue as a tracking dye, were applied to a precast 8 - 25% gradient PhastSystem polyacrylamide gel with a stacking region. Lane 1: Purified GTF-S, 447 ng. Lane 2: Purified GTF-I, 191 ng.

Separating  
Gel Boundary -



1

2

between the stacking and separating gel regions. The aggregation of GTF-I also explains why the enzyme smeared on isoelectric focusing gels (Figure 14), while GTF-S focused as two sharp bands.

Aggregation of GTF was also shown to affect enzymatic activity. The upward concavity of the GTF activity versus enzyme concentration curve in the absence of ammonium sulfate, as shown in Figure 8A, can be explained by assuming that GTF exists in a less active, dissociated form, as well as a more active, aggregated form. As the enzyme concentration increased, more GTF aggregated by a mass action effect, so that the resulting curve was concave upward. The linearizing of the GTF activity by the addition of ammonium sulfate (Figure 8B) can be explained by supposing that this salt, a potent salting-out agent (Hatefi and Hanstein, 1969), caused the aggregation of GTF to the more active form at all enzyme concentrations.

The purified enzymes were used to show that GTF-I was the enzyme responsible for this behavior. Figure 32 shows that purified GTF-S activity was linear in the absence of ammonium sulfate. However, Figure 33A shows that purified GTF-I activity was concave upward in the absence of ammonium sulfate, just as for the crude GTF preparation. As shown in Figure 33B, purified GTF-I activity was linear only when ammonium sulfate was added to the assay mixture.

Figure 32. GTF activity (initial rate) with varying purified GTF-S concentration in the absence of ammonium sulfate. The points represent the average of duplicate spottings. The assay buffer was 50 mM sodium acetate, pH 5.5, containing 20 mM sucrose and 10  $\mu$ M dextran T10. The 1.0 dilution of GTF-S was 0.204 unit/ml. The predicted line is drawn from the highest activity point through zero.



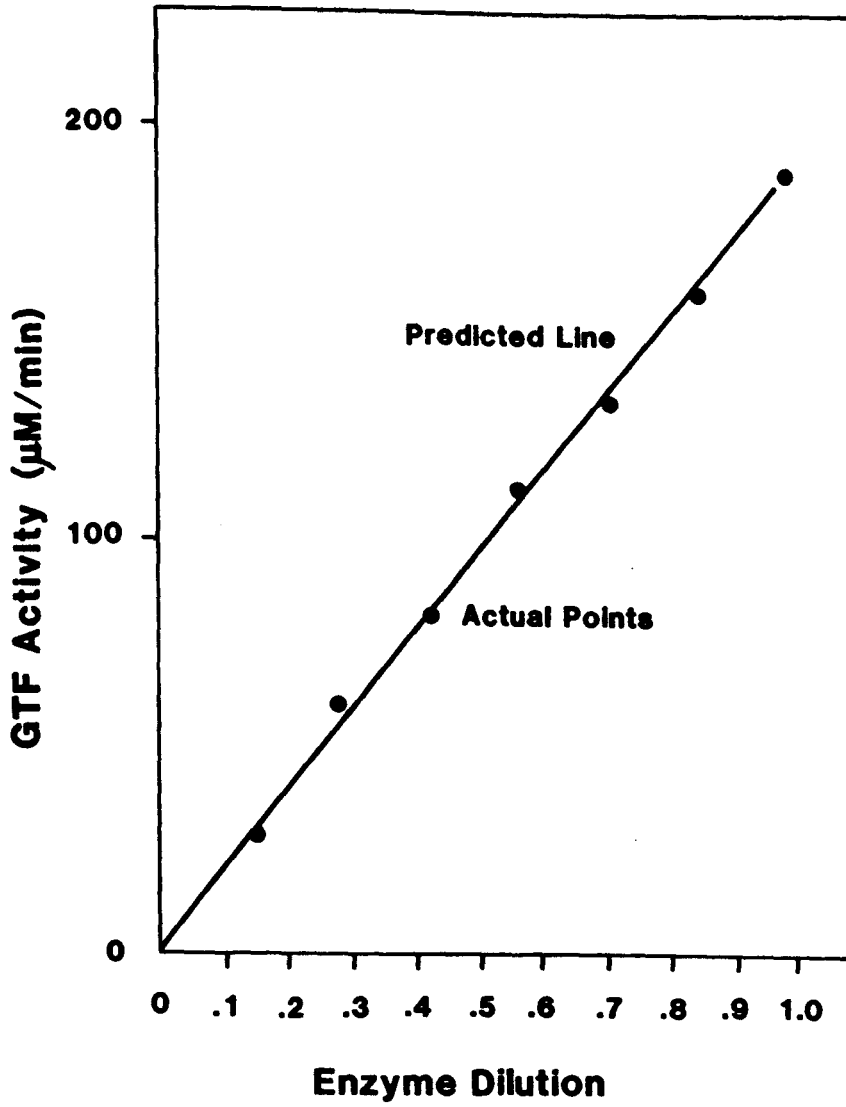
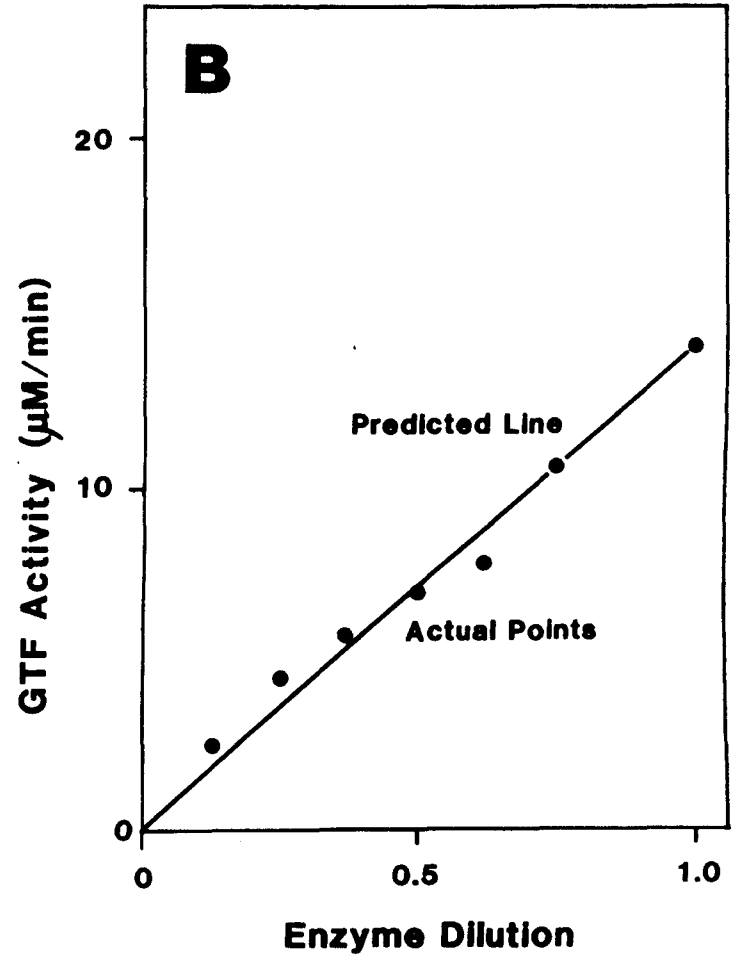
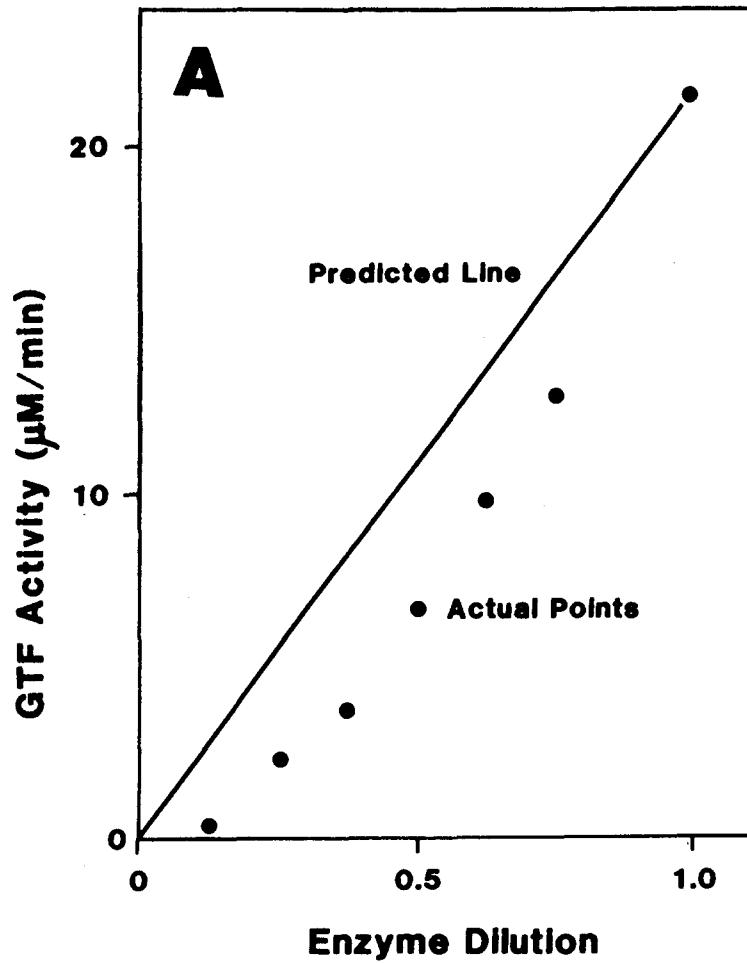


Figure 33. GTF activity (initial rate) with varying purified GTF-I concentration. The assay buffer was 50 mM sodium acetate buffer, pH 5.5, containing 20 mM sucrose and 10  $\mu$ M dextran T10. The 1.0 dilution of GTF-I was 0.061 unit/ml. The points represent the average of duplicate spottings and show the measured activity in the absence (A) and presence (B) of 1.55 M ammonium sulfate. The predicted lines are drawn from the highest activity point through zero.



The results of the maltose inhibition study can also be explained by the GTF-I aggregation. GTF-S followed Michaelis-Menton kinetics in the presence or absence of maltose, while maltose induced substrate cooperativity in GTF-I (Figure 30). This result can be explained by assuming that maltose dissociated GTF-I to the less active form, while sucrose aggregated it to the more active form. Such sucrose-induced aggregation could explain the observed substrate cooperativity.

These experiments demonstrate that the aggregation behavior of GTF-I has a significant influence on its catalytic activity.

#### F. NMR Study of GTF Dextran Products

Once the kinetic behavior of the purified GTF's had been studied, optimal conditions for using the enzymes to synthesize dextran products for the NMR study could be developed. The knowledge gained about such enzymatic properties as pH dependence and behavior of the enzymes toward substrates was used to design the product synthesis experiments.

##### 1. Goals of the NMR Experiments

The main idea of the NMR study was to determine the structures of the products formed as the GTF's progressively add glucose units to primer dextran. Because of the large rate acceleration in the presence of primer dextran, it is important to study the GTF-catalyzed modification of the

primer, a reaction which has previously been largely uninvestigated.

The basics of the method used are as follows. It is possible in the case of a GTF reaction to calculate, using the filter paper spotting assay, the ratio of glucose residues added during catalysis per glucose residue in the original primer dextran. This ratio can be calculated from the known original concentration of primer dextran and the number of  $\mu$ moles of glucose added per ml as determined by the assay. The relative peak areas in C-13 NMR spectra of the products can then be used to determine percentages of different bond types present in the products. When the amount of glucose added to the primer is compared to the percentages of bond types, conclusions can be drawn about the structures of the branches being added to the primer. Thus, products with increasing amounts of glucose added were synthesized with each enzyme and analyzed by C-13 NMR to give the desired information about the modification of primer dextran by the GTF's.

The validity of using relative peak areas from product spectra to estimate bond percentages is fully examined in the DISCUSSION section. In regard to this question, spin-lattice ( $T_1$ ) relaxation times of important peaks in the product spectra were measured and are reported later in Table 6.

## 2. Specifics of the NMR Method

Products were synthesized by incubating the individual, purified enzymes with substrates at 37°C for 16 hr. (GTF-S products) or 24 hr. (GTF-I products). Large reaction volumes (5 - 25 ml) were used in order to obtain sufficient quantities of products for NMR spectra. The large reaction mixtures for product synthesis were similar to the normal assay mixtures for kinetic studies except for a few variations.

a. Since no sucrose substrate inhibition was observed for either enzyme, a large sucrose concentration (200 mM, 6.85% w/v) was used. Thus, the percent conversion of sucrose was always less than 20%.

b. Since dextran T70 was a better primer than T10 on a weight basis, T70 was used as the primer.

c. Although the GTF-I product syntheses were done at pH 6.2 in phosphate buffer, just as for kinetics, GTF-S syntheses were not done in the pH 5.5 sodium acetate buffer used for kinetics. Potassium phosphate buffer, pH 5.7, was used in order to eliminate the possibility of acetate carbon peaks appearing in NMR spectra.

d. No [U-<sup>14</sup>C]sucrose was included in product synthesis mixtures in order to avoid the difficulties involved in handling radioactive samples and transporting them to Argonne for NMR experiments. Such a procedure would also waste expensive radioactivity in large reaction mixtures.

Therefore, the amount of glucose added to primer was determined in duplicate small assay mixtures (150 - 250  $\mu$ l), identical to the large ones except for the addition of [ $U$ - $^{14}C$ ]sucrose. The percent soluble and insoluble synthesis for each product was also determined with these small assay mixtures. GTF-S was determined to synthesize in all cases products that were, within the limits of experimental error, 100% soluble. GTF-I synthesized in all cases products that were greater than 90% insoluble.

Products were collected by precipitation with two volumes of 100% ethanol (a step which was not done for the water-insoluble GTF-I products), centrifugation at 10,000xg for 30 minutes, resuspension in water, dialysis against water, and lyophilizing. The dialysis removed sugars and buffer salts, which were found to coprecipitate with the products. This procedure gave an 85% recovery of unmodified dextran T70 in test experiments.

The spectra of several types of products were recorded. Products with various increasing ratios of glucose added per glucose in the primer were obtained by varying the concentration of T70 in reaction mixtures. Preliminary experiments were used to determine the concentration of T70 needed to obtain a particular ratio. For GTF-S, products were synthesized with ratios of 0.65:1, 1.42:1, and 6.0:1, and for GTF-I, 1.8:1 and 4.5:1. A de novo GTF-S product was synthesized in a reaction mixture containing sucrose but no

primer. However, no de novo GTF-I product could be produced, since the synthetic activity without primer was so small. A "blank," which consisted of dextran T70 incubated without enzyme in a standard reaction mixture, was isolated by the same procedure as the other products. Also, GTF-S was tested for branching activity by incubating the enzyme with T70, but without sucrose, in a standard reaction mixture, and isolating the product. The NMR spectra of all these products were recorded and used in this study.

For NMR spectra, water soluble samples were dissolved in  $D_2O$ , and water-insoluble samples were dissolved in  $D_2O$  adjusted to pD 14 with sodium deuterioxide (NaOD). At pD 14, the water-insoluble samples were soluble and formed mobile, slightly viscous solutions. Samples were run in 10 mm NMR tubes designed for high-field instruments. Spectra of water-soluble samples were run at  $70^\circ C$  because of the increased sensitivity and resolution at high temperatures (Seymour et al., 1979b), but water-insoluble samples were run at  $25^\circ C$  because they caramelized at pD 14 above room temperature. Proton-noise decoupling was used for all routine spectra with a 5 second pulse delay inserted. With the sample sizes used, this procedure gave spectra with a reasonable signal to noise ratio in about 2000 scans. For analysis of spectra, ratios of types of carbon species present were calculated using the peak areas, or integrals, corresponding to those peaks.  $T_1$  relaxation times were



measured by the inversion-recovery method (Abraham and Loftus, 1980), using a computer program for this purpose supplied by Bruker.

### 3. Results of the NMR Study

It has already been discussed in the REVIEW OF THE RELATED LITERATURE section how NMR can be used to elucidate dextran structure and at what ppm values important peaks in the spectra usually appear (Figure 5 and Table 1). The following two tables serve as the basis for the discussion of the NMR results obtained in our study. Table 4 gives the chemical shifts of the carbon species of interest determined for the GTF dextran products. Table 5 shows ratios of various bond types calculated from the values of integrals of the corresponding peaks in the GTF dextran spectra. In Table 5, the ratio of glucose added per glucose in the primer is shown for each sample where applicable. The Anomeric 1,6:1,3 column gives the ratio, expressed as percentages, of the areas of the peaks due to  $\alpha$ -1,6 and  $\alpha$ -1,3-linked anomeric carbons. The column 3 and 6 Carbons 1,6:1,3 gives the ratio of the areas of the peaks due to the 6 and 3-carbons that were bound  $\alpha$  to the anomeric carbon. Of these two ratios, the anomeric 1,6:1,3 ratio was given more weight for structural analysis, since the same carbon atom was used to calculate the percentages of  $\alpha$ -1,6 and  $\alpha$ -1,3 bonds. However, the 1,6:1,3 ratios calculated using the 3- and 6-carbon peaks were almost identical for all the

TABLE 4. Positions of Peaks in C-13 NMR Spectra of GTF Dextran Products<sup>a</sup>

Enzyme	Ratio of Glucose Added per Glucose in Primer	Anomeric $\alpha$ -1,3	Anomeric $\alpha$ -1,6	3-Carbon $\alpha$ -1,3	6-Carbon $\alpha$ -1,6	Free 6-Carbon	Sample Concentration (% w/v)
GTF-S	0.65 : 1	100.65 <sup>a</sup>	99.21	82.44	67.38	62.10	4.2
S	1.42 : 1	100.67	99.26	82.44	67.38	62.11	2.0
S	6.00 : 1	100.66	99.26	82.41	67.31	62.12	3.5
S	<u>de novo</u>	100.66	99.27	82.44	67.38	62.11	2.8
S	Branching	100.58	99.23	82.71	67.59	62.25	1.6
-----	Dextran T70 (dilute)	-----	99.20	-----	67.39	62.15	3.2
-----	Dextran T70 (concentrated)	100.53 100.78	99.16	82.64 83.20	67.59	62.25	33.3
GTF-I	1.80 : 1	101.17	98.85	83.63	66.45	61.87	2.3
I	4.50 : 1	101.21	98.95	83.71	66.53	61.95	1.1

<sup>a</sup>Chemical shifts are expressed in ppm relative to tetramethylsilane. An external chloroform standard was used to calculate the shifts. GTF-S and Dextran T70 spectra were run in D<sub>2</sub>O at 70°C, while GTF-I spectra were run in D<sub>2</sub>O at pD 14 and 25°C.

TABLE 5. Ratios of Carbon Species in GTF Dextran Products as Determined by Integration of C-13 NMR Peaks

Enzyme	Ratio of Glucose Added per Glucose in Primer	Anomeric 1,6 : 1,3	3 and 6 Carbons 1,6 : 1,3	Ratio of Free 6 : 1,3	Predicted Ratio <sup>b</sup> 1,6 : 1,3
-----	Primer <sup>a</sup>	96.2 : 3.8	96.7 : 3.3	1.13 : 1	-----
GTF-S	0.65 : 1	79.7 : 20.3	81.2 : 18.8	1.00 : 1	84.6 : 15.4
S	1.42 : 1	74.1 : 25.9	75.0 : 25.0	1.10 : 1	78.8 : 21.2
S	6.00 : 1	68.8 : 31.2	74.5 : 25.5	1.08 : 1	70.9 : 29.1
S	<u>de novo</u>	66.7 : 33.3	71.1 : 28.9	1.03 : 1	-----
S	Branching	94.8 : 5.2	93.2 : 6.8	1.00 : 1	-----
GTF-I	1.80 : 1	24.5 : 75.5	22.1 : 77.9	1.11 : 1	34.4 : 65.6
I	4.50 : 1	11.7 : 88.3	11.4 : 88.6	0.98 : 1	17.5 : 82.5

<sup>a</sup>Concentrated dextran T70 (33% w/v in D<sub>2</sub>O).

<sup>b</sup>Predicted based on GTF-S adding chains containing a 2:1 ratio of 1,6 to 1,3 bonds and GTF-I adding 100% 1,3 bonds. Primer dextran is assumed to contain a 96.2 : 3.8 ratio of 1,6 to 1,3 bonds as determined by NMR for concentrated dextran T70.

applicable samples, as shown in Table 5. The Ratio of Free 6:1,3 column gives the ratio of the area of the peak due to free-6-carbons to that of the  $\alpha$ -1,3-linked anomeric carbons. The individual spectra used to generate these tables are discussed below.

The spectrum of the product synthesized by GTF-S de novo from sucrose with no primer present is shown in Figure 34. Table 5 shows that the ratio of  $\alpha$ -1,6 to  $\alpha$ -1,3 bonds is 2 to 1 and that the free 6:1,3 ratio is 1. Both the 1,6:1,3 and free 6:1,3 ratios obtained strongly support the comb dextran structure shown in Figure 6. The spectra of products synthesized in the presence of primer can be compared to this de novo spectrum.

The spectrum of unmodified dextran T70 from the "blank" reaction mixture is shown in Figure 35. Since no  $\alpha$ -1,3 peaks are visible, the T70 is essentially a linear  $\alpha$ -1,6-linked polymer. (A more concentrated T70 sample discussed later showed that about 4%  $\alpha$ -1,3 bonds were also present.) The free-6 peak is very small, as expected for a nearly linear molecule. This T70 sample had a concentration similar to those of the glucan products synthesized by GTF and can be used for comparison to these spectra.

It has not been previously determined whether GTF-S adds to primer dextran a 2:1 ratio of  $\alpha$ -1,6 to  $\alpha$ -1,3 bonds, as it produces de novo. If it does, predicted ratios can be calculated assuming a 2:1 ratio of addition of  $\alpha$ -1,6 and

Figure 34. C-13 NMR spectrum of the de novo GTF-S product (28 mg/ml). The reaction mixture for product synthesis contained 100 mM potassium phosphate buffer, pH 5.7, 200 mM sucrose, and GTF-S (0.117 unit/ml) with no primer dextran present. The spectrum was run in D<sub>2</sub>O at 70°C with 9900 scans. The carbon species giving rise to important peaks in the spectrum are indicated.

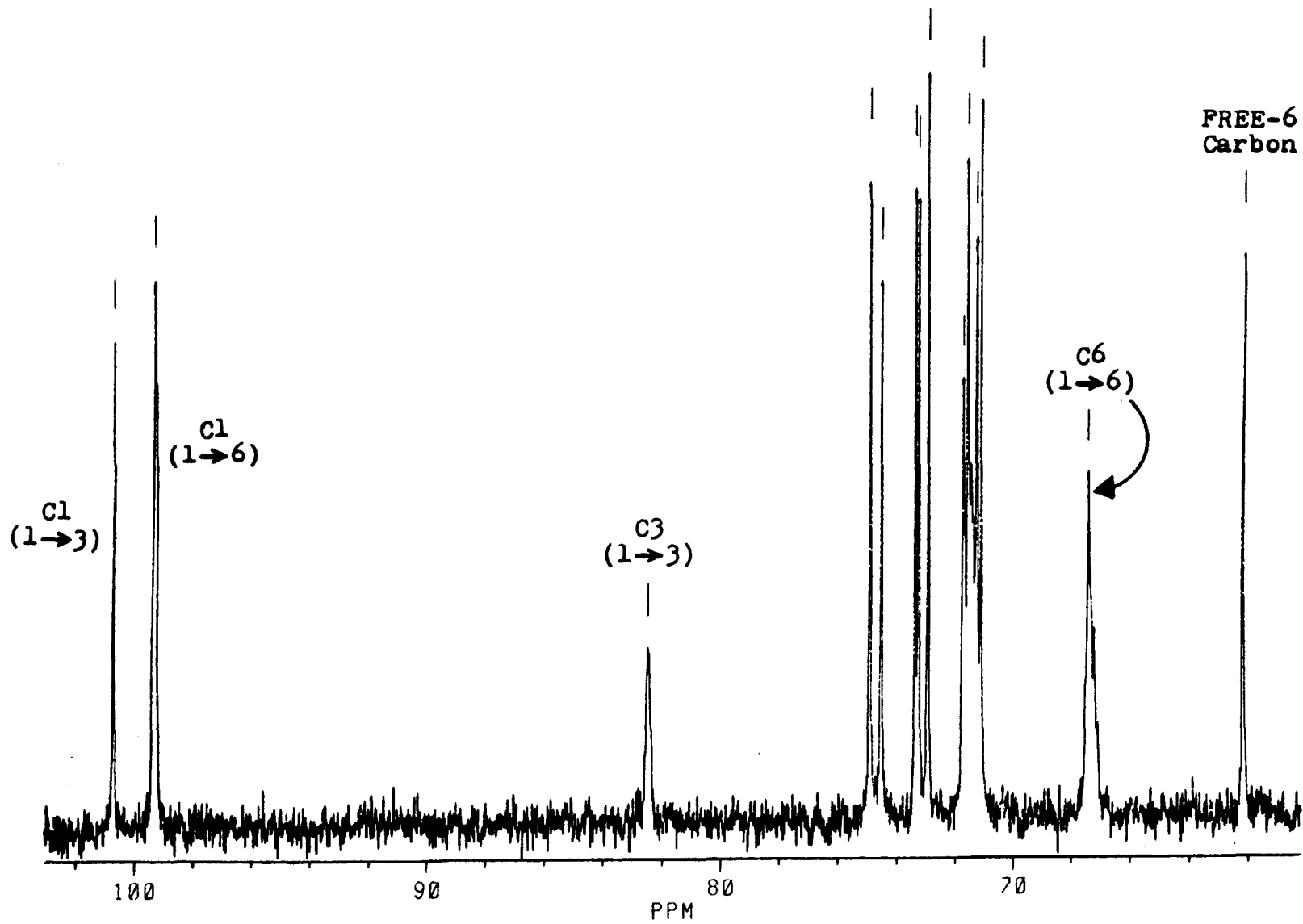
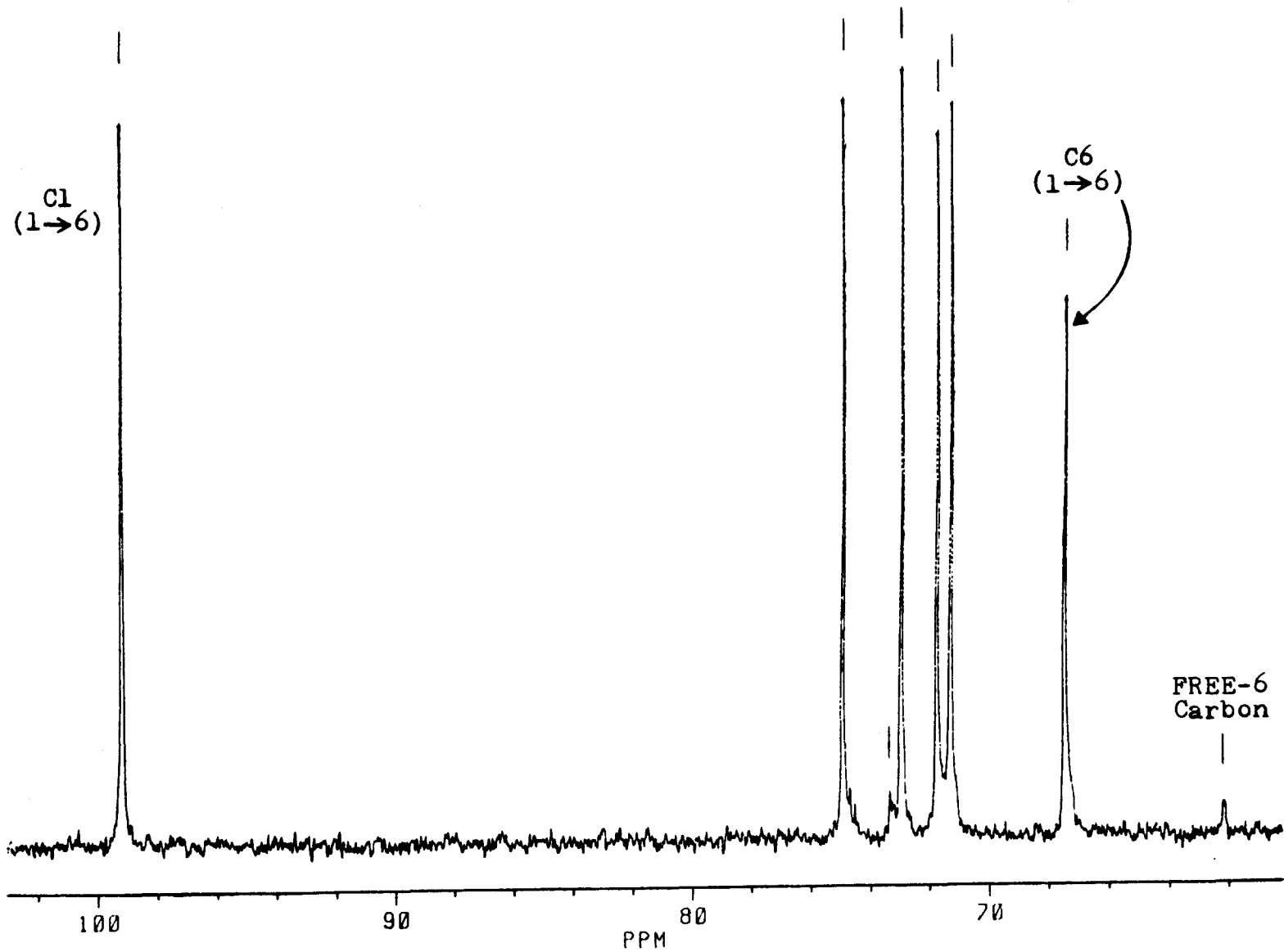


Figure 35. C-13 NMR spectrum of dextran T70 (32 mg/ml). The sample was incubated overnight in a standard GTF-S reaction mixture, but without enzyme, and isolated by ethanol precipitation the same as for other GTF-S samples. The spectrum was run in D<sub>2</sub>O at 70°C with 2910 scans.





$\alpha$ -1,3 bonds to primer dextran. These predicted ratios are tabulated in the last column of Table 5.

Products resulting from the progressive addition of glucose to primer dextran by GTF-S were studied to answer this question. The spectrum of the 0.65:1 GTF-S product is shown in Figure 36. Small peaks at the  $\alpha$ -1,3 positions are present, and the calculated 1,6:1,3 ratio is close to the predicted ratio. Furthermore, one free-6 carbon was formed for every  $\alpha$ -1,3 bond added (free 6:1,3 = 1), as predicted by the comb dextran model. The spectrum of the 1.42:1 GTF-S product is shown in Figure 37. The  $\alpha$ -1,3 peaks are larger, the 1,6:1,3 ratio is close to the predicted ratio, and the free 6:1,3 ratio is again 1. The GTF-S 6.0:1 product spectrum is shown in Figure 38. The  $\alpha$ -1,3 peaks are now quite large, the 1,6:1,3 ratio is again close to the predicted ratio, and the free 6:1,3 ratio is still 1. These spectra strongly suggest that GTF-S added, to primer dextran, branches with a structure similar to the de novo comb structure. It can also be deduced that only a few branch points were added to the primer. If numerous  $\alpha$ -1,3 branches had been added initially, a "burst" of  $\alpha$ -1,3 bond formation, reflected in a greater proportion of  $\alpha$ -1,3 bonds than the predicted ratio would suggest, should be observed, especially in the 0.65:1 and 1.42:1 spectra. However, the actual 1,6:1,3 ratio was close to the predicted ratio in all cases.

Figure 36. C-13 NMR spectrum of the 0.65:1 GTF-S product (42 mg/ml). The reaction mixture for product synthesis contained 100 mM potassium phosphate buffer, pH 5.7, 200 mM sucrose, 142.9  $\mu$ M dextran T70, and GTF-S (0.073 unit/ml). The spectrum was run in D<sub>2</sub>O at 70°C with 3570 scans.

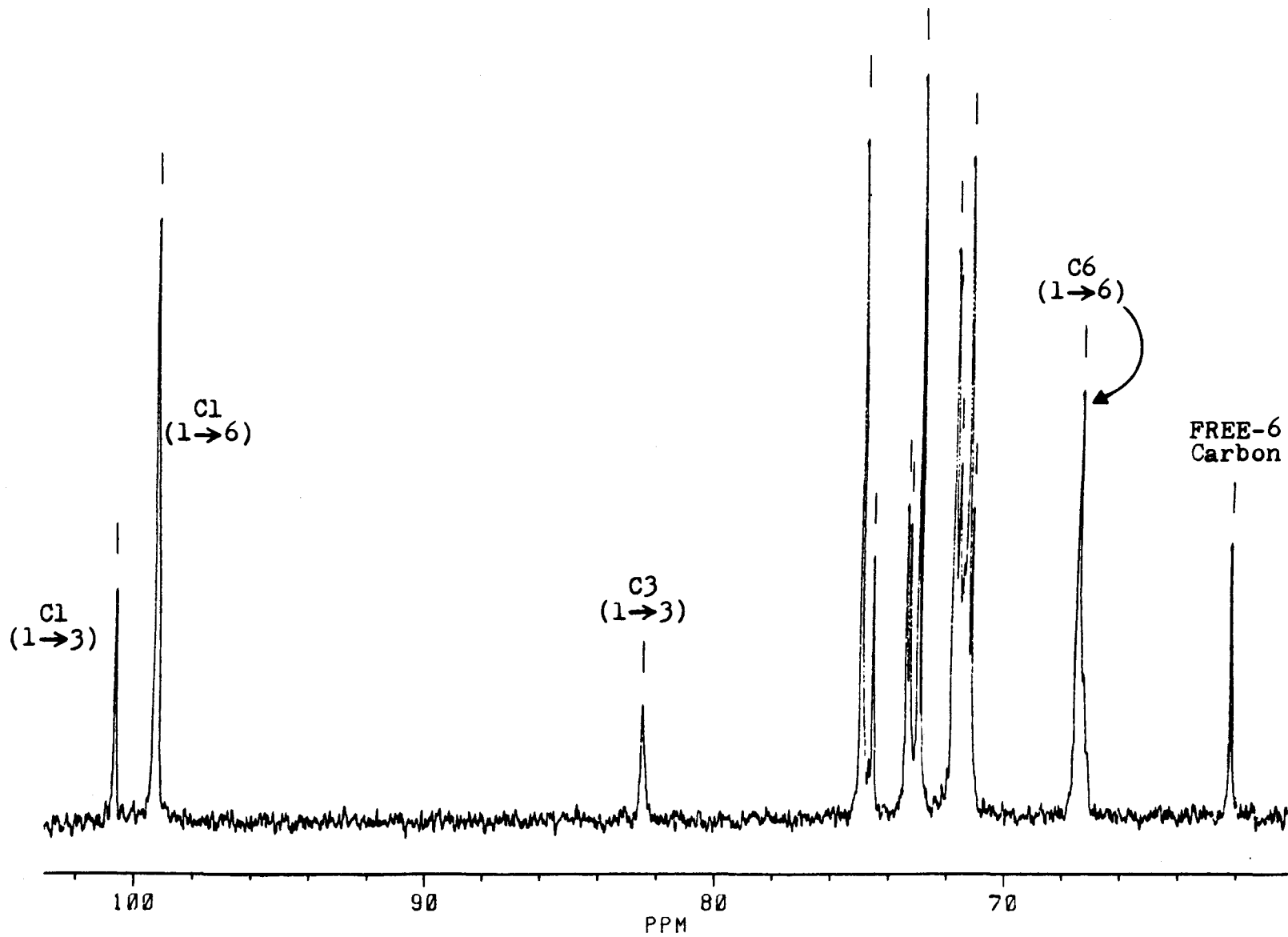


Figure 37. C-13 NMR spectrum of the 1.42:1 GTF-S product (20 mg/ml). The reaction mixture for product synthesis was the same as for Figure 33 but with 57.1  $\mu$ M dextran T70. The spectrum was run in D<sub>2</sub>O at 70°C with 9780 scans.

C1  
(1→3)

C1  
(1→6)

C3  
(1→3)

C6  
(1→6)

FREE-6  
Carbon

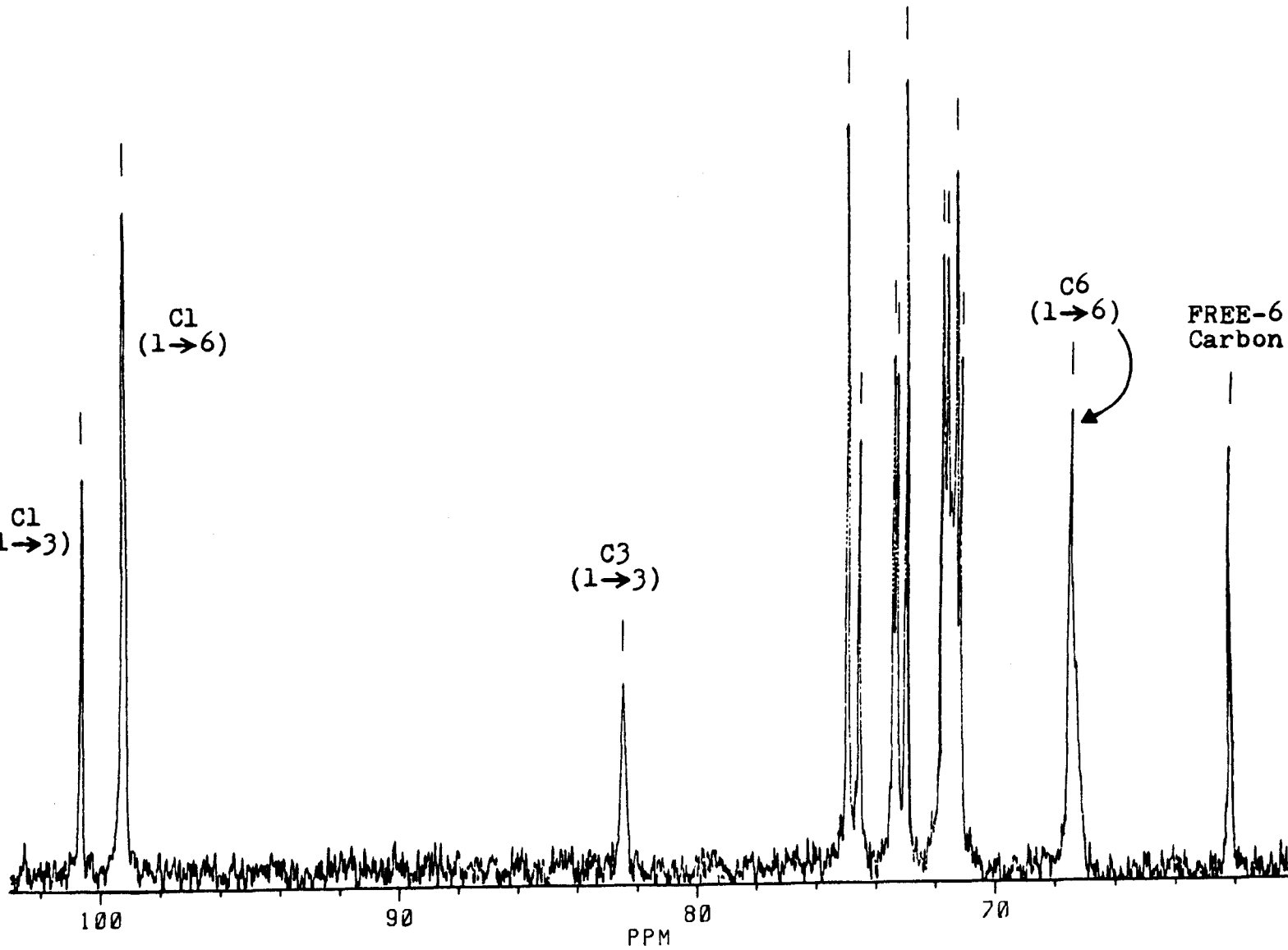
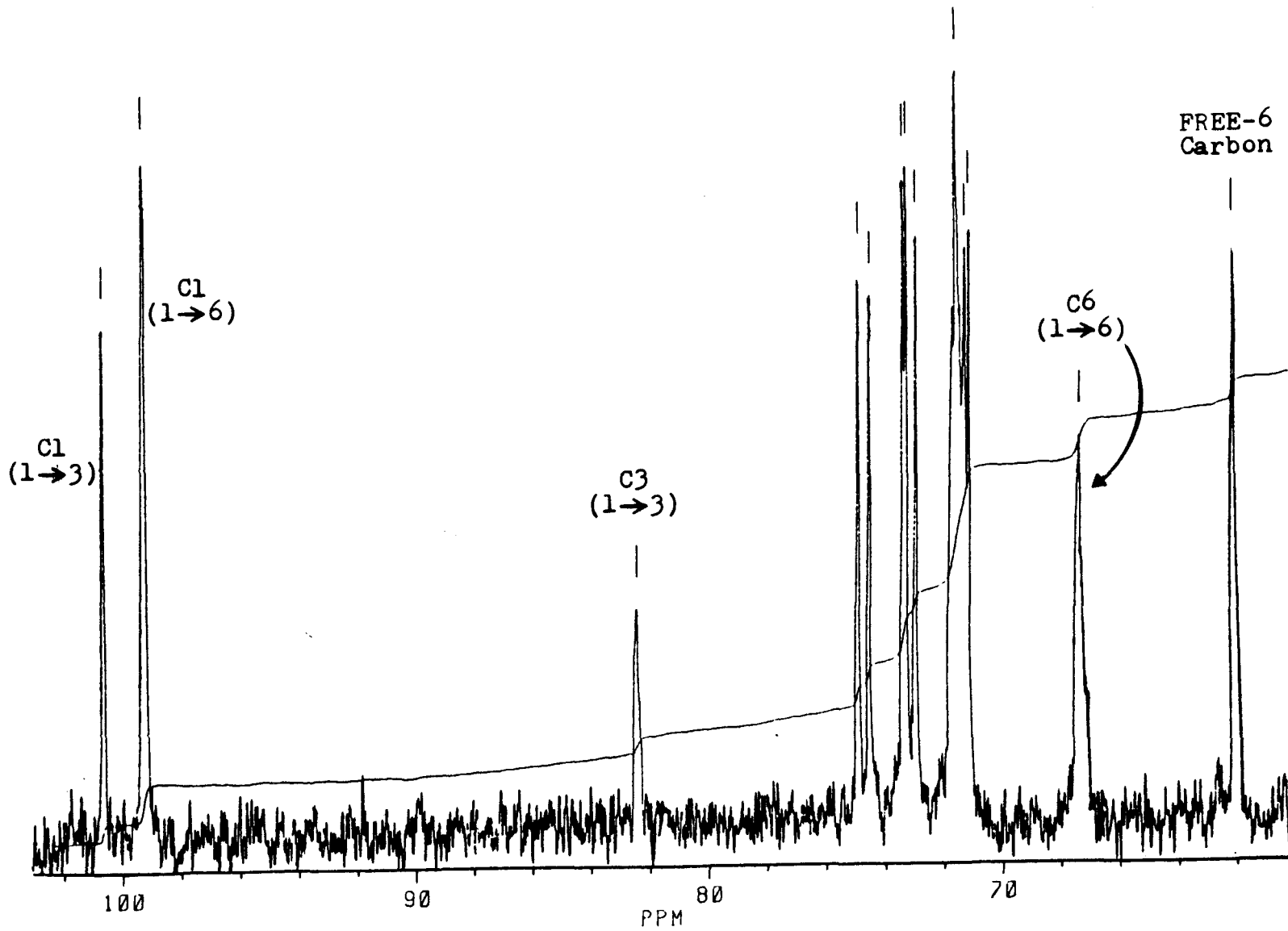


Figure 38. C-13 NMR spectrum of the 6.0:1 GTF-S product (35 mg/ml). The reaction mixture for product synthesis was the same as for Figures 34 and 35 but with 10.7  $\mu$ M dextran T70. The spectrum was run in D<sub>2</sub>O at 70°C with 2103 scans.



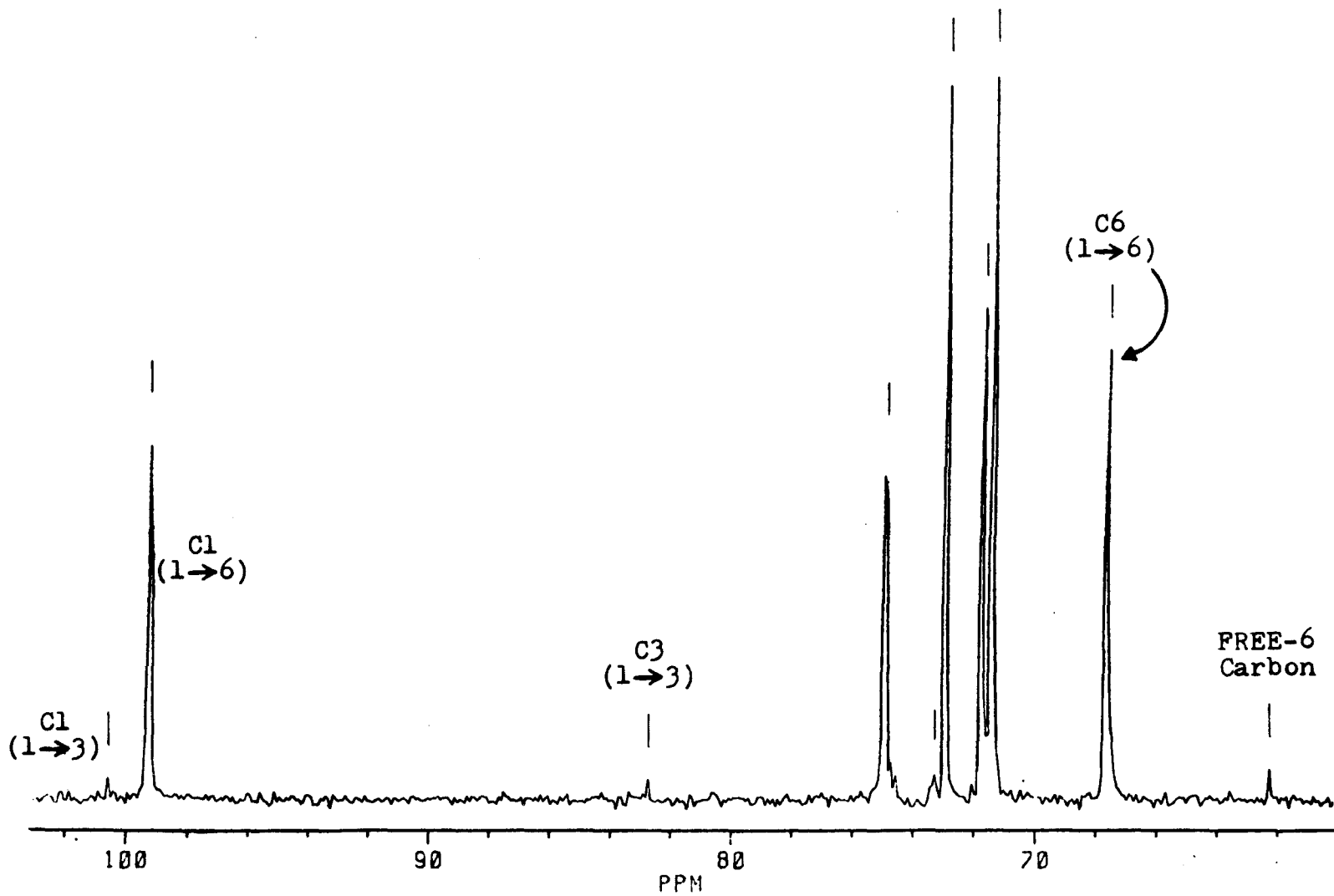
Since no peaks in the anomeric region characteristic of  $\beta$  bonds were observed, all bonds in the products were  $\alpha$ . Furthermore, no peaks were observed at ppm values characteristic of  $\alpha$ -1,2 or  $\alpha$ -1,4 bonds, so that all bonds in the products were either  $\alpha$ -1,6 or  $\alpha$ -1,3.

Since S. sobrinus GTF-S has been reported to have branching activity (McCabe and Hamelik, 1983; McCabe, 1985), in which  $\alpha$ -1,3 bonds are formed in the absence of sucrose, an NMR experiment was performed to determine the contribution of this reaction to overall  $\alpha$ -1,3 bond formation by GTF-S. The spectrum of the branching activity product is shown in Figure 39. This spectrum can be compared to the 1.42:1 GTF-S spectrum (Figure 37) since the same dextran T70 concentration (14.3  $\mu$ M) was used for both. As can be seen by comparing the relative sizes of the  $\alpha$ -1,3 peaks in the two spectra and their integrals from Table 5, the branching activity, although present, contributed a very small number of  $\alpha$ -1,3 bonds to the product, compared to the polymerization activity. The branching activity increased the proportion of  $\alpha$ -1,3 bonds in T70 by only 1.4%, from 3.8% in the primer to 5.2% in the reaction product. This experiment is the first reported comparison of the relative activities of the branching and polymerization reactions.

Products resulting from the progressive addition of glucose to the primer by GTF-I were also studied. Since these spectra were run at 25°C and pD 14, some differences



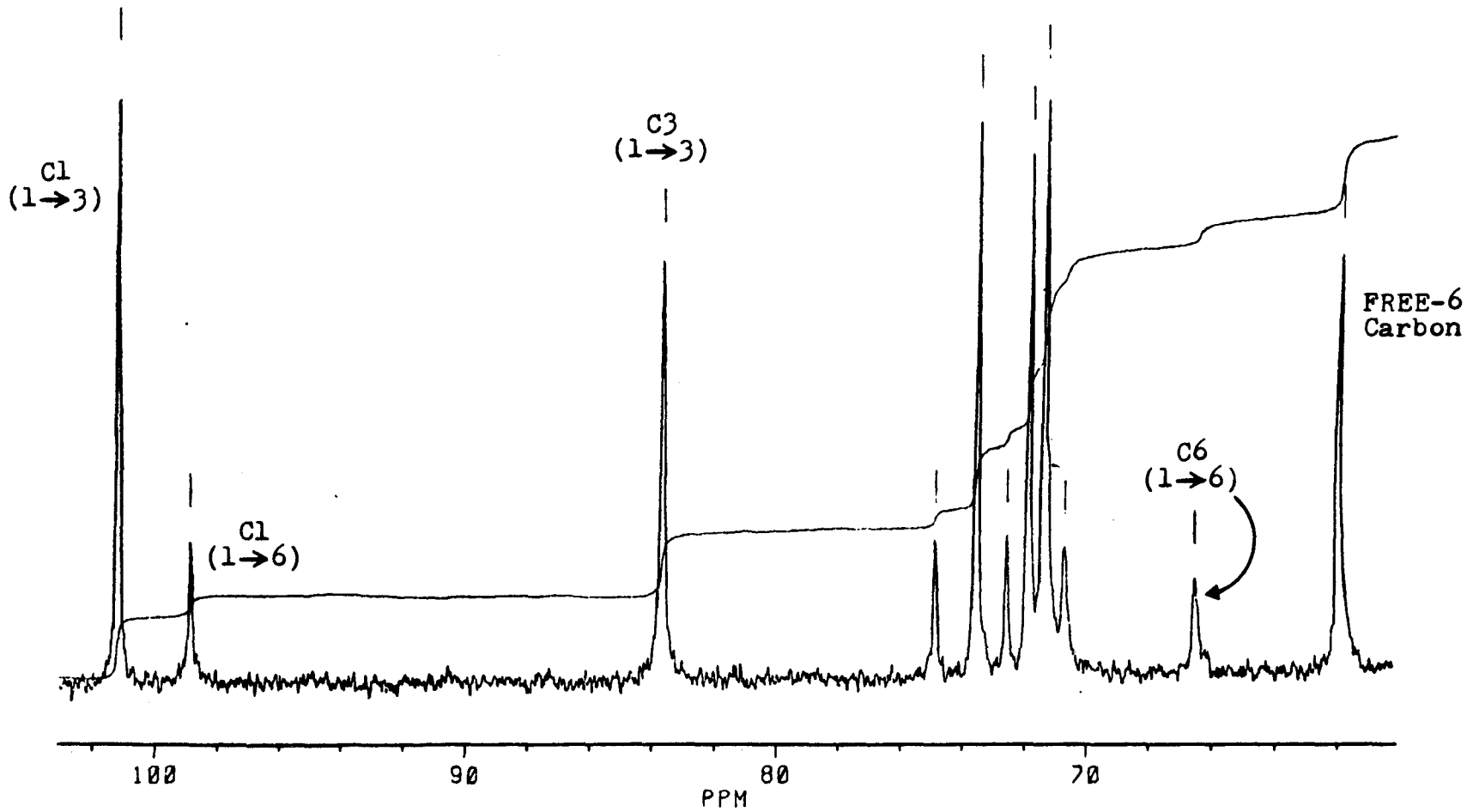
Figure 39. C-13 NMR spectrum of the product (16 mg/ml) resulting from GTF-S branching activity. The reaction mixture contained 100 mM potassium phosphate buffer, pH 5.7, 57.1  $\mu$ M dextran T70, and GTF-S (0.073 unit/ml), with no sucrose present. The spectrum was run in D<sub>2</sub>O at 70°C with 4040 scans.



in chemical shifts for the various peaks, compared to the 70°C, pD 7, spectra of GTF-S, are expected. Decreased temperatures shift the peaks of dextran spectra upfield (Seymour et al., 1979b), whereas increased pH generally shifts the peaks of simple carbohydrates (Colson et al., 1974) and dextrans (Colson et al., 1974, 1979) downfield. Since the range of ppm values covered by dextrans in C-13 NMR spectra is so large, and the offsetting pH and temperature effects are relatively small, the signals due to different carbon species in the GTF-I product spectra could still be easily identified, and the ppm values varied little from those observed for the GTF-S products, as shown in Table 4. Similar chemical shifts, for insoluble dextrans under similar conditions, have been observed in another study of products synthesized by crude, GTF-containing culture supernatants (Colson et al., 1979).

In the same way as for GTF-S, examination of the GTF-I product spectra revealed the nature of the branches added to the primer. Again, the structures of these branches have not previously been determined, but predicted ratios can be calculated assuming a 100% addition of  $\alpha$ -1,3 bonds to the primer. The spectrum of the 1.8:1 GTF-I product is shown in Figure 40. The  $\alpha$ -1,3 peaks are very large, and the 1,6:1,3 ratio is close to the predicted ratio listed in Table 5. One free-6-carbon was formed for every  $\alpha$ -1,3 bond added (free 6:1,3 = 1), as is consistent for an  $\alpha$ -1,3-linked

Figure 40. C-13 NMR spectrum of the 1.8:1 GTF-I product (23 mg/ml). The reaction mixture for product synthesis contained 100 mM potassium phosphate buffer, pH 6.2, 200 mM sucrose, 14.3  $\mu$ M dextran T70, and GTF-I (0.038 unit/ml). The spectrum was run in D<sub>2</sub>O, pD 14, at 25°C with 10,632 scans.



linear dextran. The spectrum of the 4.5:1 GTF-I product is shown in Figure 41. The  $\alpha$ -1,3 peaks are again very large, and the 1,6:1,3 ratio is close to the predicted value. The free 6:1,3 ratio is again 1. These spectra strongly suggest that GTF-I added, to primer dextran, branches consisting of a 100%  $\alpha$ -1,3-linked polymer. In the case of GTF-I, unlike GTF-S, it was not possible to determine whether few or many branch points were added to the primer, since no "burst" of  $\alpha$ -1,3 bond synthesizing activity would be observed for GTF-I in either case.

Because the spectrum of dextran T70 at 32 mg/ml (Figure 35) did not show any  $\alpha$ -1,3 peaks, even though previously reported methylation data showed the presence of about 5%  $\alpha$ -1,3 bonds in L. mesenteroides B512 dextran (Van Cleve et al., 1956; Lindberg and Svensson, 1968), the spectrum of a more concentrated T70 solution (333 mg/ml) was recorded (Figure 42). Although this spectrum does show peaks at ppm values characteristic of  $\alpha$ -1,3 bonds, the C1(1 $\rightarrow$ 3) and C3(1 $\rightarrow$ 3) peaks are each split in two. The most likely explanation for this splitting involves the observation (Seymour et al., 1979c) that, in dextrans, an  $\alpha$ -1,3 branch residue generally displays a C3(1 $\rightarrow$ 3) peak at about 82.5 ppm, whereas a linear  $\alpha$ -1,3 residue displays a C3(1 $\rightarrow$ 3) peak at a ppm value slightly greater than 83 ppm. Thus, the split C3(1 $\rightarrow$ 3) peak in Figure 42 probably indicates that T70 contains both branched and linear  $\alpha$ -1,3 residues, which

Figure 41. C-13 NMR spectrum of the 4.5:1 GTF-I product (11 mg/ml). The reaction mixture for product synthesis was the same as for Figure 37 but with 3.57  $\mu$ M dextran T70. The spectrum was run in D<sub>2</sub>O, pD 14, at 25°C with 9976 scans.

C1  
(1→3)

C3  
(1→3)

FREE-6  
Carbon

C1  
(1→6)

C6  
(1→6)

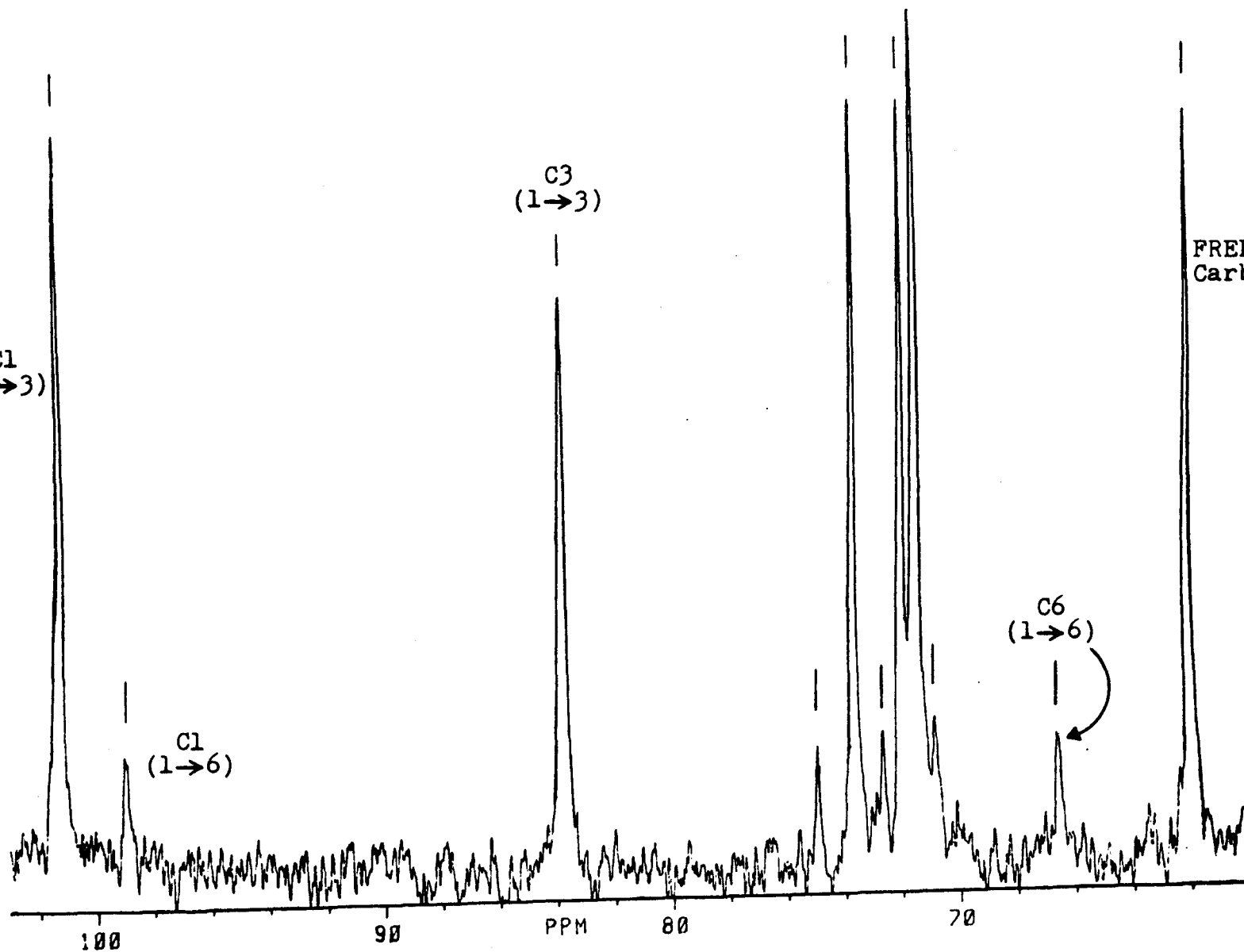
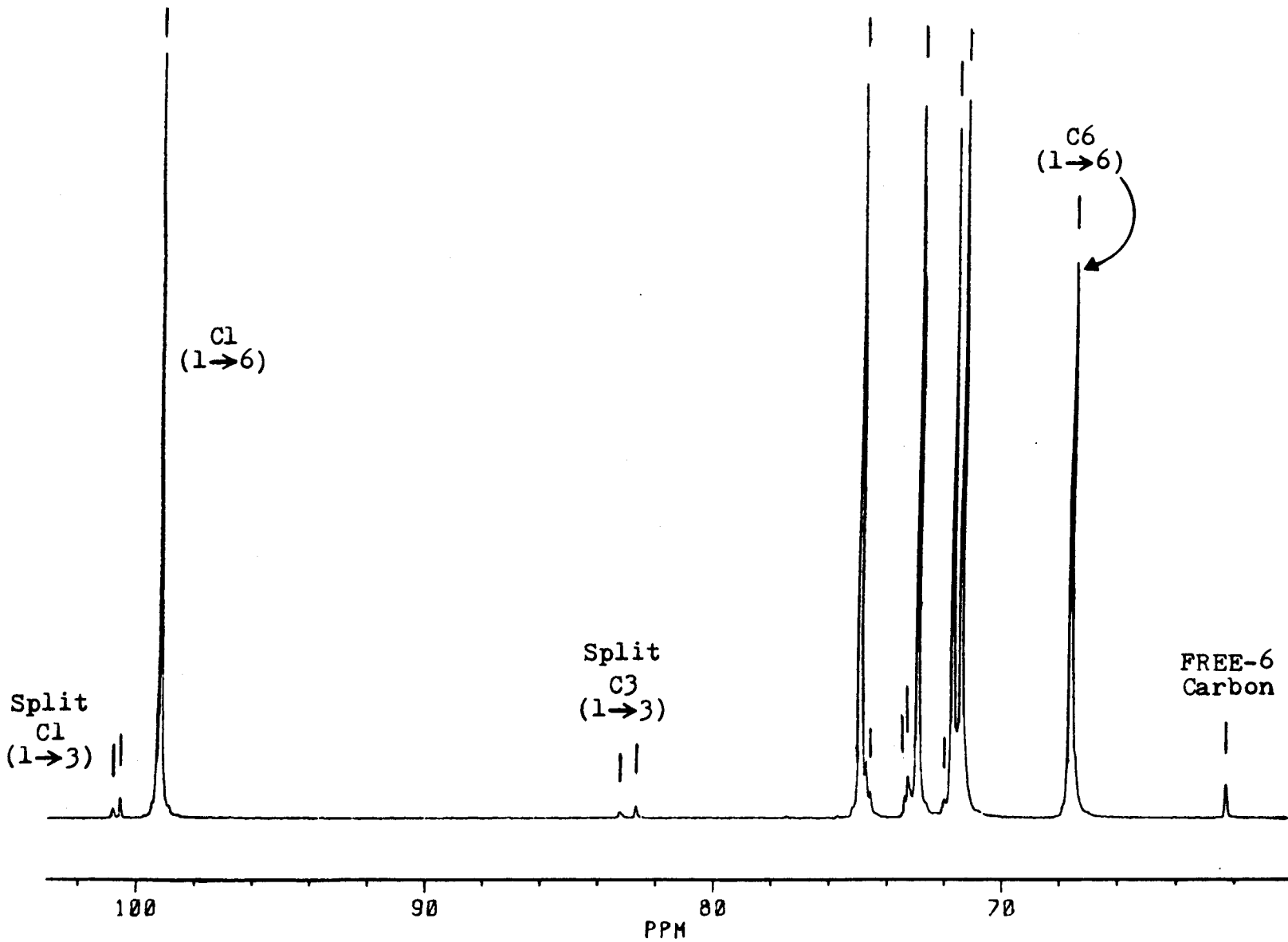




Figure 42. C-13 NMR spectrum of concentrated dextran T70 (333 mg/ml). The spectrum was run in D<sub>2</sub>O at 70°C with 2221 scans.



displayed peaks at 82.64 ppm and 83.20 ppm, respectively (Table 4). This explanation also accounts for the split C1(1→3) peaks at 100.53 and 100.78 ppm, since the Seymour (1979c) study showed that C1(1→3) peaks from linear and branched residues in other dextrans were separated by about 0.2 ppm. The percentage of  $\alpha$ -1,3 bonds in T70, calculated from the sum of the integrals of the split anomeric peaks, was 3.8% (Table 5), in good agreement with the 4.5 - 5.0% determined in the methylation studies (Van Cleve et al., 1956; Lindberg and Svensson, 1968), although methylation failed to detect the presence of the linear residues.

The fact that the C3(1→3) peak in all the GTF-S product spectra appeared at 82.41 - 82.44 ppm (Table 4), characteristic of branched  $\alpha$ -1,3 linkages, indicates that the  $\alpha$ -1,3 linkages in these products were branched, another piece of spectral data which supports the "comb" dextran structure both for the de novo GTF-S product and for the branches added to the primer by GTF-S.

#### 4. Spin-Lattice ( $T_1$ ) Relaxation Times of Carbon Species in the GTF Products

The conclusions drawn from the NMR spectra of the GTF products depend on the assumption that the peak areas in the spectra are proportional to the number of carbon atoms that give rise to the peaks. However, this assumption is not generally true for C-13 NMR spectra (Abraham and Loftus, 1980). Since the  $T_1$  times of the carbon species of interest

are critical in determining whether this assumption is true in a particular case,  $T_1$  values were determined for the 6.0:1 GTF-S and 1.8:1 GTF-I samples.

The  $T_1$  values were measured by the inversion-recovery method using a Bruker computer program designed for this purpose. In this method, a  $180^\circ$  pulse was applied, a pulse delay  $\tau$  was inserted, and a  $90^\circ$  pulse was applied to flip the z-component of the bulk magnetization vector onto the y-axis for measurement. This pulse sequence was repeated several times to obtain a spectrum that measured the accumulated y-components, which were actually the z-components flipped through  $90^\circ$ , for each carbon species. Several such spectra were recorded at different  $\tau$  values, and the resulting curve followed the equation:

$$M_z = M_z^0(1 - 2\exp(-\tau/T_1)) \quad (5)$$

where  $M_z$  is the z-component of the bulk magnetization vector at time  $\tau$  after the  $180^\circ$  pulse, and  $M_z^0$  is this component at equilibrium (Abraham and Loftus, 1980). Thus, the  $T_1$  value can be calculated by constructing a plot of peak intensity (which is a measure of  $M_z$ ) vs.  $\tau$  and determining the best-fit curve in the form of equation (5). The Bruker program automatically ran the spectra, performed the curve-fitting, and printed out values for  $T_1$  with standard deviations.

A sample computer printout of a peak intensity vs.  $\tau$  graph, including the best-fit curve in the form of equation

(5), is shown in Figure 43 for the C3(1→3) peak from the 1.8:1 GTF-I sample.

$T_1$  values for the carbon nuclei of interest in the spectra of the 6.0:1 GTF-S sample and the 1.8:1 GTF-I sample are recorded in Table 6. All the  $T_1$  times were 0.52 second or less, far below the 5 second pulse delay used in obtaining the spectra. Thus, since the nuclei were all almost completely relaxed before the next pulse, the  $T_1$  times had little effect on the relative peak areas.

Figure 43. Sample computer printout of the peak intensity vs.  $\tau$  graph for the C3(1 $\rightarrow$ 3) peak from the 1.8:1 GTF-I sample. The best-fit curve in the form of equation (5) (see text) is shown on the graph. The table at the top of the figure shows a printout of the individual peak intensity values and the calculated  $T_1$  time.

TAU	CURSOR	FREQ	PPM	INTEGRAL	INTENSITY
3.0000	3706	6259.096	82.936	11.611	54.620
2.0000	3706	6259.096	82.936	12.140	58.235
1.0000	3707	6257.875	82.920	12.352	56.660
.5990	3707	6257.875	82.920	12.563	54.155
.3999	3707	6257.875	82.920	11.579	49.693
.1999	3707	6257.875	82.920	8.510	37.221
.0999	3708	6256.654	82.904	4.695	27.237
.0499	3708	6256.654	82.904	3.577	14.522

MINIMUM T1= .050    MAXIMUM T1= 20.000 3

CURSOR	FREQ	PPM	T1	STD DEV
3706	6259.096	82.9362	.18826	.021616

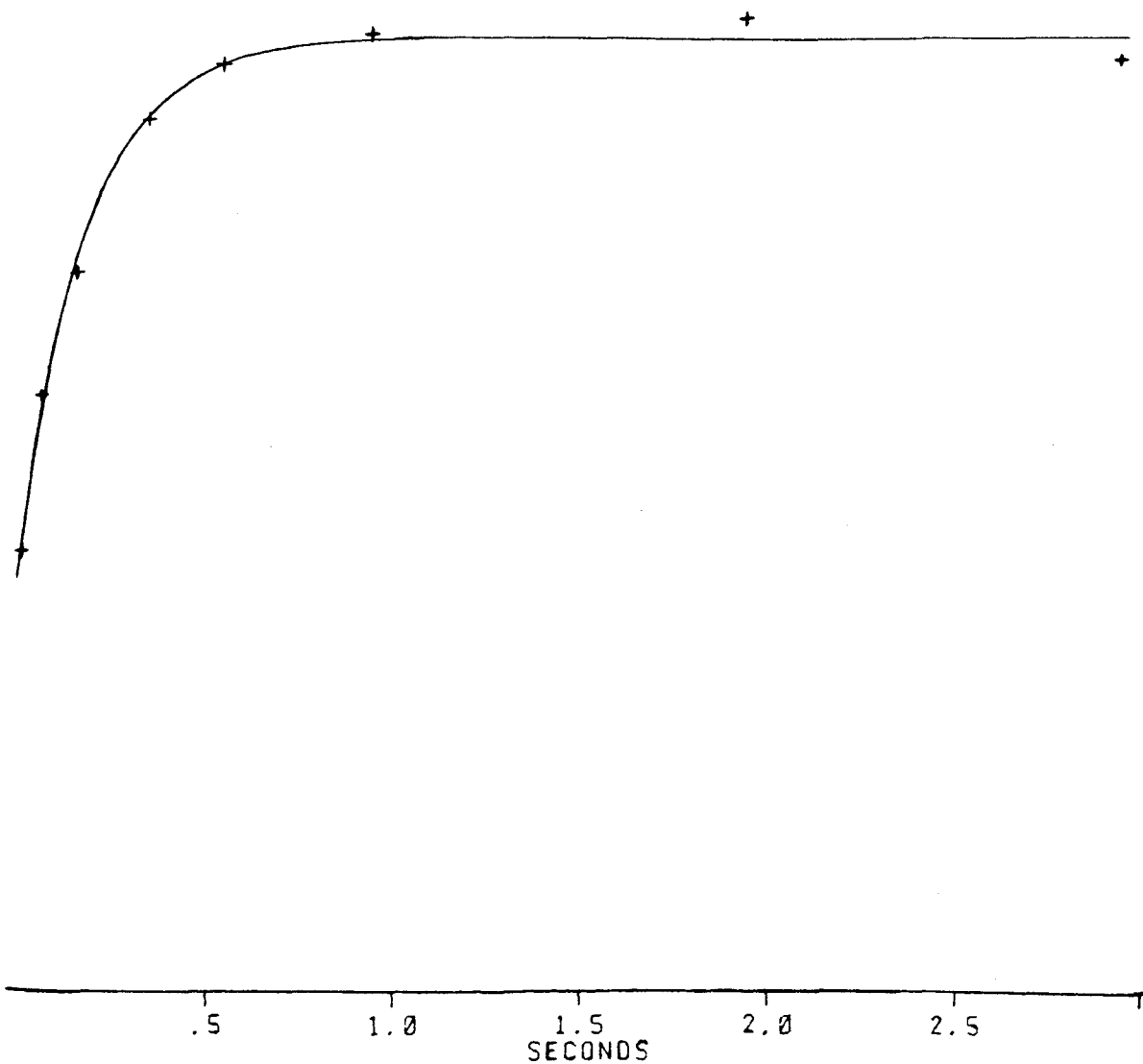


TABLE 6. Spin-Lattice ( $T_1$ ) Relaxation Times (in seconds) of Carbon Species in GTF Dextran Products

Sample	Anomeric $\alpha$ -1,3	Anomeric $\alpha$ -1,6	3-Carbon $\alpha$ -1,3	6-Carbon $\alpha$ -1,6	Free 6-Carbon
GTF-S 6.0 : 1 <sup>a</sup>	0.34 $\pm$ 0.05 <sup>b</sup>	0.31 $\pm$ 0.02	0.28 $\pm$ 0.06	0.10 $\pm$ 0.03	0.52 $\pm$ 0.01
GTF-I 1.8 : 1 <sup>a</sup>	0.20 $\pm$ 0.02	0.14 $\pm$ 0.09	0.19 $\pm$ 0.02	0.11 $\pm$ 0.13	0.13 $\pm$ 0.03

<sup>a</sup>Ratio of glucose added per glucose in primer dextran.

<sup>b</sup>Calculated  $T_1$  time  $\pm$  standard deviation in seconds as computed by the Bruker curve-fitting program.



## CHAPTER V

### DISCUSSION

#### A. Importance of Studies of GTF

Enzymatic studies of the oral streptococcal GTF enzymes are of importance for several reasons. One of these is the physiological role of the enzymes in the formation of dental plaque and its associated pathological oral lesions. However, the enzymes are also interesting from a biochemical point of view, since they are large, multifunctional enzymes that synthesize complex polysaccharide products. The enzymes are unusual in that they do not utilize a nucleotide-activated sugar for transfer to the growing oligosaccharide chain (Sharon, 1975). The nucleotide activation step occurs in the plant, where sucrose is synthesized from UDP-glucose and fructose (Sharon, 1975). Therefore, the bond between glucose and fructose contains a relatively large amount of chemical energy, and sucrose itself can be considered as an "activated" form of glucose. Study of the GTF's can provide new information about how enzymes polymerize glucose residues in a system other than the well-studied amylose and glycogen synthesis systems. Particularly, the oral streptococcal GTF polymerization reactions serve as models for enzyme-catalyzed dextran synthesis, a process that has not been well-studied.

In order to study GTF catalytic activity, it is necessary to have available an adequate supply of highly purified GTF enzymes. The project reported in this dissertation developed largely from the realization that, despite the importance of studying oral streptococcal GTF's, an acceptable technique for purifying the enzymes had not, as yet, been developed. Thus, the first goal of this work was to develop such a technique for the GTF's of S. sobrinus 6715-7.

#### B. Growth of Bacteria

The first matter to be settled was the choice of a suitable bacterial culture medium that would provide a dextran-free crude GTF preparation. Commonly used streptococcal broths, such as Trypticase Soy and Todd-Hewitt, contain traces of sucrose, and yield salt-resistant, dextran-dependent GTF aggregates (Spinell and Gibbons, 1974; Janda and Kuramitsu, 1976; Schachtele et al., 1976; Hamada and Torii, 1978), which make purification of the GTF's very difficult. Although the use of a sucrose-free "chemically defined" culture medium resolves this problem (Janda and Kuramitsu, 1976; Schachtele et al., 1976), over 40 individual components must be included in such media to support bacterial growth, and the growth obtained is not as rapid or consistent as with complex media (Terleckyj et al., 1975). The complex TTY broth of Hamada and Torii (1978), which contains the sucrose-free protein source Trypticase Peptone,

was shown to yield a dextran-free GTF preparation in our study (Figure 7), and was, therefore, chosen as the culture medium for S. sobrinus.

In addition, we obtained our S. sobrinus 6715-7 parent strain (catalog number 27351) from the highly regarded American Type Culture Collection (ATCC) in order to assure the quality of the culture. Other investigators have often used for GTF studies relatively uncharacterized laboratory strains or mutants obtained from different sources.

### C. GTF Assay

The nonlinearity of the GTF assay in dilute buffer was discovered when yields of GTF activity much greater than 100% were consistently obtained in the ammonium sulfate precipitation step. It was thought that this finding could be explained by aggregation of GTF to a more active form in the concentrated ammonium sulfate fraction as compared to the unconcentrated supernatant, a conclusion borne out by further experimentation. Thus, to determine percent yields in the purification, ammonium sulfate was added to the assay mixture to artificially aggregate GTF, a procedure which linearized the activity (Figures 8 and 33). The tendency of other investigators to use dilute assay buffers has undoubtedly led to overestimation of percent yields in other purification schemes.

Another problem that was consistently encountered in the assay was sampling error due to the insoluble nature of

reaction products synthesized in the presence of GTF-I. As discussed in the RESULTS, it was found that this problem could be resolved by restricting assay times to keep products soluble, or by sonicating reaction mixtures before spotting on the filter papers for scintillation counting.

Thus, accurate and reproducible assays were obtained only by careful control of assay conditions and procedures.

#### D. The GTF Purification Procedure

Due to the failure of classical techniques to purify GTF's adequately, it was determined before beginning work on a purification scheme that the new procedure would involve affinity chromatography. In general, affinity techniques are capable of providing a very high degree of purification because the methods depend on specific interactions of the protein to be purified with ligand molecules attached to a column.

Several types of affinity resins have been reported that are capable of binding GTF's: insoluble dextran mixed with gel filtration beads (McCabe and Smith, 1977; Figures and Edwards, 1979), dextran T10 covalently attached to epoxy-activated Sepharose (Russell, 1979), and unmodified Sephadex beads (Russell, 1979; McCabe, 1985; Mooser et al., 1985). We attempted to synthesize a resin consisting of dextran T500 covalently linked to epoxy-activated Sepharose, but chromatography with this resin proved unsuccessful. Since one of our goals was to develop a purification scheme

that was simple and universally applicable, Sephadex, available directly from commercial suppliers, was chosen as the affinity resin.

An ideal affinity chromatography procedure would involve purification of the protein of interest directly from a complex biological mixture, with no other steps required. Although Sephadex was capable of binding GTF's directly from the culture supernatant, GTF-S and GTF-I were not separated by elution with a gradient of the eluting agent maltose.

Thus, the additional step of ion-exchange chromatography was used for this separation. Since the GTF's have acidic pI values, a diethylamino ethyl (DEAE) resin was used. Since DEAE-cellulose and DEAE-Sephadex can have relatively poor flow characteristics, often require special preparation before pouring, and shrink substantially in salt solutions, DEAE-Sepacel was chosen as the resin because it is supplied preswollen and ready for use, and is beaded for good flow characteristics and minimal shrinkage in salt. Thus, after the ammonium sulfate concentration step, a KCl gradient successfully separated GTF-S and GTF-I on the ion-exchange column with minimal activity loss, provided ion exchange was done before affinity, as discussed in the RESULTS.

For affinity, batch processing was used because of the large volumes of the ion-exchange fractions, and coarse

Sephadex beads were used because they poured into columns very easily after enzyme binding. To keep proteins from aggregating and binding nonspecifically to GTF, 1.0 M KCl was included in the buffers for all affinity steps. Also, room temperature elution at a very slow flow rate was used to maximize release of bound GTF's from the columns. Another reason for performing the elution at room temperature was that the maltose solution was more viscous at 4°C.

Because of the desire to avoid the use of dextran or strong denaturants in eluting the enzymes, maltose was tried as an eluting agent, and found to be as effective as dextran T10 for this purpose. The rationale for using maltose was that it is known to compete with dextran for the GTF acceptor binding site (Fukui and Moriyama, 1983) and should, therefore, remove GTF's from Sephadex columns by a competitive effect. Once purified enzymes were obtained, this idea was verified by the finding of mixed inhibition of purified GTF-S by maltose (Figure 28). Since a mixed inhibitor can bind to the enzyme-substrate complex, as shown in equation (4) in the RESULTS, the inhibitor must bind at a site other than the substrate-binding site. Thus, according to the classical kinetic treatment, the mixed inhibition mechanism of maltose is consistent with maltose binding in a site other than the sucrose-binding site. In the case of GTF, this alternate site is the acceptor-binding site, the site at which dextran binds. Although large concentrations of

maltose were required for elution because of the high  $K_i$  and  $K_i'$  values, such concentrations were easily achieved in the aqueous eluting buffer. Unlike dextran, which has been used as an affinity chromatography eluting agent by others (McCabe and Smith, 1977; Figures and Edwards, 1979), maltose was easily dialyzed out of the enzyme preparations after the affinity step. Furthermore, the use of maltose avoided the enzyme denaturation that occurs when the chaotropic agent guanidine hydrochloride is used for elution (Russell, 1979; McCabe, 1985; Mooser et al., 1985). Thus, the use of maltose as an eluting agent is a significant improvement of our purification scheme compared to other reported techniques.

The reason for the low yield of GTF-I in the affinity step (1.5%), as compared to 23% for GTF-S, is not known. GTF-I may not have been eluted from the column as effectively, or may have lost activity during elution or dialysis. However, the purification scheme gave a reasonable yield overall (5.1%) with a high degree of purification, and provided sufficient amounts of enzymes for the enzymatic studies performed in this work.

It is important to demonstrate the purity of any enzyme obtained from a new purification scheme. Investigators reporting purifications of GTF's have often been very vague about this point, and sometimes do not even report percent yields (Russell, 1979; Fukui et al., 1982). Our preparations were shown to be pure by SDS PAGE, with no

contaminating bands present even on an overloaded, silver-stained gel (Figure 11). Furthermore, no contaminating dextranase, invertase, or fructosyltransferase activities were detected in the GTF preparations, even with overnight incubation of reaction mixtures (Figures 16 and 17). Thus, the final preparations were shown to be sufficiently pure to yield valid results in enzymatic studies.

Although our purification scheme was applied only to the GTF's of S. sobrinus, the enzymes from S. mutans could probably also be purified by modifying the technique. Although the S. mutans GTF system is apparently somewhat more complex than that of S. sobrinus, the basic technique of separating GTF forms by ion exchange (and other steps if necessary), followed by final purification with affinity chromatography, should be applicable.

As alluded to earlier, our purification scheme as developed solved many of the problems associated with other reported schemes:

1. Highly purified enzymes were obtained that were free of contaminating enzyme activities;
2. Both GTF-I and GTF-S were obtained as separate preparations;
3. A small number of steps were required for purification, and a reasonable yield was obtained.



4. No dextran was introduced into the enzyme preparations, either during bacterial growth (Figure 7), or during elution from the affinity columns;

5. The enzymes were never exposed to strong denaturants that destroy the native enzyme conformations. These last two improvements were made possible by the use of the novel affinity chromatography eluting agent maltose.

Furthermore, the techniques involved in our purification of the S. sobrinus GTF's are simple and reproducible, and can be applied by other researchers in the GTF field, since all of the supplies needed are readily obtained from commercial suppliers. The development of this successful and universally applicable purification scheme fulfills a critical need in enzymatic studies of GTF.

#### E. Physical and Kinetic Properties of the GTF's

The physical and kinetic studies of the GTF's were undertaken to describe the properties of the purified enzyme preparations and to obtain information necessary to design product synthesis experiments for the NMR study.

Both enzymes were shown to consist of large, single polypeptide chains (Figures 11 and 12). Since they are relatively stable enzymes, able to be stored for long periods concentrated at 4°C, it might be predicted that the large structure is stabilized by numerous intrachain disulfide cross-links. However, published amino acid analyses (Shimamura et al., 1982; Ciardi, 1983; Furuta et al., 1985)

indicate that there may be as few as 4 - 6 cysteine residues per enzyme molecule. Thus, the structures must also rely heavily on secondary and tertiary structures for stabilization. The aggregation behavior of GTF-I may also play a role in its stability.

Both enzymes showed two isozyme forms on SDS PAGE (Figures 11 and 12). Isozyme forms of GTF have been reported for all three well-studied oral streptococci--S. sobrinus, S. mutans, and S. sanguis. As discussed earlier, some of these isozyme forms are due to proteolytic degradation, but the origin of the S. sobrinus forms is not known. Isoelectric focusing also showed two bands of GTF-S (Figure 14). Native PAGE showed two major GTF-S bands, but some very faint bands also appeared between the two major ones (Figure 31). A native PAGE gel of S. sobrinus GTF-S in another study (McCabe, 1985) also showed two major bands of GTF detected by a PAS activity stain. However, the area between these major bands, where the faint protein bands in Figure 31 appear, also stained for GTF activity (McCabe, 1985). The reason for this "smearing" of GTF-S activity on native gels has not been determined, although it may be due to a lack of resolution of minor GTF-S bands on SDS PAGE and IEF gels. Determination of the origin of the S. sobrinus isozymes and a study of their properties would be an interesting area for future GTF research.

As far as kinetic behavior is concerned, the GTF's showed results consistent with other studies with respect to pH activity ranges and  $K_m$  values for sucrose. Although both enzymes exhibited some activity in the absence of dextran, GTF-I showed an absolute requirement for primer in order to synthesize high molecular weight, insoluble products. Also, GTF-I showed a dextran substrate inhibition that was absent for GTF-S (Figures 19 and 20). These latter two observations about GTF-I lead to the speculation that streptococci may be able to control the production of the dental plaque matrix by controlling the availability of primers, which, in vivo, are presumably synthesized by GTF-S enzymes. Although the presence of primer is required to initiate insoluble glucan synthesis, the activity of GTF-I can be "shut off" when too much primer is present. Such a mechanism may prevent the "overgrowth" of dental plaque in the oral cavity, a situation which would be detrimental for both host and bacteria.

Informative data were also obtained from the study of the variation of kinetic constants with pH (Figures 25, 26, and 27). Both GTF-S and GTF-I were shown to possess at least two ionizable active-site groups, which had  $pK_a$ 's of 4.1 and 6.7 (GTF-S) and 4.7 and 6.6 (GTF-I). These groups are involved in the association of enzyme and sucrose. From the variation of  $\log(V_{max})$  with pH (Figure 27), which showed a dependence on only one ionizable group, it appears that

one of the ionizable groups is the nucleophile that binds glucose in the covalent glucosyl-enzyme complex. This group loses its ionization potential when it binds the glucosyl moiety. Because of the potential for perturbed  $pK_a$  values, it is impossible to state, without further study, the identity of the active site groups, or which of the two groups is bound to the glucosyl moiety. However, as discussed in the REVIEW OF THE RELATED LITERATURE, by analogy with the Bacillus subtilis fructosyl-transferase (Chambert and Gonzyl-Treboul, 1976) and sucrose phosphorylase (Voet and Abeles, 1970; Mieyal and Abeles, 1972), and according to kinetic hydrolysis data of model organic compounds and the denatured glucosyl-GTF complex (Iwaoka and Mooser, 1983), it is likely that the glucosyl group is bound to a carboxyl residue. In fact, both of the ionizable groups may be carboxyls as in lysozyme, which has two active-site carboxyls with  $pK_a$  values of 4.2 and 6.1 (Banerjee et al., 1973). The presence of a histidine as one of the ionizable groups cannot be ruled out, however, since photooxidation of crude GTF's from S. sobrinus, S. sanguis, and S. cricetus in the presence of methylene blue led to chemical modification of histidine and inactivation of the enzymes in one study (Koga and Inoue, 1981).

One property of GTF that was determined based on results from several different physical and kinetic experiments was that the aggregation of GTF-I has a significant

effect on its catalytic activity. It has previously been shown that the GTF activity in crude enzyme preparations is present in the form of enzyme aggregates (Kuramitsu, 1975; Schachtele et al., 1976). It has also been shown that salts can affect this aggregation behavior. Monovalent salts disaggregate dextran-independent GTF aggregates (Germaine et al., 1977 and Figure 7). On the other hand, ammonium sulfate has been reported to increase insoluble glucan synthesis by crude S. mutans GTF preparations (Newman et al., 1980), an effect attributed to the aggregation of the pI 7.5 GTF-S of S. mutans and the resultant production of water-insoluble products in the presence of ammonium sulfate (Asem et al., 1986; Baba et al., 1986). However, we demonstrated that the S. sobrinus GTF-S cannot be made to synthesize insoluble products in the presence of ammonium sulfate, even though the enzyme remains active. Instead, the unexpected result emerged that it is S. sobrinus GTF-I that is most affected catalytically by ammonium sulfate.

We have demonstrated, as discussed in the RESULTS, that ammonium sulfate causes aggregation of GTF-I to a more active form than the disaggregated enzyme. The aggregation of GTF-I was demonstrated both by the physical techniques of gel IEF (Figure 14) and native PAGE (Figure 31). The fact that GTF-I aggregation affects its catalytic activity was demonstrated kinetically by the upward concavity of the GTF-I activity versus enzyme concentration graph, as shown

in Figure 33A, and by the linearizing of this curvature by the addition of ammonium sulfate, as shown in Figure 33B. Another relevant kinetic result was that, for GTF-I, induction of sucrose substrate cooperativity in the presence of the inhibitor maltose (Figure 30) could be explained by assuming that maltose disaggregated GTF-I, while sucrose aggregated it to the more active form. Substrate cooperativity with sucrose has not previously been reported for any oral streptococcal GTF enzyme, and was observed in our study only in the presence of inhibitor. Thus, our results indicate that the aggregation of S. sobrinus GTF-I has a significant effect on its catalytic activity.

#### F. Carbon-13 NMR Study of Dextran Products

##### 1. Nature of Experiments Needed to Study GTF-Catalyzed Dextran Synthesis

Since the primary physiological function of the GTF enzyme system of oral streptococci is the synthesis of the dental plaque matrix, in vitro studies of the structures of products synthesized by GTF are needed in order to describe how the enzymes synthesize this matrix in vivo. Many studies of GTF products have been performed by growing the bacteria in a suitable culture medium, removing the bacteria, concentrating the supernatant, and using this crude GTF preparation to synthesize products for structural studies (Usui et al., 1975; Hare et al., 1978; Colson et al., 1979; Trautner et al., 1982; Davis et al., 1986). Although

such studies are informative with regard to describing general types of glucans that can be synthesized by GTF, the studies are limited by the fact that the products studied are synthesized by unresolved mixtures of enzymes, so that the contribution of each enzyme to the final structure cannot be determined. It has been shown that the proportions of GTF-S and GTF-I produced by oral streptococci vary widely with growth conditions (Walker et al., 1984; Hardy et al., 1986), so that the final glucan structures synthesized by a crude GTF preparation are a function not only of the organism producing the GTF but also of the way the organism was grown. For this reason, it is absolutely essential to study the products synthesized by the individual enzymes in order to determine how the enzymes work together in vivo to synthesize the dental plaque matrix.

Furthermore, due to the large GTF rate acceleration observed in the presence of primer dextran, it is of interest to study the structures of products synthesized not only de novo from sucrose alone, but also in the presence of primer. The structures produced by GTF's in the presence of primer have been largely uninvestigated.

## 2. Choice of C-13 NMR as the Dextran Structure Analysis Technique

The purification scheme developed in our work effectively separated and purified GTF-S and GTF-I and gave quantities of enzymes sufficient to synthesize milligram

quantities of glucan products with each enzyme individually. The goal of our NMR work was to study the primer modification reaction using these purified enzymes. Since primer modification involves a dynamic polymerization process, no one sample was sufficient to adequately describe the means by which GTF's modify the primer. Several samples at various stages of polymerization needed to be analyzed in order to follow the enzymatic process. Thus, the analysis technique used needed to fulfill two major requirements: it had to detect and quantitate various linkage types in the glucans, and it had to be suited to analyzing the multiple samples required for the study. Obviously, the technique also needed to be sufficiently sensitive to yield the desired information with the available sample sizes.

Classical chemical techniques have several undesirable characteristics for the type of analysis required. Depolymerization techniques, such as acid hydrolysis and acetylation, do not give percentages of linkage types because glycosidic bonds are destroyed in the reactions. Although periodate oxidation can give some information about quantities of linkage types present, it cannot unequivocally distinguish between some linkage types. Smith degradation is more exact with regard to the identities of linkage types present, but it is an experimentally difficult technique in which problems of selectivity in the acid hydrolysis step, as well as the occurrence of unwanted side reactions, can



make the data difficult to interpret (Aspinall, 1982). Methylation analysis could potentially be used in the GTF product study, since it can theoretically give accurate determinations of the percentages of linkage types present in a sample. However, it has been observed that dextrans are, in general, difficult to exhaustively methylate and to hydrolyze completely after methylation, so that inaccurate data can easily be obtained unless methylation conditions, including the need for multiple methylation steps, are carefully controlled (Seymour et al., 1977, 1979d). Also, methylation analysis shares the great disadvantage of all chemical techniques: the experiments are time-consuming and labor-intensive and are not, therefore, well-suited to processing large numbers of samples, as required for following an enzymatic polymerization reaction.

On the other hand, NMR is a relatively rapid technique that can easily process many samples in a reasonable period of time. It had already been shown, particularly by the Seymour group, that NMR could give information about dextran structure similar to that obtainable with methylation analysis. NMR even has the added advantage of being able to distinguish  $\alpha$  and  $\beta$  anomeric configurations. In dextran structure analysis, C-13 NMR shows more diagnostic resonances and better separation of peaks than proton NMR.

Therefore, as a result of all these considerations, Carbon-13 NMR was determined to be the only available

carbohydrate analysis technique reasonably capable of giving the information required for the envisioned study of GTF-catalyzed primer modification reactions.

### 3. Significance of the Results Obtained from the NMR Experiments

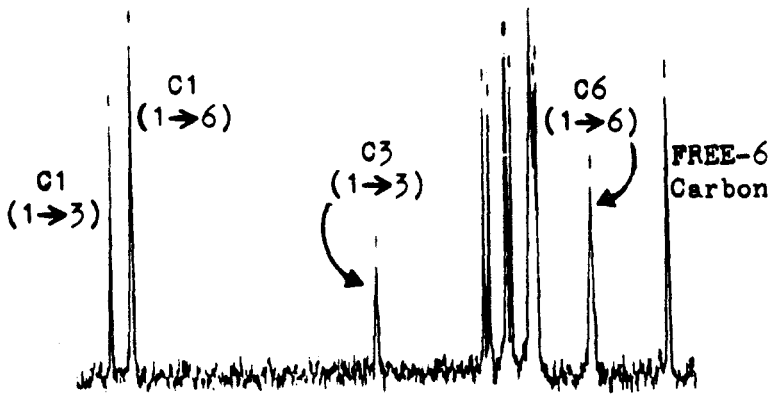
In order to study the primer modification reaction, products in various stages of polymerization were synthesized using the purified GTF-S and GTF-I preparations. In all cases, the spectra obtained exhibited chemical shift values for various linkage types similar to those of previously studied dextrans (Table 1). Particularly, C1(1→6), C1(1→3), C3(1→3), C6(1→6), and free-6-carbon resonances were easily distinguishable in all spectra. Therefore, several conclusions of significance could be drawn by analyzing the spectra.

The spectrum of the de novo GTF-S product (Figure 34), which had not been previously reported, strongly supports the comb dextran model for this dextran (Figure 4A). This model cannot be unequivocally proved as yet, because present techniques for analyzing branch length and the distribution of branches along the backbone in dextrans are not entirely adequate. However, it is virtually impossible to draw a reasonable structure containing a 2 to 1 ratio of  $\alpha$ -1,6 linkages to  $\alpha$ -1,3 branch linkages that does not contain stretches of comb structure. This observation, combined with the usual finding of predominantly single glucose

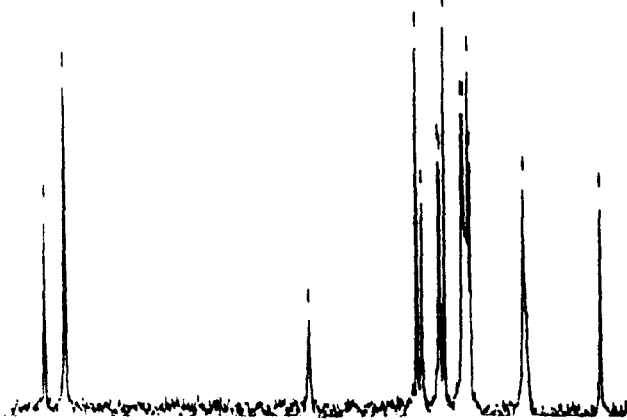
residue branches in highly branched dextrans (Abbot et al., 1966; Sidebotham, 1974; Kenne and Lindberg, 1982) makes the comb dextran structure very reasonable. Characteristics of our NMR spectrum that support this structure are the findings of a 2:1 ratio of  $\alpha$ -1,6 to  $\alpha$ -1,3 bonds, the presence of a C3(1 $\rightarrow$ 3) peak at 82.44 ppm, characteristic of branched  $\alpha$ -1,3 linkages, and a 1:1 ratio of  $\alpha$ -1,3 bonds to free-6-carbons.

The unresolved question of whether GTF-S adds a similar structure to primer dextran was also answered by the Carbon-13 NMR spectra. The increase in the sizes of the C1(1 $\rightarrow$ 3), C3(1 $\rightarrow$ 3), and free-6-carbon peaks as more glucose residues were added to the primer is very obvious visually when the spectra are shown together in a single figure (Figure 44). The calculations in Table 5 represent a quantitation of this visual observation. When the bond ratios determined from the NMR spectra of the 0.65:1, 1.42:1, and 6.0:1 GTF-S products are compared to the predicted ratios listed in Table 5, it is evident that GTF-S added, to the primer, branches with a 2:1 ratio of  $\alpha$ -1,6 to  $\alpha$ -1,3 bonds. The actual percentage of  $\alpha$ -1,3 bonds added was within 5% of the predicted value in all cases. This finding, together with the presence in the spectra of a C3(1 $\rightarrow$ 3) peak at 82.41 - 82.44 ppm (Table 4), characteristic of branched  $\alpha$ -1,3 residues, and a 1:1 ratio of  $\alpha$ -1,3 bonds to free-6-carbons, as shown in Table 5, supports the contention that

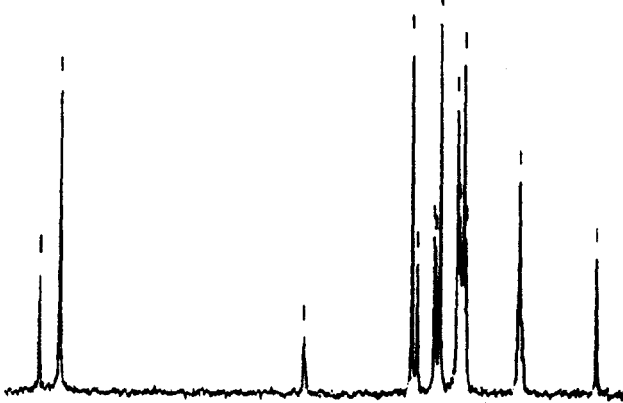
Figure 44. C-13 NMR spectra of dextran T70 and the 0.65:1, 1.42:1, and 6.0:1 GTF-S samples. As the number of glucose residues added to the primer increases, the C1(1→3), C3(1→3), and free-6-carbon peaks increase in size accordingly. The calculations in Table 5 represent a quantitation of this visual observation.



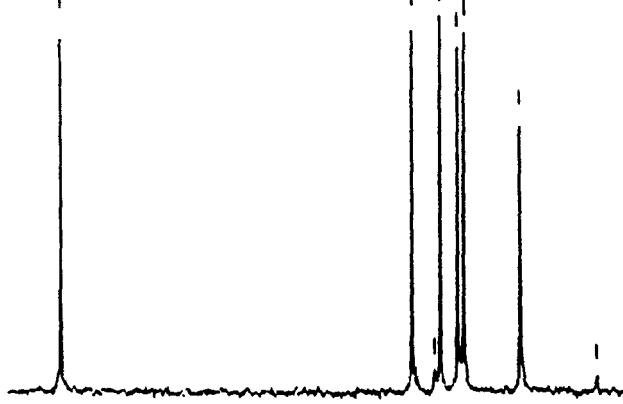
6.0:1  
GTF-S



1.42:1  
GTF-S



0.65:1  
GTF-S



DEXTRAN  
T70

100 90 ppm 80 70

the structures of the branches added to the primer by GTF-S were similar to the de novo synthesized comb structure. This experiment is the first reported demonstration that the presence of primer does not change the percentage of linkage types synthesized by GTF-S. Furthermore, the formation of  $\alpha$ -1,3 linkages was due almost exclusively to polymerization activity, since the branching activity was shown to be very small by comparison (Figure 39 and Table 5).

It was also determined from spectra of the 1.8:1 and 4.5:1 GTF-I products (Figures 40 and 41) that the branches added to the primer by this enzyme were linear,  $\alpha$ -1,3-linked polymers. In these spectra, the actual  $\alpha$ -1,6 to  $\alpha$ -1,3 ratios were further from the predicted ratios than for the GTF-S spectra. As shown in Table 5, the NMR spectra actually indicate, as a result of experimental error, that the enzyme added slightly more than 100%  $\alpha$ -1,3 bonds, a physical impossibility. However, the 7 - 10% differences between the actual and predicted ratios are still within a reasonable experimental error, and indicate the addition of 100%  $\alpha$ -1,3 bonds to the primer. The finding of 1:1 ratios of  $\alpha$ -1,3 bonds to free-6-carbons (Table 5) also supports the linear,  $\alpha$ -1,3-linked structure for the branches. At the low temperature (25°C) that had to be used to record the spectra of the GTF-I samples, the peaks due to linear and branched  $\alpha$ -1,3 residues are not resolved, according to the data of

Seymour et al. (1976, 1979c). However, for a 100%  $\alpha$ -1,3-linked polymer, only linear residues are possible.

In addition to studying the structures of the GTF reaction products, the structure of primer dextran was also investigated by NMR. Although the spectrum of the concentrated T70 solution (Figure 42) shows peaks at ppm values characteristic of  $\alpha$ -1,3 bonds, the C1(1 $\rightarrow$ 3) and C3(1 $\rightarrow$ 3) peaks are each split in two. As discussed in the RESULTS, the most likely explanation for the splitting is that the primer contains both linear and branched  $\alpha$ -1,3 residues. This finding of the presence of linear  $\alpha$ -1,3 residues in Leuconostoc mesenteroides B512 dextran was not detected in previous methylation studies (Van Cleve et al., 1956; Lindberg and Svensson, 1968). The presence of  $\alpha$ -1,3 residues in primer dextran is not essential for priming activity, since a chemically synthesized, 100% linear  $\alpha$ -1,6-linked glucose homopolymer has been shown to be an effective primer (Walker and Scheurch, 1986). However, a complete description of the primer structure requires inclusion of these residues.

The proposed structures of the products produced by the GTF primer modification reactions, as determined by the C-13 NMR study, are summarized in Figure 45 for GTF-S and in Figure 46 for GTF-I. For GTF-S, comb structures are added as branches to the 3-hydroxyl positions of the primer. As discussed in the RESULTS, the data indicate that relatively

Figure 45. The structure of the product produced in the GTF-S primer modification reaction, as determined by C-13 NMR. Branches with a structure similar to the de novo synthesized comb dextran product are attached during catalysis to the 3-hydroxyl groups of primer dextran. (○) glucose residues; (—)  $\alpha$ -1,6 linkages; (■)  $\alpha$ -1,3 linkages.



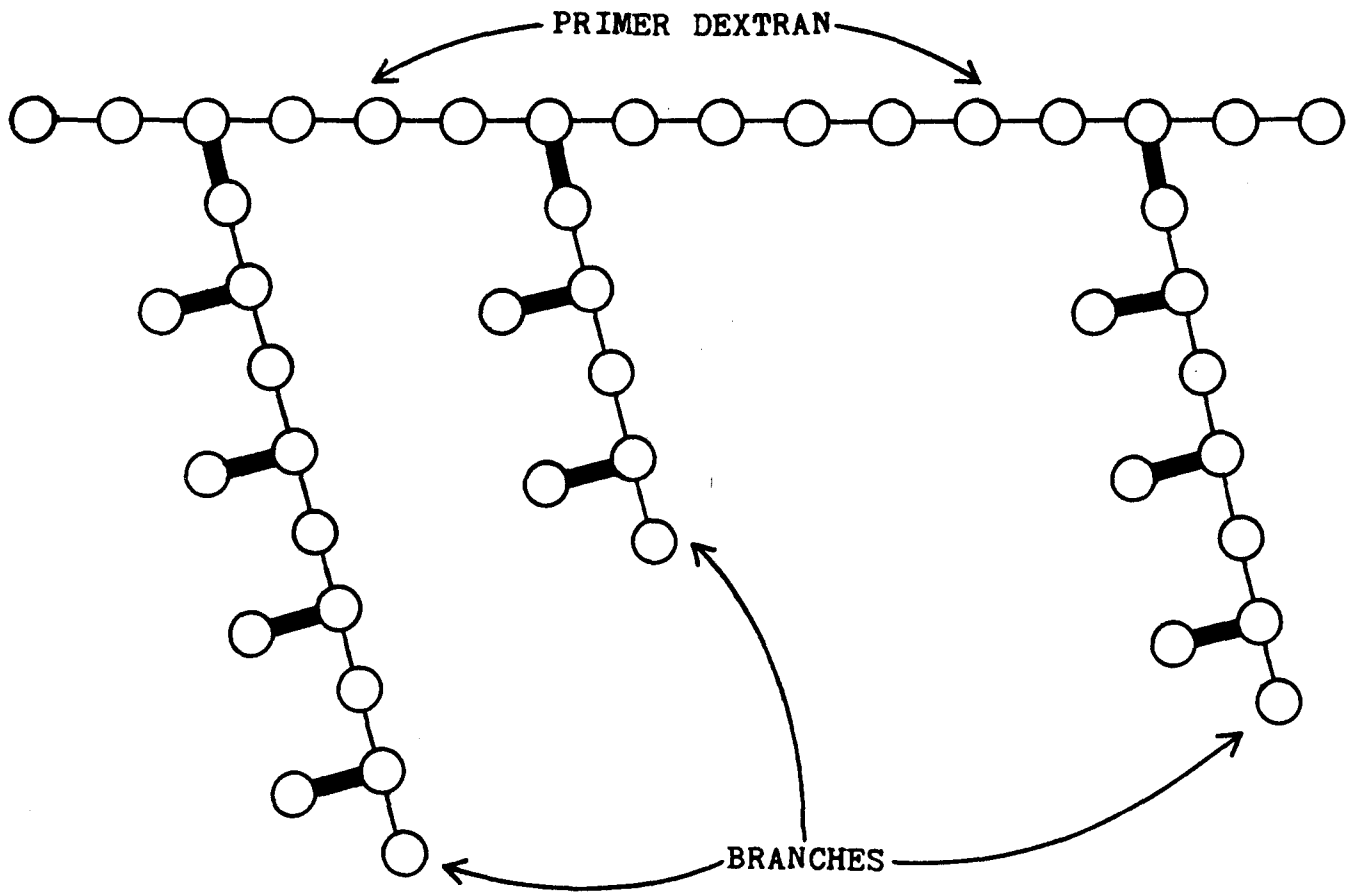
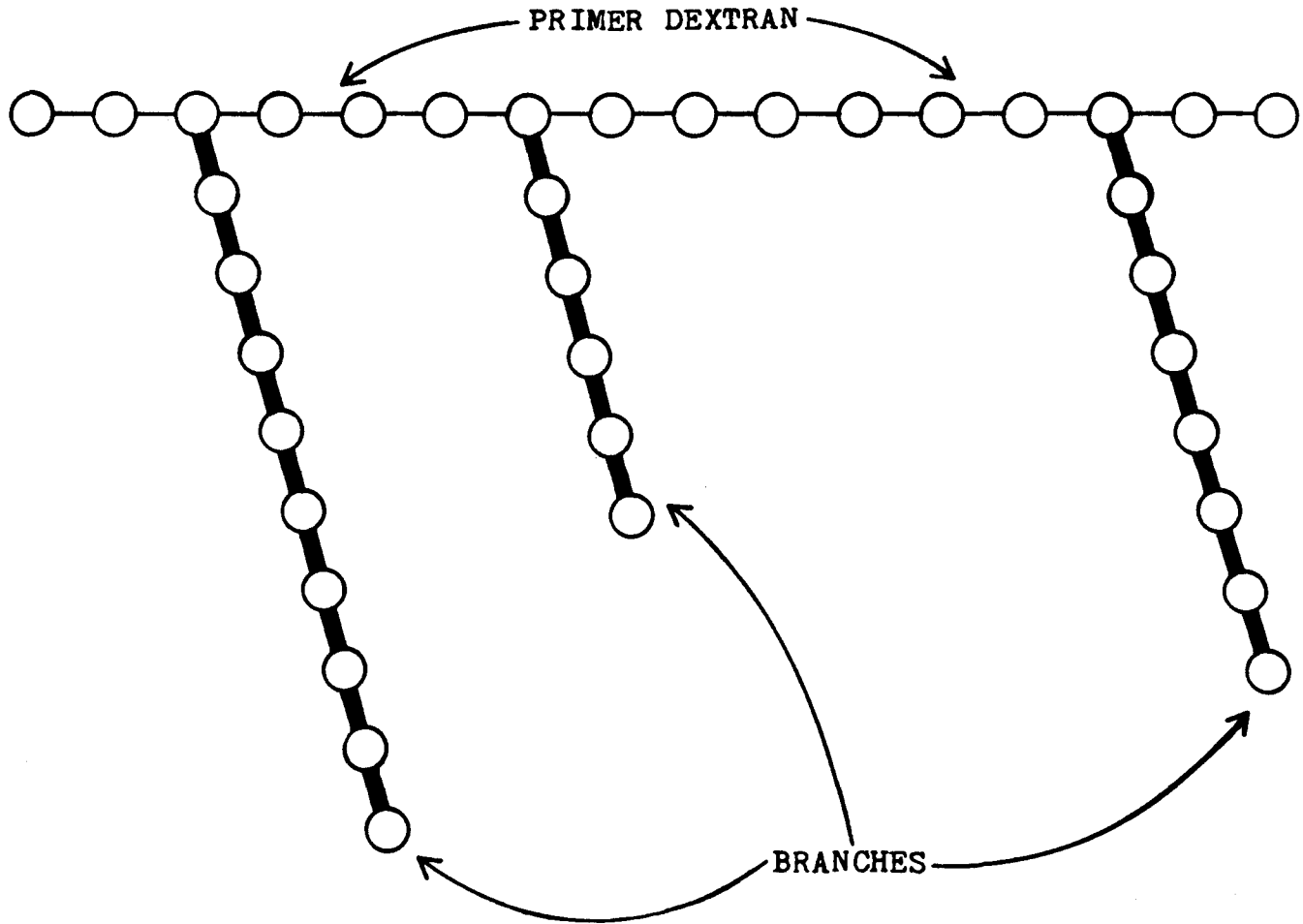


Figure 46. The structure of the product produced in the GTF-I primer modification reaction, as determined by C-13 NMR. Branches with a linear,  $\alpha$ -1,3-linked structure are attached during catalysis to the 3-hydroxyl groups of primer dextran. (○) glucose residues; (—)  $\alpha$ -1,6 linkages; (■)  $\alpha$ -1,3 linkages.



few of the primer 3-hydroxyl groups are modified. For GTF-I, linear  $\alpha$ -1,3 chains are added as branches to the 3-hydroxyl positions of the primer. In this case, it could not be determined from the NMR data whether few or many primer 3-hydroxyl positions were modified. Thus, the NMR technique has provided a description of many features of the primer modification reactions of the GTF's.

#### 4. Validity of Using C-13 NMR Peak Areas to Estimate Proportions of Carbon Species in Glucans

All the structural arguments discussed above depend on the assumption that the peak areas in the C-13 NMR spectra are proportional to the number of carbon atoms giving rise to the peaks. However, for C-13 NMR spectra, this assumption is not generally true, as it is for proton NMR. There are four basic requirements in NMR for obtaining quantitative peak areas (Abraham and Loftus, 1980).

1. The radiofrequency pulse must be strong enough to irradiate all nuclei in the molecule equally well. Otherwise, nuclei that are not irradiated sufficiently will not give a large enough peak intensity.

2. The computer must have enough data storage capacity to give accurate measurements of peak shape and size. If there is not enough storage space, the tops of peaks may be "cut off," resulting in an underestimate of peak size.

Both of these problems are functions of the instrument and have been resolved by the manufacturers of modern NMR

spectrometers. However, there are also two requirements that are dependent on molecular characteristics, and cannot be controlled by instrument design.

3. The spin-lattice ( $T_1$ ) relaxation times of different atoms in a molecule must be equivalent, or variations in the degree of relaxation of various nuclei between pulses will lead to quantitatively inaccurate peak areas. Since the  $T_1$  times of carbon atoms can vary widely, this variation is the major cause of nonquantitative peak areas being obtained in C-13 NMR spectra. The problem can be resolved by inserting a pulse delay of at least five times the longest  $T_1$  value in order to allow nuclei to relax essentially completely before the next pulse.

4. The nuclear Overhauser enhancement (NOE) effects for various nuclei in the sample must be equivalent. In routine C-13 NMR spectra, NOE effects result in an enhancement of signal intensity as a result of a change in the populations of the ground and magnetically excited  $^{13}\text{C}$  nuclear states caused by proton-noise decoupling. A  $^{13}\text{C}$  nucleus experiencing maximum NOE gives a peak with an area almost three times as large as the area in the absence of NOE effects. If various carbon atoms in a molecule display different NOE's, quantitative peak areas will not be obtained.

Although the latter two requirements represent potential problems in basing structural arguments on C-13 NMR

spectra, quantitation in the GTF glucan spectra is expected to be valid for several reasons. As shown in Table 6, the  $T_1$  times of all the carbon atoms in the products were 0.52 sec or less. Since the pulse delay for spectral recording was ten times this value (5 sec), the nuclei were almost completely relaxed before the next pulse was applied. Thus,  $T_1$  time variation had little effect on relative peak areas.

As for the NOE problem, it is known that a maximal NOE is obtained for molecules that relax exclusively by the efficient dipolar relaxation mechanism. For large polysaccharides, dipolar relaxation is, by far, the major relaxation mechanism observed (Casu, 1985). This mechanism is expected to be very efficient for the carbon nuclei in GTF glucans because of the large size of the molecules and because each carbon atom contains at least one covalently bonded hydrogen atom, a very favorable situation for dipolar relaxation to occur. The efficiency of this relaxation mechanism is evidenced by the very short  $T_1$  times of the carbon atoms in the GTF products. Thus, since the carbon atoms in the glucans are expected to exhibit nearly maximal, and therefore equivalent, NOE's, NOE effects are expected to have little influence on relative peak areas.

These predictions of the validity of quantitation in C-13 NMR spectra of glucans have been proven to be true by several experimental results.

1. Studies comparing proportions of bond types in dextrans determined by C-13 NMR and by methylation have shown that the results are similar, usually to within about 0 - 7% (Usui et al., 1975; Colson, et al., 1979; Seymour, 1979c). In the rare cases where a larger difference has been observed, it has not been established whether the difference is due to error in methylation analysis or in the NMR technique.

2. The 33.3% composition of  $\alpha$ -1,3 bonds in our NMR spectrum of the de novo GTF-S product is similar to values of 36% (Furuta et al., 1985) and 26.3% (Shimamura et al., 1982) obtained by methylation of de novo products of the GTF-S from the same bacterial strain. At least some of this minor discrepancy can be explained by the fact that the strain 6715 bacteria used in each of these studies were obtained from different sources, and the purification schemes used to obtain the GTF-S preparations were all different. Also, due to the difficulty of dextran methylation, differences could result from errors in the methylation analyses.

3. The value of 3.8%  $\alpha$ -1,3 bonds obtained in our concentrated T70 spectrum (Figure 42) is only slightly different from the values of 4.5% (Lindberg and Svensson, 1968) and 5.0% (Van Cleve et al., 1956) obtained by methylation analysis for L. mesenteroides B512 dextran.

4. The  $\alpha$ -1,3 bond to free-6-carbon ratio in all our NMR spectra was 1, as predicted for the products synthesized by GTF-S and GTF-I.

Thus, all these considerations justify our use of quantitative C-13 NMR data for drawing structural conclusions regarding the GTF-synthesized glucan products.

#### 5. General Applicability of the NMR Method

The NMR method described here is by no means restricted to studying the polymerization reactions of the GTF's from S. sobrinus. Since the GTF's of all oral streptococci synthesize similar products, any of these GTF systems could be studied by this method. Furthermore, the capacity of NMR to distinguish between  $\alpha$ -1,2,  $\alpha$ -1,3, and  $\alpha$ -1,4 bonds means that the technique could be used to study the reactions of Leuconostoc and Lactobacillus GTF's as well. The ease of the method as compared to methylation analysis is striking. The NMR technique is, therefore, well-suited to studying the dynamic process of polymerization, which requires analysis of several samples, not just to studying products synthesized in long incubations.

The NMR technique was shown to be sufficiently sensitive so that reasonable amounts of products synthesized by laboratory-scale quantities of enzyme gave adequate spectra for structural studies. The sensitivity was demonstrated by the fact that only 5.2%  $\alpha$ -1,3 bonds could be detected in the GTF-S branching activity sample (Figure 39), which had a



concentration (16 mg/ml) similar to those of other samples in the study. The concentrated T70 sample (Figure 42) demonstrated that the sensitivity could be increased even more by using larger sample sizes. The validity of using peak areas for quantitative purposes was also demonstrated by  $T_1$  measurements and by correlation with previous methylation data. Since dextrans synthesized by the enzymes of any species are expected to have carbon nuclei that relax efficiently by dipolar relaxation and have short  $T_1$  times, the quantitation methods should also be generally applicable.

The scope of the NMR technique could be expanded, and some of the minor limitations that exist could be overcome, by using techniques complementary to those described. First, two-dimensional NMR could be used to provide better resolution of certain peaks, such as the peaks due to linear and branched  $\alpha$ -1,3 residues. These peaks are separated in one-dimensional spectra by less than 1 ppm at high temperatures, and are not resolved at low temperatures. Also, the peaks in the 70 - 75 ppm region, which increase in complexity as glucose residues are added to the primer, could be assigned and better resolved by two-dimensional NMR techniques. These peaks might provide additional useful structural information.

Second, sucrose enriched with  $^{13}\text{C}$  at various positions of the glucose moiety could be used to increase the sensitivity and specificity of the NMR spectra. Although the

synthesis of sucrose from commercially available Carbon-13 labeled glucose and unlabeled fructose is extremely complex and would involve both organic synthesis and enzymatic techniques, such a synthesis is theoretically possible. This labeled sucrose could be used as a GTF substrate or as the starting material to synthesize  $^{13}\text{C}$ -labeled primer dextran with L. mesenteroides B512 dextransucrase. Numerous GTF product synthesis experiments could be performed with these  $^{13}\text{C}$ -labeled compounds. Due to the very low natural abundance of the  $^{13}\text{C}$  nucleus, if  $^{13}\text{C}$ -enriched primer were used in a GTF reaction, only the contribution of the primer would be observed in the C-13 NMR spectrum of the glucan product, whereas labeled sucrose substrate would allow visualization only of the branches. Such techniques could provide additional information about the primer modification reactions. For example, the structures of products synthesized very early in the reactions (products with ratios of glucose added per glucose in the primer of 0.1:1 or less) could be studied. Furthermore, the number of branch points added to the primer dextran molecule could be determined more accurately than is possible using the natural abundance spectra. Combined use of [ $^{13}\text{C}$ ]sucrose substrate and two-dimensional NMR could even theoretically be used to calculate rate constants for the GTF reaction, as has been done by  $^{31}\text{P}$  NMR for the adenylate kinase and phosphoglyceromutase reactions (Mendz et al., 1986). In this method, NMR is used to

follow the enzyme reaction as it occurs directly in the NMR tube. These techniques would require a great deal of experimental development, but are theoretically possible, based on our C-13 NMR results.

At the present stage of development, however, our one-dimensional NMR results have provided a wealth of information about the primer modification reactions of S. sobrinus GTF's, and have answered several of the questions about the structures of the products produced in these reactions.

Thus, the work described in this dissertation has advanced the field of GTF research in at least three major ways. First, new and significant information has been obtained about the physical properties, kinetic properties, and polymer synthesis reactions, especially the primer modification reactions, of S. sobrinus GTF's. Second, a successful purification scheme for the GTF enzymes has been developed that will allow other investigators also to obtain acceptable enzyme preparations for further GTF studies. Finally, an NMR technique of general applicability has been developed that will allow study of any enzyme-catalyzed dextran synthesis reaction in any system from which purified GTF enzymes are available.

## CHAPTER VI

### SUMMARY

Streptococcal glucosyltransferase (GTF) enzymes, which are postulated to be virulence factors in the formation of dental caries, synthesize high molecular weight, polyglucose polymers either from sucrose alone or by transferring glucosyl units from sucrose to  $\alpha$ -1,6-linked "primer" dextran. The primer-dependent enzymes GTF-S and GTF-I, which synthesize soluble and insoluble products, respectively, were purified 6870 and 3970 fold from the culture fluid of the cariogenic bacterium Streptococcus sobrinus 6715-7 by anion-exchange chromatography and affinity chromatography on Sephadex G-25. Use of the novel affinity chromatography eluting agent maltose, which elutes GTF's from Sephadex by a competitive effect rather than by denaturation of the enzymes, represents an improvement over previously reported purification schemes in which dextran or the chaotropic agent guanidine hydrochloride was used for elution.

GTF-S and GTF-I each showed two isozyme bands on SDS polyacrylamide gels. All isozymes were single polypeptide chains of molecular mass 155-180 kD. GTF-S also showed two bands of pI 4.18 and 4.33 on gel isoelectric focusing, but GTF-I did not focus well due to enzyme aggregation. The activities of both enzymes were stimulated by primer dextran,

but GTF-I showed dextran substrate inhibition at high dextran concentrations. In the presence of primer dextran, GTF-S and GTF-I had  $K_m$  values for sucrose of 4.6 and 1.6 mM, respectively. Maltose was a mixed inhibitor of GTF-S, with  $K_i$  and  $K_i'$  values of 0.058 M and 0.227 M, respectively. Maltose also inhibited GTF-I, but induced sucrose substrate cooperativity, so that the form of inhibition could not be determined. Graphs of  $\log(V_{max}/K_m)$  vs. pH showed that the association of GTF and sucrose was controlled by two ionizable groups with  $pK_a$  values of about 4.7 and 6.6. A  $\log(V_{max})$  vs. pH graph for GTF-I showed a dependence upon a single ionizable group, which needed to be unprotonated for activity and had a  $pK_a$  value of 4.55.

It was shown by native polyacrylamide gel electrophoresis, gel isoelectric focusing, and various kinetic experiments that, unlike GTF-S, GTF-I aggregated from a kinetically less active, dissociated form, to a more active, aggregated form. Aggregation occurred with increasing enzyme concentration or upon addition of 1.55 M ammonium sulfate to the assay mixture. This aggregation necessitated addition of ammonium sulfate to assay mixtures in order to obtain an assay that was linear with increasing enzyme concentration.

Natural abundance  $^{13}C$  NMR spectra of reaction products in progressive stages of polymerization were used to follow the GTF reaction in the presence of primer dextran. It was

shown that GTF-S modified the primer by adding branches containing  $\alpha$ -1,6 and  $\alpha$ -1,3 bonds in a 2 to 1 ratio. The branches had a structure similar to that of the comb dextran product synthesized de novo from sucrose alone. The sucrose-independent branching activity of GTF-S was shown to make only a very small contribution to the overall formation of  $\alpha$ -1,3 bonds. GTF-I was shown to add branches consisting of linear,  $\alpha$ -1,3-linked polymer. Measurements of spin-lattice ( $T_1$ ) relaxation times, all of which were 0.52 sec or less, demonstrated the validity of using relative peak areas in the C-13 NMR spectra to estimate the proportions of different carbon species present. Thus, this NMR technique was shown to be an effective substitute for time-consuming methylation analysis in the study of enzyme-catalyzed dextran synthesis reactions.

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**APPENDIX A**

## APPENDIX A

In this dissertation, the kinetic constants  $K_m$  and  $V_{max}$  were determined by the method of direct linear plots, a technique first described by Eisenthal and Cornish-Bowden (1974). APPENDIX A describes this graphical method.

Several methods exist for calculating enzyme kinetic constants from  $s$  and  $v$  values. The most popular methods involve linear transformations of the Michaelis-Menton equation and determination of the best-fit line by the least squares method. Another technique involves minimizing the sum of squares of errors, and requires a weighting scheme for the experimental observations. Such methods require the assumption that errors in kinetic experiments are normally distributed and that the correct weights are known. In practice, it is often impractical to gather enough experimental points to verify the assumption of normality, and, often, not enough information is available to correctly assign weights. Furthermore, the normal distribution may simply not apply to enzyme kinetics in many cases, particularly because of the common occurrence of "outliers" in kinetic measurements.

The method of direct linear plots (Eisenthal and Cornish-Bowden, 1974; Cornish-Bowden and Eisenthal, 1974, 1978) was developed in order to remedy some of these



problems. It is a non-parametric method and, therefore, does not depend on assumptions of normality. The only assumption required is that errors in kinetic measurements are as likely to be positive as negative, a much less stringent requirement than the assumption of a normal distribution of errors.

The method involves algebraic transformation of the Michaelis-Menton equation:

$$v = \frac{V_{\max} s}{K_m + s} \quad (6)$$

to the form:

$$V_{\max} = v + (v/s)K_m \quad (7).$$

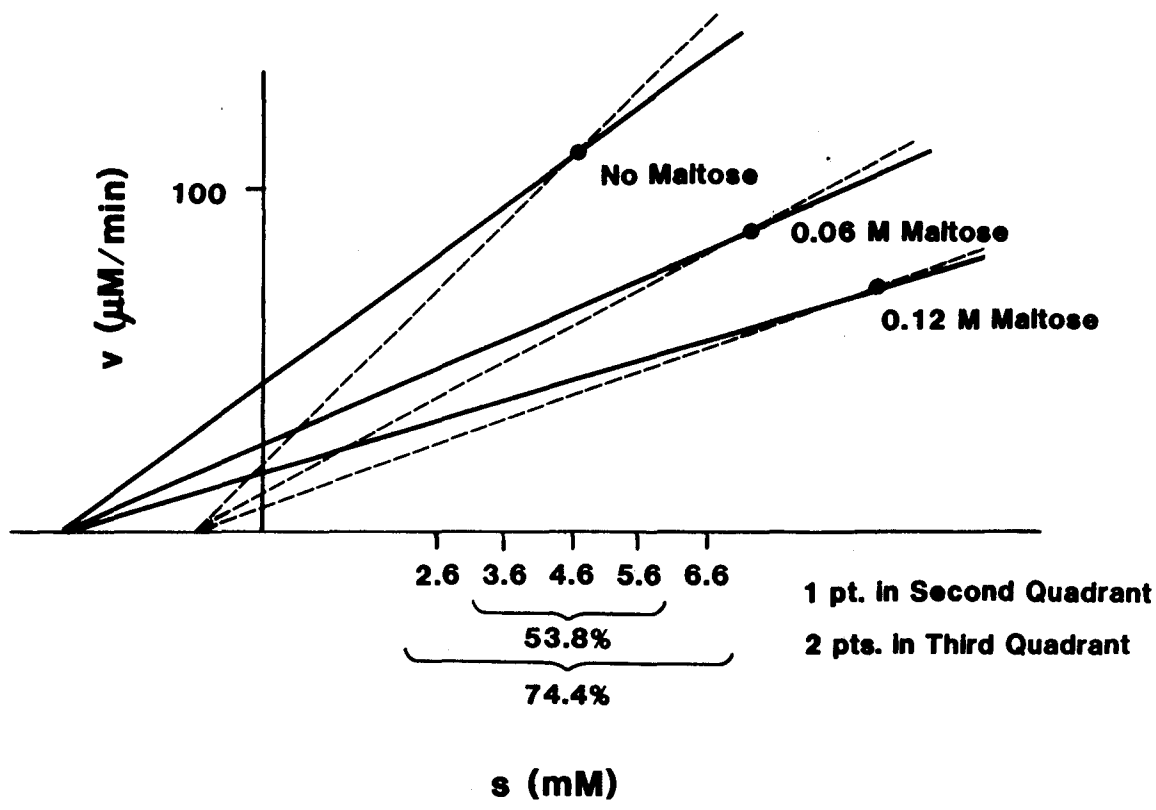
If  $V_{\max}$  and  $K_m$  are treated as the variables, this equation gives a line with a y-intercept of  $v$  and an x-intercept of  $-s$ . Thus, if experimental values of  $-s$  and  $v$  obtained in a kinetic experiment are plotted on the x- and y-axes, respectively, each  $-s$  and  $v$  pair will determine a line with a slope of  $v/s$ . From equation (7) it can be seen that all these lines should theoretically intersect at a point with coordinates  $(K_m, V_{\max})$ . In practice, due to experimental error, each pair of lines will generate an intersection that varies somewhat from the true  $(K_m, V_{\max})$  point. For  $n$  pairs of  $-s$  and  $v$  values,  $\frac{1}{2}n(n-1)$  intersections will be obtained. These intersection points will tend to cluster around the true  $(K_m, V_{\max})$  point if the Michaelis-Menton equation is being followed. Therefore, the median values of  $K_m$  and  $V_{\max}$

obtained from the intersection points are taken as estimates of the true values of these constants.

Figure 47 shows a direct linear plot of typical data for GTF-S in the presence and absence of maltose. Two sample substrate concentrations are shown on the x-axis, with lines drawn through the corresponding values of  $v$  on the y-axis. Actually, thirteen substrate concentrations were used in the no maltose experiment, so that 78 intersections were generated. As shown on the x-axis, the median of these points gave a  $K_m$  value of 4.6 mM, with 53.8% of the intersections lying within  $\pm 1$  mM of the median and 74.4% lying within  $\pm 2$  mM. This clustering of intersections around the median demonstrates that the Michaelis-Menton equation was followed. Three extreme outlier points, one in the second quadrant and two in the third quadrant, were observed. The points in the third quadrant must be treated as though the coordinates had large positive values, in order to avoid statistical bias (Cornish-Bowden and Eisenthal, 1978). Thus, these third quadrant points were artificially "shifted" into the first quadrant before determining medians.

A similar analysis of kinetic data in the presence of maltose was performed, and the median points obtained are indicated in Figure 47. The fact that the median intersection points from the no maltose, 0.06 M maltose, and

Figure 47. Direct linear plot of kinetic data for GTF-S in the presence and absence of maltose. The intersections generated by sample -s and v pairs are shown. For the no maltose experiment, 13 substrate concentrations were actually used, and these generated 78 intersections. The percentages of these intersections that fell within 1 or 2 mM of the median  $K_m$  value are indicated on the x-axis. This clustering of intersection points around the median indicates that the Michaelis-Menton equation was followed. The fact that the no maltose, 0.06 M maltose, and 0.12 M maltose median intersection points determine a line sloping downward to the right indicates that maltose is a mixed inhibitor.



0.12 M maltose experiments determine a line sloping downward to the right indicates that maltose is a mixed inhibitor.

The method of direct linear plots is capable of giving a two-dimensional confidence interval about the median point ( $K_m$ ,  $V_{max}$ ) (Cornish-Bowden and Eisenthal, 1974). However, the procedure for determining such confidence intervals is rather confusing and does not give confidence intervals for  $K_m$  and  $V_{max}$  individually. A simpler, but equally valid, procedure has been developed by Porter and Trager (1977). This method gives nonparametric confidence intervals for  $K_m$  and  $V_{max}$  individually by using Kendall's K statistic (Kendall, 1970). The method is very similar to calculating confidence intervals for a normal distribution, except that the nonparametric distribution is used instead of a normal curve. Using the  $\frac{1}{2}n(n-1)$  intersection points from a kinetic experiment, nonparametric confidence intervals of about 68.2%, corresponding in a normal curve to "plus or minus one standard deviation," can be calculated from tabulated values of the K statistic. Such confidence intervals are shown in Table 3, for the determination of  $K_m$ 's at varying enzyme concentrations. It can be noted that, due to the nonparametric nature of the analysis, the confidence intervals obtained are not exactly 68.2%, and the intervals are not evenly distributed about the estimated  $K_m$  value. Thus, intervals such as 4.60 +0.40/-0.38 mM for a 69.4% confidence interval are obtained.

The direct linear plot method, with the confidence intervals of Porter and Trager (1977), was compared to parametric methods by a sophisticated computer analysis of simulated kinetic experiments (Cornish-Bowden et al., 1978). For these simulated experiments, when assumptions of normality did not hold, the nonparametric method was shown to be superior to parametric methods in estimating kinetic constants. Even when assumptions of normality were valid, which is rarely or never the case in real kinetic experiments, the nonparametric method was still almost as effective as parametric analysis. Furthermore, the nonparametric method is much less sensitive to outliers, the occurrence of which is universal in enzyme kinetic experiments. Thus, for routine kinetic experiments to determine  $K_m$  and  $V_{max}$  values, the nonparametric direct linear plot method has been shown in general to be superior to parametric methods.

APPROVAL SHEET

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The dissertation is, therefore, accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

2/24/89

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