<u>Glycosidase Induction in</u> *Pseudomonas stutzeri*

<u>and</u>

<u>Properties of One of Its Amylases, Maltotetraohydrolase</u>

by

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To My Loving Husband, Leonard,

and My Daughter, Lithe,

Without Whose Understanding and Encouragement

This Work Could Not Have Been Completed.

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Glycosidase Induction in *Pseudomonas stutzeri* and Properties of One of Its Amylases, Maltotetraohydrolase.

by Dawn Wilson Sebesta

Pseudomonas stutzeri has been shown to produce extracellular amylases of different specificity, as well as a carbohydrase which hydrolyzed oligosaccharide but not starch. *Pseudomonas stutzeri* NCIB 11359 and NRRL B-3389 were adapted to, and then cultured in five different growth media. Both culture collections showed similar patterns in cell morphology and glucosidase secretion. Maltotetraohydrolase was produced in several of the media, a ´typical´ α-amylase was exclusively produced in one of the defined media, and no glucanase in yet another medium. The endo-α-amylase which produced primarily maltose was present during early exponential cell growth and no longer detected by late exponential growth. When maltotetraohydrolase was produced, it was found throughout exponential growth, and in one of the media remained at maximum levels of activity into stationary phase.

Maltotetraohydrolase hydrolyzed a suspension of commercial amylose in water producing 71-99% maltotetraose in the oligosaccharide fraction. Studies with maltotetraohydrolase demonstrated endo-amylase activity with the substrates blue amylose and with several β-amylase limit dextrins. A limit dextrin was produced which could be further hydrolyzed by α-amylase. [1]H-nmr studies showed that no α-1,6-glycosidic linkages were hydrolyzed and that the α-anomer was produced from the hydrolysis of α-1,4-linkages. Between 18-24% of the glycosidic linkages in amylopectin and 11-20% in β-limit dextrin were hydrolyzed. Maltotetraose was the primary product from hydrolysis of glucans but from the β-limit dextrins a high proportion of the smaller oligosaccharides was produced. Maltotetraohydrolase limit dextrin representing 38-54% of wheat amylopectin was purified and it had a molecular weight of 7000-15000; a degree of branching of 15%; ca. 13 branches per molecule; and an average chain length of 7. HPLC showed it to have a narrow weight distribution. Comparison of wheat and potato amylopectin indicated potato amylopectin to have more evenly distributed branching and probably less densely branched clusters.

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GLYCOSIDASE INDUCTION IN *PSEUDOMONAS STUTZERI*

AND PROPERTIES OF ONE OF ITS AMYLASES, MALTOTETRAOHYDROLASE.

CHAPTER 1.   INTRODUCTION.

The bacterium *Pseudomonas stutzeri* secretes a unique amylase
which hydrolyzes starch, to produce a high proportion of maltotetra-
ose. This organism has been cultured in several different media and
the induction or suppression of this amylase, maltotetraohydrolase
[E.C. 3.2.1.60] followed. Maltotetraohydrolase from two of the media
was partially purified and several properties of the enzyme studied.

This amylase, maltotetraohydrolase, hydrolyzes starch to produce
a unique limit dextrin. From the results of hydrolysis of starch and
of β-limit dextrins, it has been possible to demonstrate the unique
endo-activity of this enzyme and to compare the fine structure of two
amylopectins.

1.1. **Pseudomonas stutzeri.** Pseudomonads are unicellular, aero-
bic bacteria widely distributed in soil and water. Their general pro-
perties and taxonomy have been described (1, 2, 3). They are motile
gram-negative rods between 0.5 to 1 μm by 1.5 to 4 μm in length, with
typically long, straight axes, occasionally curved but never helical.
The genus *Pseudomonas* is noted for its catabolic versatility. Pseudo-
monads do not require growth factors, most can grow in simple mineral
media with ammonium ions or nitrate, and a single organic compound as
the sole carbon and energy source (4). Although the species are noted
for their biochemical versatility, few are able to metabolize either

large molecules or many sugars (3, 5).

Pseudomonas stutzeri is frequently identified by the unusual colony structure of freshly isolated strains (1). These have a characteristic rough, wrinkled, reddish-brown appearance, which after repeated subculturing frequently become smooth, butyrous and pale (6). *P. stutzeri* is one of the few pseudomonads which is able to utilize starch as the sole carbon and energy source (2). This is one of the most valuable diagnostic properties for differentiation of *P. stutzeri* from other species (1). Of the 17 strains of *P. stutzeri* tested, two were found to be unable to grow on starch, one was also found to be unable to grow on maltose (1). All of the amylase positive strains secrete the unique amylase, maltotetraohydrolase, which hydrolyzes starch to produce a high proportion of maltotetraose (7). The maltotetraohydrolase from one strain, *P. stutzeri* 320 (NRRL B-3389), has been studied both here and in several other laboratories (7-15).

Pseudomonas stutzeri NCIB 11359 and NRRL B-3389 (believed to be the same strain but from different culture collections) has been cultured here in complex media (containing protein digests and/or growth factors) and in defined media (containing only minerals and starch). Changes in cell morphology, growth patterns, and the secretion of glycosidases, particularly maltotetraohydrolase, were followed in each of the media.

1.2. General Characteristics of Bacteria.

   1.2.1. The Growth Cycle. Bacterial growth involves two distinct but related processes, that of an increase in cell mass as

well as an increase in the number of cells.   In a closed system these
can be measured by changes in culture density, either by counting the
cells or by measuring the cell mass (16-19).   In this report changes
in cell mass have been measured, indirectly, by measuring changes in
optical density as described (and discussed) in Chapter 5.5.5.

The bacterial growth cycle can be divided into three major
phases (lag, exponential growth, and stationary) with two transitional
periods separating the phases (18-20).   The lag phase may occur after
the addition of inoculum to medium and is a period during which no
growth can be detected.   During the exponential growth phase, some-
times called log phase, the culture density increases logarithmically
with time.   Growth ceases at the growth end point and enters the
stationary phase either when an essential nutrient has been exhausted
in the medium or with the accumulation of toxic products.

Even though growth cannot be detected during lag phase, many
other processes are occurring as the cells prepare for exponential
growth.   This preparation period, the lag phase, is most apparent when
the inoculum is taken from cells in stationary growth phase, or is
made into a medium different from that of previous growth.   No lag
phase is apparent when the inoculum is made from cells taken from ex-
ponential growth (19) or when growth is limited by exhaustion of the
carbon source (18).   During the transition between lag and exponential
phases, cells increase in size in preparation for replication (21).

In exponential growth each cell continues to synthesize cell
material and to divide at the same rate at the parent cell.   The rate
of synthesis of new material, *i.e.* the specific growth rate, depends

on the particular bacterium and on the conditions of growth. Growth conditions are influenced by such factors as medium, temperature, rate of aeration, as well as the history of the cells. In complex media the lag phase is usually shorter and the growth rate higher than in minimal mineral media (17). When over-aeration occurs during lag phase, it may inhibit growth, particularly in mineral media (21). Cells which have been adapted to a particular medium will also have a shorter lag phase and higher growth rate. In cells adapted to a particular medium, extraneous metabolic pathways are presumably suppressed and those essential for efficient use of the medium are induced. In studies reported here, the adaptation of *P. stutzeri* to different media, and the induction or suppression of maltotetraohydrolase and other glycosidases were followed during the adaptation.

Although cell growth does not occur during stationary phase, it is not a static period. Extracellular enzymes and polysaccharides may be produced during this phase. Those cells already producing exopolysaccharides (including slime) generally continue to do so, frequently in increasing amounts after exponential growth (22). In *Pseudomonas* species, the rate of exopolysaccharide has been shown to be greatest during stationary phase, with no polysaccharide being produced until late exponential phase (23). *P. stutzeri* was also reported to sometimes produce an exopolysaccharide (24).

Extracellular enzyme secretion generally follows one of two patterns. Secretion may accompany growth and decline in early stationary phase, or, more frequently, is minimal during exponential growth but accumulates in large amounts during the stationary phase (25). This is the case with most bacterial α-amylases (25) and in

several species of *Bacillus*, the most efficient inducer tested for amylase induction was found to be maltotetraose (20). Production of extracellular enzymes or polysaccharides depends on the conditions of growth and, in particular, the medium. In a defined, minimal medium extracellular enzyme production may accompany growth whereas in a complex medium both extracelluar enzyme and polysaccharide are more frequently produced in stationary phase (23, 25).

1.2.2. The Effect of Media on Bacteria. In general, bacteria respond to their microenvironment by adapting their morphology, their products and their growth rate (18, 20, 26, 27). Encapsulation or slime formation is frequently associated with a high nitrogen source in the medium, low temperature, or the carbohydrate source. Cell size and shape can vary depending upon the age of the culture, concentration of substrate, and composition of the medium.

Of particular importance is the induction of a variety of proteins including carbohydrases, proteases, and perhaps permeases. Extracellular protease activity can be regulated by the amount of exogeneous amino acid and peptides (28). The complex media used by Robyt, Ackerman and others (7-13, 29) for growth of *P. stutzeri* contained 1% protein digest and was, therefore, likely to induce proteases.

In order for microorganisms to utilize polymers in their environment they must secrete exoenzymes which can degrade the substrate into oligomers small enough to cross the cell membrane. G_4-amylase is an extracellular enzyme secreted by *Pseudomonas stutzeri*. Extracellular enzymes, or exoenzymes, may refer to any enzyme found outside of

the cytoplasmic cell membrane (25).   In gram-negative bacteria this can include enzymes found in the periplasmic space (located between the cytoplasmic cell membrane and the outer membrane and cell wall), enzymes bound to the outer membrane or cell wall, as well as enzymes secreted into the culture supernatant.

In gram-negative bacteria the outer membrane acts as a permeability barrier to molecules larger than disaccharides.   Inducible permeases are believed to be required for transport of the maltodextrins into the bacterium (30).   In most bacteria, saccharides with a molecular weight greater than 700 are excluded by porins in the outer membrane, although saccharides smaller than 4000 daltons have been shown to diffuse freely into plasmolysed *Pseudomonas aeruginosa* (31). A similar situation exists in *E. coli* in which maltose induces a specific porin for regulation of maltodextrins (31) as well as a periplasmic maltodextrin binding protein (28).   The binding protein may have a dual role, of not only retaining the substrate in the periplasmic space, but in stimulating the maltose/maltodextrin transport system (30, 32).

Transport of some carbohydrates is dependent on ion transport. In *E. coli* and *Salmonella typhimurium*, mellibiose transport is linked to Na^+ transport (32, 33).   In this work it has been shown that the induction of maltotetraohydrolase in *P. stutzeri* is influenced by the sodium content of the medium.

1.2.3. Induction of Glycosidases in Pseudomonas stutzeri.

Robyt and Ackerman (7) were the first to report on the induction of maltotetraohydrolase in *P. stutzeri*.   The amylase was secreted at the

end of exponential growth (8h) with increasing levels of activity for several days thereafter. With continued subculturing the levels of activity increased. Using the same culture conditions and the same or a similar complex medium, others (9) have reported maximum amylase concentration at the beginning of stationary phase, with subsequent loss in activity during the stationary period.

Wober (29) followed growth of *P. stutzeri* on maltose, maltodextrins and soluble starch, and the induction of maltotetraohydrolase, pullulanase, maltodextrin phosphorylase, and amylomaltase. He used a complex medium at pH 6 (Robyt and others have used pH 7 (7-13)) and measured cell-bound enzymes as well as extracellular enzymes secreted into the culture supernatant. Although maltose and maltodextrins induced maltotetraohydrolase during exponential growth in Wober's medium, soluble starch did not induce synthesis until the stationary phase, and then the amylase remained cell-bound.

The growth of *P. stutzeri* and induction of maltotetraohydrolase in five media, all at pH 7, are reported here. Each of the media contained starch, and only enzyme secreted into the culture supernatant was examined. In two of the media, in which G4-amylase secretion was suppressed, other glycosidases could be detected, including one which appeared to be a classical α-amylase. This α-amylase hydrolyzed starch to produce a mixture of oligomers as well as maltose. Kainuma *et al* (34) also observed two different amylases were secreted by single bacterial culture. They found a classical α-amylase was secreted by *Aerobacter aerogenes* along with a maltohexaose-producing α-amylase.

In all media studied here, if maltotetraohydrolase was secreted, it was detected during exponential growth and generally followed growth. In some of the media the enzyme activity decreased during the stationary phase, in two of the media there was little or no loss in activity.

1.3. **Amylases**.

1.3.1. **Classifications**. Amylases hydrolyze the α-1,4-glucosidic linkages in α-glucans. They may be classified in a number ways (25, 35-38), the most frequent method of classification describes both the principal product from hydrolysis of starch and the anomeric configuration of the product. By this method amylases generally fall into one of the following categories: α-amylases which produce maltose and oligosaccharides in the α-anomeric configuration, β-amylases which produce principally β-maltose, and glucoamylases which produce β-glucose.

Alpha-amylases [EC 3.2.1.1] hydrolyze starch by an endo-mechanism whereby α-1,4-glycosidic bonds are hydrolyzed whether along extended glucosidic straight chains or between branches. Initially large product molecules as well as α-maltose are produced. After extended hydrolysis only short chain oligosaccharides (principally maltose with some maltotriose and glucose) and low molecular weight α-limit dextrin remain. The α-limit dextrins have at least four glucosyl residues per molecule, generally a degree of polymerization (DP) of 5-10, and contain the α-1,6-bonds from the original glucan.

Beta-amylases [EC 3.2.1.2] hydrolyze starch to produce β-maltose from the non-reducing termini of α-1,4-glucosyl chains. Hydrolysis is

arrested by modification of the glucosyl chain including an α-1,6 branch point in the starch molecule. This exo-mechanism produces maltose during all stages of the hydrolysis and, from starch, leaves a high molecular weight β-limit dextrin.

Glucoamylase [EC 3.2.1.3], like β-amylase, hydrolyzes starch by an exo-mechanism but produces β-D-glucose instead of β-maltose. Both exo-enzymes cause inversion of configuration at the C1 carbon, giving the β-anomer from an α-1,4-bond. Glucoamylase, however, also hydrolyzes α-1,6-linkages, although at a slower rate, so that little or no limit dextrin remains, depending upon the type of glucoamylase.

There are at least three oligosaccharide-producing amylases which appear to be exceptions to these categories. These are maltotriohydrolase [EC 3.2.1.-], maltotetraohydrolase [EC 3.2.1.60] and maltohexaohydrolase [EC 3.2.1.98]. All three have been described as exo-α-amylases (12, 14) and produce α-maltotriose, α-maltotetraose, and α-maltohexaose respectively, by an exo-mechanism. In other properties they differ considerably.

Maltotetraohydrolase (G_4-amylase) does not further degrade the initial oligosaccharide product at low concentrations of amylase (7, 12). Maltotriohydrolase (G_3-amylase) does slowly hydrolyze G_3, while maltohexaohydrolase (G_6-amylase) produces G_4 and G_2 from the respective primary products, G_3 and G_6 (14). G_6-amylase also partially hydrolyzes β-limit dextrin and to produce branched oligosaccharides (39). This has been attributed to an ability to accomodate an α-1,6-branch in the binding site. Of the exo-amylases only G_4-amylase appears to have endo-activity as well as exo-activity (14). Studies

of this endo-activity are described in Chapter 4.

1.3.2. Use in the Description of the Structure of Starch.

Although starch has been the topic of extensive investigation for many years, and the subject of several recent reviews (40-43), the exact structure and composition of starch is not known. Chemically, starch is principally an α-1,4-glucan containing <5% α-1,6-linked glucosyl branches points. There are two major types of molecule, amylose and amylopectin, and possibly a third intermediate type of molecule. The proportion of the different types of molecules as well as the average molecular weight of each type of molecule varies not only with the source of the starch granule but also with the maturity and growth conditions of the plant.

Amylose was once thought to be a linear molecule of α-1,4-glucosyl residues. With the crystallization of β-amylase, free of other amylolytic components, amylose was shown to be incompletely hydrolyzed by β-amylase. This was not due to retrogradation of amylase but due to α-1,6 branch linkages which are not bypassed by this exoamylase. Amylose from different starches has since been found to contain an average of 2 to 20 branches per molecule (44, 45), <0.6% branching. Potato amylose, used here for preparation of maltotetraose, contains an average of 7 to 12 branches per molecule (44, 45) or <0.2% branching, depending on the source of the potato starch.

Amylopectin contains about 4 α-1,6-linked glucosyl residues for each 100 α-1,4-linked residues, depending on the source of amylopectin (46). Beta-amylase hydrolyzes the outer portions of the chains in amylopectin, producing from 55% to 58% maltose and leaving a high

molecular weight β-limit dextrin (46). The β-limit dextrin contains maltosyl and maltotriosyl stubs at the outer branch points (47). From this information, the average chain length of various amylopectins and the average chain length of the limit dextrins or internal chains can be calculated.

Alpha-amylase hydrolyzes amylopectin much more extensively than β-amylase and produces low molecular weight oligosaccharides. The size of the branched α-limit dextrin depends upon the source of the α-amylase, with the branch point conferring some resistance to amylolysis of neighboring α-1,4-linkages. Most of the α-limit dextrins have one branch, however some have two or three branches. The multibranched α-limit dextrins have no more than one glucosyl residue between branch points and indicate that the distance between some branch points differs considerably from the average of 7 or 8 units between branches (46, 48, 49).

The "cluster" model for amylopectin, for which there is a growing consensus, explains many of the characteristics of amylopectin both in solution and in the starch granule. First proposed by French (48) in 1972, the model has since been modified by Robin *et al* (50), by Manners and Matheson (51) and most recently by Hizukuri (45) and additional detail incorporated into the model.

The cluster model contains regions of high density branching from which extend parallel chains. The parallel chains are probably found as double helices in the starch granule, are resistant to acid hydrolysis and form crystallite regions (52). These chains probably form the Nageli amylodextrins which have chain lengths of 10-18

glucosyl units (53-55).   The pattern of growth rings in the starch granule, the high viscosity, and angular dependence of scattered light are all consistent with an elongated molecule containing heterogeneous branching (50, 52, 56).

Debranching enzymes have generally shown amylopectin molecules to contain two predominant chain lengths, one of about 15-20 units and a second of about 45-50 units long (50, 57, 58).   Some of the shorter, 15-20 unit chains must include chains which were branched B chains in the amylopectin molecule (45, 57).   The longer chains are expected to be B chains which are part of and interconnect two clusters or branched regions.   In addition there may be a third population of B chains of 60 units in length, interconnecting three clusters (45, 50, 58).

Debranching enzymes, in conjunction with β-amylase, have been used to show that the ratio of outer unbranched A chains to branched B chains is 1:1 (51).   Partial debranching leads to a change in the A:B ratio and suggests that some of the B chains probably carry more than one A chain.   Models incorporating these various structural features of amylopectin have recently been reviewed (40, 42).

1.4.   Maltotetraohydrolase.   *Pseudomonas stutzeri* is the only organism known to produce maltotetrahydrolase (G_4-amylase).   Production of this unique amylase probably ensures its preferential use of starch since many organisms cannot readily utilize maltotetraose. Robyt and Ackerman (7) noted that contamination of the culture medium with other microorganisms caused a rapid loss in G_4-amylase activity. G_4-amylase production was enhanced by phosphate, inhibited by glucose in the medium, and reduced when maltose replaced soluble starch in the

medium (7). Wober (29) found the reverse to be true, and found that G_2 induced G_4-amylase secretion, whereas none was detected in cultures grown in soluble starch.

In Wober's medium, G_4-amylase remained cell bound when induced by soluble starch and was not secreted into in the culture supernatant. Although G_4-amylase may be induced in *Pseudomonas stutzeri* it is not necessarily excreted into the culture supernatant. Dellweg *et al* (9) found G_4-amylase to be bound to the outer membrane as well as in the supernatant. Dellweg *et al* (24) also characterized an extracellular polysaccharide secreted by *Pseudomonas stutzeri*. This polysaccharide "sometimes interfered with purification of G_4-amylase". In this thesis only extracellular enzyme which was secreted into the culture supernatant was examined. Exopolysaccharide did sometimes interfere with purification of the enzyme and an unsuccessful attempt was made to develop a medium in which exopolysaccharide was not produced.

Robyt and Ackerman (7) were the first to purify and characterize G_4-amylase. Since then others (13, 14, 24) have described additional properties of the enzyme. During the initial stages of hydrolysis, maltotetraose is produced exclusively from starch or glycogen. Hydrolysis of short chain malto-oligomers occurs only at the fourth residue from the nonreducing terminal of the α-1,4-glucosyl chain. A limit dextrin is produced from amylolysis of amylopectin or glycogen, reminescent of the limit dextrins produced by β-amylase. However, unlike β-amylase, the α-anomer is produced by maltotetraohydrolase. Maltotetraohydrolase (G_4-amylase) was the first exo-α-amylase to be described.

G_4-amylase is adsorbed to cornstarch (12) and dextrans, including Sephadex. Soluble dextran is a competitive inhibitor of G_4-amylolysis, and Sephadex has been used as as an affinity adsorbant for purification of G_4-amylase (9, 13).

Native G_4-amylase consists of dimers whose subunits have a molecular weight between 48,000 and 58,000 (10, 13). There are at least 2 isozymes (7, 12). It is not a glycoprotein (10). It is most active at pH 8.0 (7, 13) and, between pH 6-9, is not affected by the addition or absence of calcium ion (11, 13). It is most active at 45°C; and although it is stable between 20-40°C there is a rapid loss in activity above 40°C (7).

In studies reported here, it was proposed to examine the possibility of using high field nmr spectroscopy to investigate the action of amylases, including G_4-amylase, on various substrates. By this means it was proposed to examine the anomeric specificity, linkage specificity, degree of hydrolysis, degree of branching, number of hydrolyses per branch, and changes in average chain length. Others (9, 10, 14) had observed that G_4-amylase contained endo-activity and that it was able to hydrolyze the α-1,4-linkages in pullulan and in cross-linked blue starch. This has been further investigated in order to define and to analyze the extent of endo-amylolysis and the G_4-limit dextrins. In this way it might be possible to obtain new information of the fine structures of amylopectins, and to relate these to the cluster model of this type of polysaccharide.

Chapter 2. Microbial Cultures and Extracellular α-1,4-Glycosidases.

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CHAPTER 2. MICROBIAL CULTURES AND EXTRACELLULAR α-1,4-GLYCOSIDASES.

2.1. **Introduction.** Robyt and Ackerman (7) were the first to report on the induction of an extracellular maltotetraohydrolase (G_4-amylase) in *Pseudomonas stutzeri*. They found that the G_4-amylase was produced only during the stationary phase of growth at increasing concentrations through 3 days in stationary phase. Other researchers (9, 12) using of the same strain, NRRL B-3389, in similar media found extracellular G_4-amylase to be at maximum concentration in early stationary phase, presumably produced during exponential growth. Wober (29) using the same strain but a different medium reported that starch induced no extracellular G_4-amylase but only cell-bound G_4-amylase. The induction of G_4-amylase in similar complex media and in other media is now reported. A comparison was made between *P. stutzeri*. NRRL B-3389 and *P. stutzeri*. NCIB 11359 which was obtained from another culture collection. Both responded similarly in five different media. The compositions of the media are given in Chapter 5, Section 5.5.3, and the reasons for their selection are presented under Results and Discussion, Section 2.2.

In several complex media, either reduced levels of G_4-amylase activity or no extracellular amylase activity could be detected. In one of the complex media, *P. stutzeri*. NCIB 11359 secreted no G_4-amylase but did produce an extracellular glycosidase which degraded maltooligosaccharide but not starch.

P. stutzeri was cultured in three defined media which differed in the addition or absence of Ca^{2+} and Na^+ cations. Calcium ion is essential for activity in most α-amylases (36) and has been reported

to either greatly stimulate G_4-amylase activity (10) and/or stabilize activity to some conditions (11, 13). In this report *P. stutzeri* which had been adapted to a defined medium containing Ca^{2+} but no sodium ion was found to secrete a classical maltose-producing amylase but no G_4-amylase. With the addition of Na^+ to the defined medium, and adaptation, cells produced G_4-amylase and no G_2-forming amylase was detected. Cotransport of sodium ion is known (30, 33) to be required for the active transport of several carbohydrates across the cell membrane of several kinds of bacteria.

2.2. Results and Discussion.

2.2.1. Cultures in Trypticase I Medium. The bacterium *Pseudomonas stutzeri* NCIB 11359 was first cultured in Trypticase I medium, a medium similar Robyt and Ackerman's (7) except that BDH Analar soluble starch was substituted for Baker soluble potato starch. The procedures of Robyt and Ackerman were followed except that cultures were grown in an orbital shaker using conical flasks, a system in which aeration may have been significantly lower.

Cell growth was not followed in these cultures but it was observed that the cultures became very turbid and viscous with a considerable amount of foaming after several days of culture.

Amylase activity was measured by the turbidimetric assay, an assay which is not readily expressed in International Units. The assay did, however, show a linear increase in activity (0.009 turbid. U/ml/day) between 1 and 7 days of culture on the orbital shaker. Robyt and Ackerman (7) had reported an increase in activity from 8 h

through 4 days of culture, and reported on the purification of amylase from culture grown 3 days.   Two cultures, a small scale culture of 255 ml and a large scale culture of 4896 ml, were grown and the maltotetraohydrolase purified as described in Chapter 3.

2.2.2. Cultures in Defined ´Ca´ Medium.   Since high quantities of maltotetraohydrolase had not been produced by *Pseudomonas stutzeri* in the Trypticase medium, Dr. J. Bunker suggested culture in a defined medium.   A defined medium containing calcium ion, a cofactor for many amylases (36), was used for three batch cultures of *P. stutzeri* NCIB 11359.

2.2.2.1.   Culture A.   *Pseudomonas stutzeri* was adapted through nine subcultures in Ca medium.   See Chapter 5.5 for general procedures.   A white sediment formed in the boiling tube sub-cultures which appeared to have normal *P. stutzeri* growth when smeared on an agar petri plate. Subcultures in the flasks were very milky and had many cells along the culture-flask rim.

Cell growth and amylase activity as measured by the Boehringer assay are shown in Figure 2.1, "Ca, Culture A".   During the first three hours there appeared to be almost no cell growth.   During this period the turbidity of the culture decreased slightly, probably due to amylolysis of the starch in the medium. Between 3h and 4h after inoculation there was a small, exponential rise in biomass, then a slight plateau in growth. It is uncertain wheather the plateau was caused by the decrease in aeration at 4h or due to a transition period while the cells produced a logarithmic increase in amylase. When the biomass was only 0.03 mg/ml and amylase activity had reached a maximum

Ca, Culture A

Figure 2.1
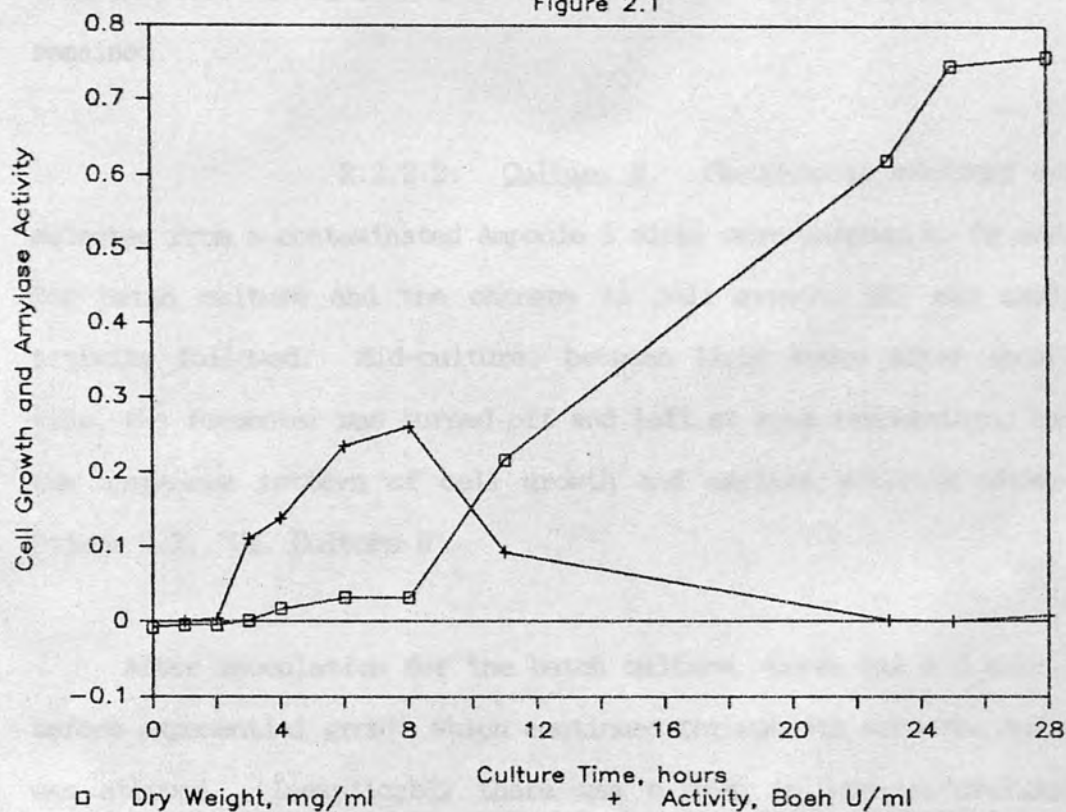
Fig. 2.1. Growth and the amylase activity of *Pseudomonas stutzeri* NCIB 11359 in Ca medium, Culture A. Growth was measured turbidimetrically at 650 nm and the dry weight calibrated from the biomass of a Na culture. Amylase activity was determined by the Boehringer Assay.

(at 8h), only then did exponential growth continue increasing through 11h and probably until 14h. From 23h onwards the cells were in the stationary phase and reached a maximum biomass of 0.76 mg/ml.

Amylase activity was detected only during exponential growth phase. Activity rose exponentially from 2h through about 4h, reaching a maximum of 0.260 Boeh U/ml at 8h, early in exponential growth, and

then decreased sharply. By stationary phase, little amylase activity remained.

2.2.2.2. <u>Culture B</u>. *Pseudomonas stutzeri* cells selected from a contaminated Ampoule I slope were adapted to Ca medium for batch culture and the changes in cell growth, pH, and amylase activity followed. Mid-culture, between 11-26 hours after inoculation, the fermenter was turned off and left at room temperature, hence the anomalous pattern of cell growth and amylase activity shown in Figure 2.2, "Ca, Culture B".

After inoculation for the batch culture, there was a 3 hour lag before exponential growth which continued through 11h when the culture was stopped. Inexplicably there was a drop in biomass overnight. Upon reinitiation of the culture there was another 3 hour lag before exponential growth from 29h-33h. The specific growth rate, 0.11 h^{1-}, was the same during both periods of exponential growth. The semilog graph of biomass vs time shows stationary phase beginning at 35h.

Gram-negative rods were observed throughout the culture growth. During exponential growth many of the rods were in head to tail chains, some up to 20 cells long. During stationary phase many of the cells formed large clumps which rapidly settled in the culture medium.

The pH of the culture decreased from 7.08 to 5.35. A plot of the difference in pH from the original pH 7.08, on the semilog graph showing changes in biomass, showed that the changes in pH directly paralleled the changes of lag, exponential, lag, exponential, and stationary phases in cell growth, i.e., the pH changes were directly

Ca, Culture B
Figure 2.2
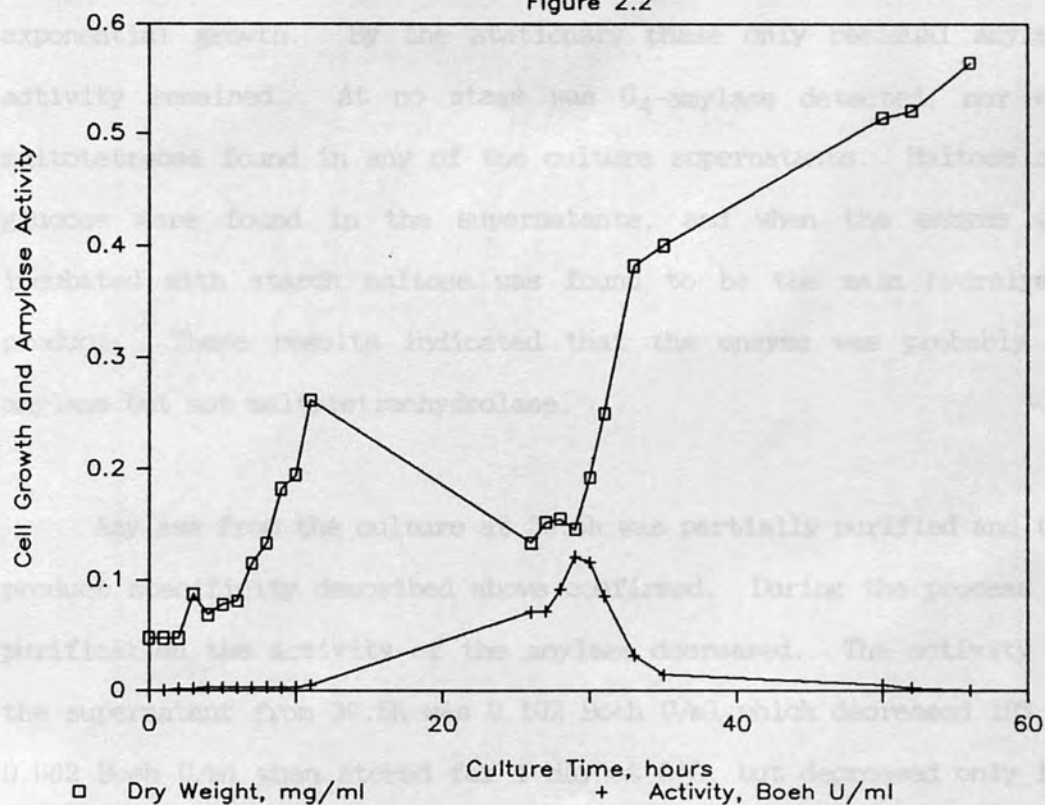
Fig. 2.2. Growth and the amylase activity of *Pseudomonas stutzeri* NCIB 11359 in Ca medium, Culture B. Growth and amylase activity were measured as described in Fig. 2.1. At 11 h the fermenter was turned off for 15 hours. A portion of the culture was harvested at 30 h for partial purification of the amylase.

related to cell growth.

During the first 10 hours, even when growth was exponential, amylase activity remained very low at <0.003 Boeh U/ml. However, amylase activity rose overnight when the culture was stopped, then rose exponentially from a maximum of 0.12 Boeh U/ml at a specific growth rate of 0.12 h^{1-} an hour after the culture was reinitiated.

Activity then decreased at 29h, at the onset of the second stage of exponential growth. By the stationary phase only residual amylase activity remained. At no stage was G_4-amylase detected, nor was maltotetraose found in any of the culture supernatants. Maltose and glucose were found in the supernatants, and when the enzyme was incubated with starch maltose was found to be the main hydrolysis product. These results indicated that the enzyme was probably an amylase but not maltotetraohydrolase.

Amylase from the culture at 30.5h was partially purified and the product specificity described above confirmed. During the process of purification the activity of the amylase decreased. The activity of the supernatant from 30.5h was 0.102 Boeh U/ml which decreased 19% to 0.082 Boeh U/ml when stored for 1 day at 8°C, but decreased only 12% (to 0.091 Boeh U/ml) upon freezing and thawing. A four-fold concentration by ultrafiltration occurring over 1 day at 4°C gave only 0.077 Boeh U/ml with only 0.001 Boeh U/ml passing through the G10T membrane. The pH of the concentrate was 6.85 and is unlikely to have caused the loss in amylase activity. Gram's stain of the concentrate showed that gram-negative cells were present, which may have been releasing proteases and thereby decreasing amylase activity. No further studies were done.

2.2.2.3. <u>Culture C</u>. A second ampoule was received from the NCIB culture collection and these *Pseudomonas stutzeri* were revived in Soy medium and then adapted to Ca medium for a batch culture study. The changes in cell growth, shown as dry weight, and in amylase activity, measured by the Boehringer assay, are summarized in Figure 2.3, "Ca, Culture C".

Ca Culture C
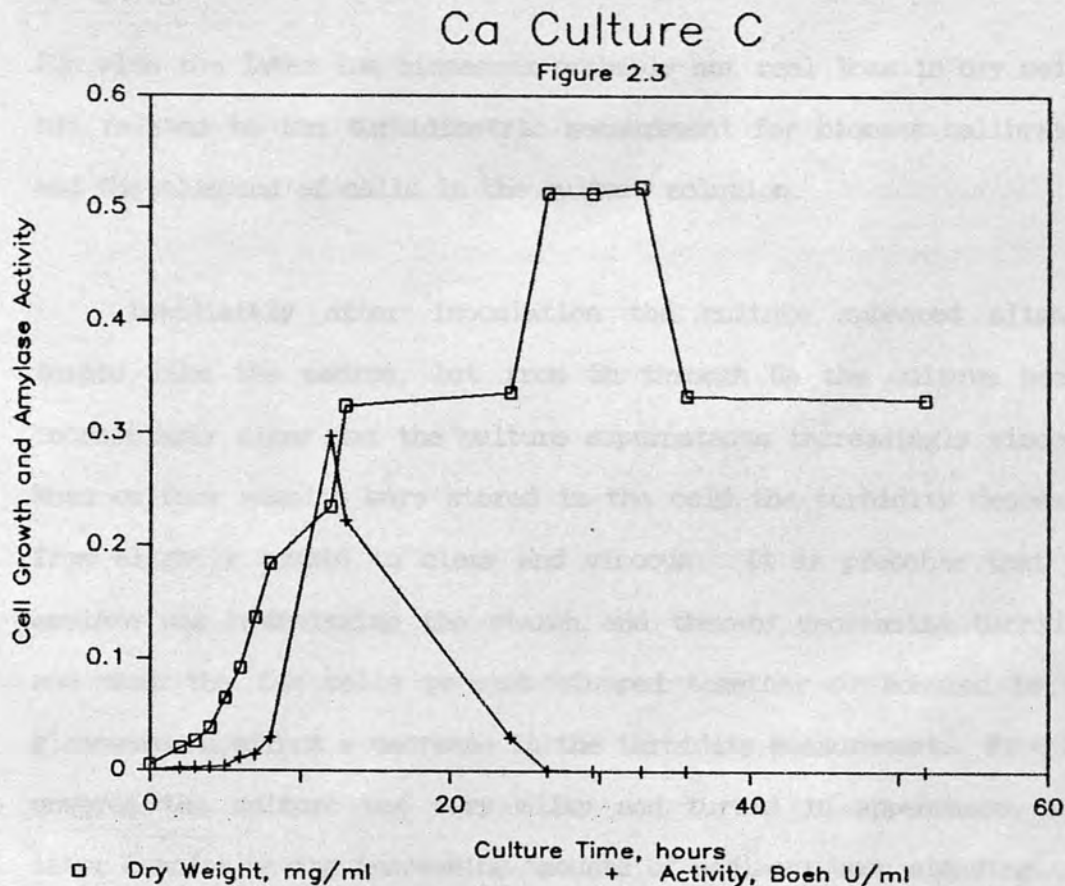
Figure 2.3

Fig. 2.3. Growth and the amylase activity of *Pseudomonas stutzeri* NCIB 11359 in Ca medium, Culture C. Growth and amylase activity were measured as described in Figure 2.1. A portion of the culture was removed at 13 h for partial purification of the amylase.

Exponential growth was maximal (specific growth rate, 0.18 h^{1-}) between 2h and 8h. During this period the pH dropped slightly from 6.9 to 6.8. Cells continued to grow through 26h but at a slower rate, during which time amylase activity reached a maximum of 0.298 Boeh U/ml and then decreased to 0.0005 Boeh U/ml. As in Culture B changes in pH and in cell mass were closely parallel. The pH difference from the original pH (7.0) decreased logarithmically from 2h-13h to pH 6.3; at 24h the pH was 5.4, only slowly decreasing to pH 5.2 by 52h. Cell growth was stationary from 26h-33h but was probably stationary through

52h with the later low biomasses probably not real loss in dry weight but related to the turbidimetric measurement for biomass calibration and the clumping of cells in the culture solution.

Immediately after inoculation the culture appeared slightly turbid like the medium, but from 3h through 8h the culture became increasingly clear and the culture supernatants increasingly viscous. When culture samples were stored in the cold the turbidity decreased from slightly turbid to clear and viscous. It is probable that the amylase was hydrolyzing the starch and thereby decreasing turbidity and that the few cells present clumped together or adhered to the glassware to effect a decrease in the turbidity measurement. From 24h onwards the culture was very milky and turbid in appearance, with later samples having increasing amounts of sediment upon standing.

During early growth little amylase was detected, <0.003 Boeh U/ml, but from 4h through 12h, during exponential growth, activity rose logarithmically at 0.256 h^{1-} to a maximum concentration of 0.298 Boeh U/ml, thereafter decreasing sharply during a second phase of exponential growth. It is unlikely that the loss of amylase activity was due to the presence of an inhibitor, as dilution of the enzyme solution gave an appropriate decrease in activity when assayed. Initially the enzyme hydrolyzed starch to produce predominately maltose, but after extended hydrolysis glucose and intermediate molecular weight oligosaccharides were also detected. Maltose and some glucose were found in culture supernatants sampled through 13 hours, but not in the supernatant sampled at 24h.

Just after maximum amylase concentration, at 13h, a portion of

the culture was harvested. During the process of removal of the cells, amylase was found closely associated with the cells as well as in the extracellular fluid. When centrifuged at 2075x*g* the amylase activity of the loose pellet left in the remaining supernatant (the pellet-supernatant recentrifuged) was 2.04 Boeh U/ml, 18 times greater than that of the slightly turbid supernatant that was decanted (0.114 Boeh U/ml). When the turbid supernatant was sterilized by hollow fiber filtration, the filtrate contained 85% of the amylase activity (0.102 Boeh U/ml). However, the cell concentrate and wash contained a higher amylase concentration, 0.169 Boeh U/ml, than the turbid super-natant had contained. In the short period required for hollow fiber filtration, the cells must have been either rapidly producing amylase, or amylase which was loosely cell-bound, or both. The amylase activity of the hollow fiber filtrate remained constant upon freezing and thawing, but showed a 7% loss in activity when stored at 4°C for 26 hours.

2.2.2.4.   Summary of Cultures in ´Ca´ Medium.
Pseudomonas stutzeri can be adapted to grow in a defined medium and to produce an amylase extracellularly. The adaptation by the cells and the subsequent production of amylase was shown *by P. stutzeri* from two sources and was not the result of a unique mutation of the cells. The amylase concentration is transient with no activity remaining during the stationary phase of the cell growth cycle.

Amylase was produced only during very early (Culture A) to mid-exponential growth phase (Cultures B and C), reaching a maximum concentration well before the cultures had attained 50% of their final biomass. The amylase activity then decreased sharply and was no

longer detected by the stationary growth phase.   The decrease in
activity was not due to an inhibitor but was probably due to the
presence of proteases which also probably caused the loss in activity
during storage and purification of the enzyme.   The amylase was stable
to freezing.   The amylase is produced extracellularly but may also be
adsorbed to the cells.

The amylase appeared to be a ´classic´ endo-α-amylase, hydrolyz-
ing starch to produce primarily maltose and, with extended amylolysis,
glucose and other oligosaccharides.   Maltose and glucose were present
in the culture supernatant during exponential growth but were no
longer detected by stationary phase.

Neither maltotetraose nor maltotetraohydrolase could be detected
at any time during these cultures of cells which had been well adapted
to Ca medium.   However, these same cells could be readapted to Soy
medium and shown to produce maltotetraohydrolase.

The cells showed normal growth on agar petri plates, forming
wrinkled, rough colonies, and appeared to be of normal shape, though
somewhat small, gram-negative rods.   During exponential growth they
were sometimes found in long head to tail chains.   During stationary
phase, the cells were closely aggregated in large masses and in the
culture solution would sediment as large white clumps.   This aggrega-
tion may be associated with the acidity of the culture solution which
had dropped below pH 6 by stationary phase. Changes in pH were found
to be closely related to the growth pattern, with pH decreasing as
biomass increased.

2.2.3.   Cultures in Complex Trypticase II Medium. Two batch cultures of *Pseudomonas stutzeri* were grown in the Trypticase II medium and a glycosidase from the culture supernatants partially purified.

2.2.3.1. <u>Culture A</u>. Figure 2.4., "Trypticase II, Culture A", shows the changes in dry weight and in Boehringer amylase activity during 82 hours of culture.

Dry weight was calculated (Chapter 5, calibrated using a Na culture) from the turbidity of the culture. Shortly after inoculation the turbidity increased logarithmically at a specific growth rate of 0.10 h^{1-} reaching a maximum density of 1.49 mg/ml at 28h. Growth was exponential from 0h through 9h after inoculation (determined graphically from a plot of log biomass vs time). The erratic pattern found at late exponential/early stationary phase can probably be attributed to asynchronous bacterial growth (17) since the subcultures were made at irregular intervals. The fall in turbidity at 82h (expressed as dry weight in Fig. 2.4) corresponds to a drop in pH and probably results from cell lysis.

The Boehringer assay showed low levels of enzyme activity increasing logarithmically at a specific rate of 0.10 h^{1-} between 4h-≈12h, during exponential growth phase. Enzyme induction continued from 12h to at least 30h, during stationary growth phase, reaching a maximum activity at 48h of 0.063 Boeh U/ml. Even though the assay showed hydrolysis of the Boehringer substrate, an oligosaccharide, there did not appear to be amylase present since incubation of enzyme with starch showed no hydrolysis either when assayed by the turbidimetric

Trypticase II, Culture A

Figure 2.4

Cell Growth and Glycosidase Activity (y-axis)
Culture Time, hours (x-axis)

□ Dry Weight, mg/ml   + Act, Boeh U/ml x 10
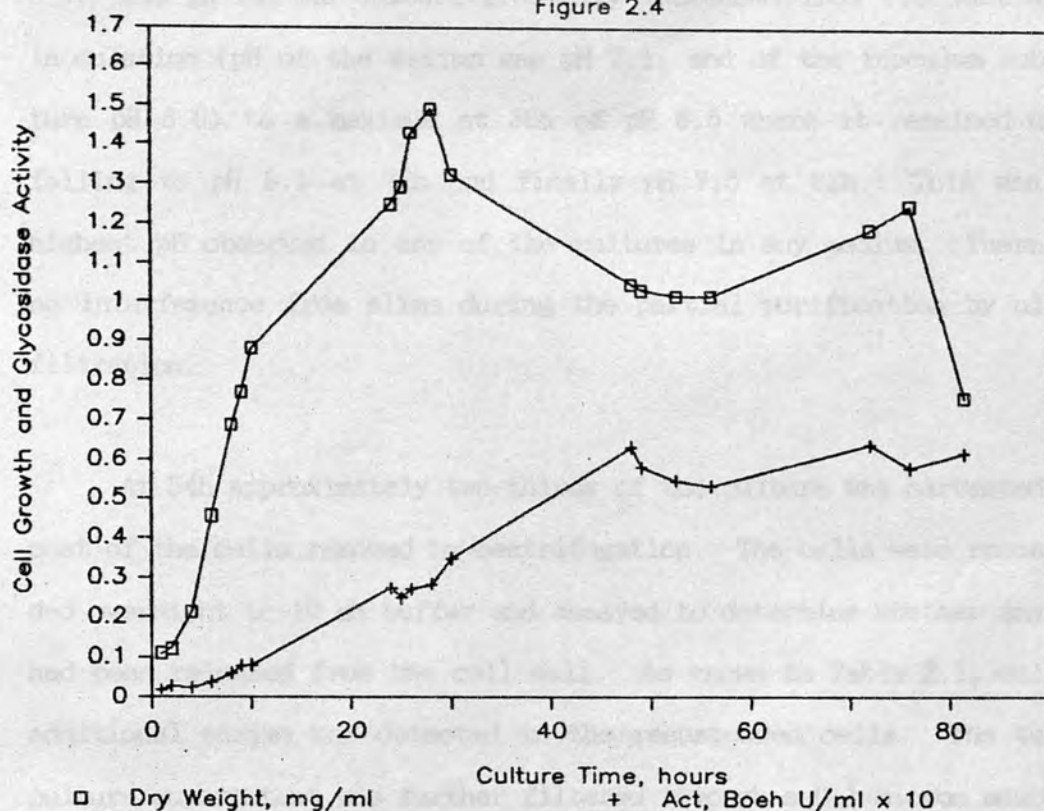
Fig. 2.4. Growth and the glycosidase activity of *Pseudomonas stutzeri*
NCIB 11359 in Trypticase II medium, Culture A. Cell growth was mea-
sured as described in Fig. 2.1. Glycosidase activity was measured by
the Boehringer assay. A portion of the culture was removed at 54 h
for partial purification of the glycosidase.

method or when tested for product specificity using tlc. In addition,
tlc of the culture supernatants showed primarily very high molecular
weight polysaccharide and possibly a little glucose.

Slide preparations of the culture at several stages in growth
showed large, 'fat' gram-negative rods, separate, and only late in the
culture were irregular masses observed which were thought to be
polysaccharide.

The pH of the culture gradually increased from 7.2 just after inoculation (pH of the medium was pH 7.1, and of the inoculum subculture pH 8.0) to a maximum at 30h of pH 8.5 where it remained until falling to pH 8.1 at 72h and finally pH 7.5 at 82h. This was the highest pH observed in any of the cultures in any medium. There was no interference from slime during the partial purification by ultra-filtration.

At 54h approximately two-thirds of the culture was harvested and most of the cells removed by centrifugation. The cells were resuspended overnight in 10 mM buffer and assayed to determine whether amylase had been released from the cell wall. As shown in Table 2.1, only 3% additional enzyme was detected in the resuspended cells. The turbid culture supernatant was further filtered through a 0.1 micron membrane and the cells thus concentrated were further washed with 1.5 M NaCl-buffer solution. There was no apparent interference from slime. The results are summarized in Table 2.1.

Table 2.1. Purification Steps of Trypticase Culture A.

Step	Volume ml	Boehringer Activity U/ml	Total Units
Centrifugation of supernatant	545	0.58	31
Cell wash of pellet	120	0.11	1
0.1 micron ultrafiltrate	480	0.52	25
Ultrafiltration wash	87	0.29	2

Most of the enzyme activity (78%) was recovered in the ultra-filtrate and amylase was apparently not removed from the cell walls (3% was found on the cell pellet and 6% in the ultrafiltrate wash). Wober (29) reported finding cell-bound amylase after culturing *Pseudomonas stutzeri* NRRL B-3389 but here there was no hint of amylase activity which could be removed by isotonic shock of the cells or by washing in a high salt concentration.

2.2.3.2. <u>Culture B</u>. Adaptation to the Trypticase II medium was continued from Culture A into a second batch culture, Culture B. Figure 2.5, "Trypticase II, Culture B", shows a slightly higher cell mass, a maximum of 1.6 mg/ml, than was produced in Culture A but a reduced level of Boehringer glycosidase activity.

Stains of culture samples showed gram-negative rods, many in head to tail chains or in clusters of cells, an observation which was associated with slime. The petri smear of one of the subcultures showed light-coloured smooth colonies. The culture pH followed a pattern similar to that in Culture A, increasing slowly during growth and reaching a maximum of pH 8.3 at 43.5h.

The subculture used for the inoculation of Culture B was in late exponential growth (cell mass 0.97 mg/ml at 28.5h, 0.14 mg/ml at 20h) when the culture was initiated. Since inoculating with cells in exponential growth usually initiates a culture in exponential phase (17), the logarithmic growth phase of Culture B probably occurred during the first 16 hours of culture when the specific growth rate was 0.085 h^{1-}.

Trypticase II, Culture B
Figure 2.5

Legend: □ Dry Weight, mg/ml   + Act, Boeh U/ml x10

X-axis: Culture Time, hours
Y-axis: Cell Growth and Glycosidase Activity
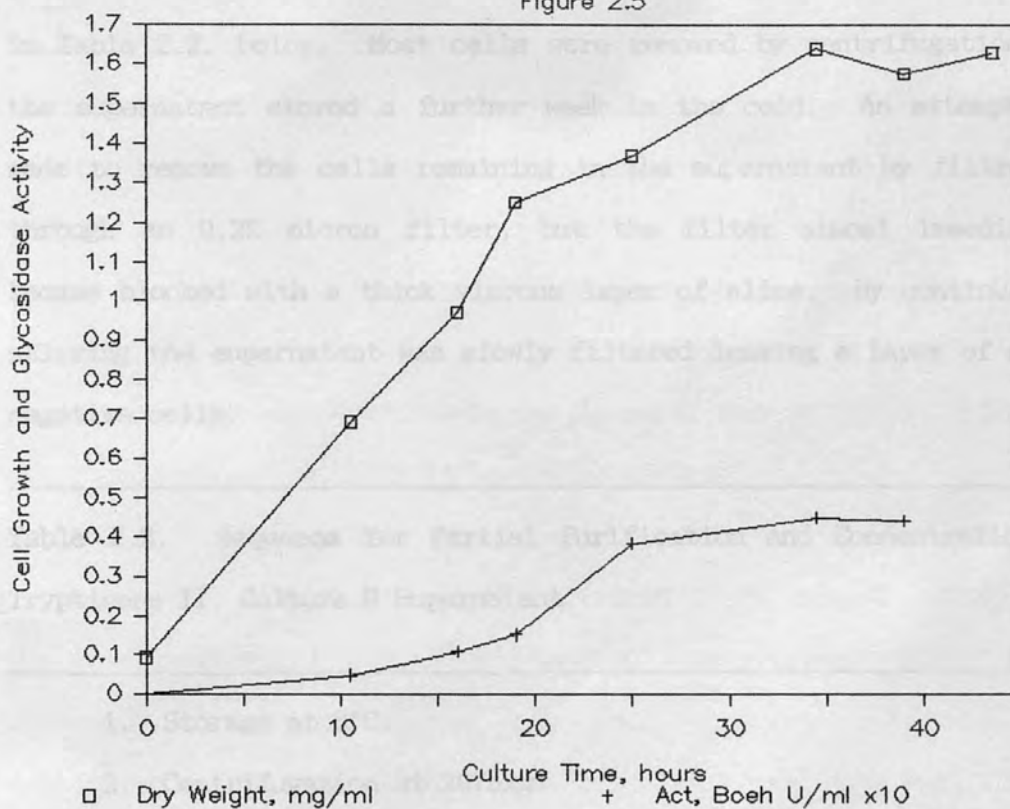
Fig. 2.5. Growth and the glycosidase activity of *Pseudomonas stutzeri* NCIB 11359 in Trypticase II medium, Culture B. Cell growth was measured as described in Fig. 2.1. Glycosidase activity was measured by the Boehringer assay.

The glycosidase giving Boehringer activity was produced logarithmically (at $0.065 \ h^{1-}$) between 10h-25h during late exponential and early stationary growth phases. In stationary phase a maximum enzyme activity of 0.04 Boeh U/ml was reached, and remained high until harvest of the culture at 45h.

Unfortunately the sterile seal was violated at 45h, so the study was stopped and the culture stored in the cold for later partial

purification, concentration and amylase studies which are summarized in Table 2.2. below. Most cells were removed by centrifugation and the supernatant stored a further week in the cold. An attempt was made to remove the cells remaining in the supernatant by filtration through an 0.22 micron filter, but the filter almost immediately became blocked with a thick viscous layer of slime. By continuously stirring the supernatant was slowly filtered leaving a layer of gram-negative cells.

Table 2.2. Sequence for Partial Purification and Concentration of Trypticase II, Culture B Supernatant.

1. Storage at 8°C.
2. Centrifugation at 2075x*g*.
3. Storage at 5°C.
4. Filtration using 0.22 micron filter.
5. Ultrafiltration and Concentration using PM-10 membrane.

The 0.22 micron filtrate was analyzed for amylase activity and found to have 0.028 Boeh U/ml (44% recovery from harvest), but no amylase activity when assayed by the alkaline ferricyanide method or for amylase specifity. Tlc of the filtrate showed very high molecular weight material and some glucose and maltose.

The 0.22 micron filtrate was then concentrated 17-fold by ultra-filtration using a PM-10 membrane. The PM-10 filtrate, 560 ml, contained only 0.001 Boeh U/ml. The turbid PM-10 concentrate was centrifuged and the clear supernatant, 35.5 ml, found to have 0.459

Boeh U/ml (43% recovery from harvest), but only 0.075 U/ml when assayed by the alkaline ferricyanide method. Analysis for product specificity showed small amounts of glucose and maltose from the starch incubate but maltose clearly present in the PM-10 concentrate.

2.2.3.3. Summary of Trypticase II Cultures. Pseudomonas stutzeri was adapted to and cultured in Trypticase II medium. Both cultures showed vigorous growth, producing a very turbid solution and a glycosidase that apparently is not an amylase.

In both cultures the cells appeared to be normal and to grow vigorously, the cell morphology changing from a typical rod shape to filamentous, irregular shapes only after 72 hours. In Culture B considerable slime was present which also interfered with purification of the extracellular glycosidase. During exponential growth the culture solutions became alkaline, rising above pH 8 in stationary phase, to a high of pH 8.5 in Culture A.

A glycosidase was produced during late exponential and early stationary growth phases, remaining at a maximum concentration throughout stationary phase. The glycosidase was not found to be cell surface bound. It was not maltotetraohydrolase nor was it the amylase produced in the Ca media. Starch was a very poor or perhaps not a substrate for the glycosidase; this was surprising, since it was the main carbon source for the bacterial cells which were able to grow vigorously and appeared to be healthy. The oligosaccharide, p-nitro-phenylmaltoheptaoside (G_7-pNP), present in the Boehringer assay was a substrate for the enzyme. The Boehringer assay relies on the degradation of G_7-pNP to G_3-pNP or smaller in order for the coupled enzyme,

α-glucosidase, to release the chromophore p-nitrophenol. This suggests that the enzyme may be either an oligosaccharidase since it did not hydrolyze starch, or a maltodextrin phosphorylase since the substrate was in phosphate buffer (the starch control was also in phosphate buffer). Wober (29) homogenized P. stutzeri and found an intracellular maltodextrin phosphorylase and also an amylomaltase, both of which were induced during exponential growth phase. Either of these glycosidases could degrade the Boehringer substrate although they are not expected to be found extracellularly.

In order to be certain that the specificity was not a function of concentration, controls of G_4-amylase from other culture media, at comparable Boehringer activity, were incubated in starch-phosphate solutions and found to give sharp increases in reducing sugar concentration and detection of G_4 by tlc. Unlike cultures in which malto-tetraohydrolase was produced, tlc of the Trypticase II culture supernatant appeared to have considerable high molecular weight polysaccharide and little oligosaccharide. Only after 17-fold concentration of the enzyme-culture supernatant was maltose readily detected in the enzyme solution, probably produced from hydrolysis of residual starch in the enzyme concentrate.

2.2.4. Comparison of Amylase Induction in Different Media.

Low concentrations of maltotetraohydrolase had been produced in Trypticase I medium (0.25 U/ml) and in early trials with Ca medium; yet, in the batch cultures in Ca medium and in Trypticase II medium no G_4-amylase was detected. Trypticase I and Trypticase II differed only in the starch (BDH Analar soluble starch versus BDH GPR potato starch) and in the batch number of their trypticase peptones.

A complete list of ingredients is found in 5.5.3. Others (7, 9, 12) have reported that 3.6-9.4 U/ml of G_4-amylase were produced in media which differed from the Trypticase media only in batch or source of starch, trypticase peptone or yeast extract. Since Wober (29) found no differences in growth of *P. stutzeri* in different branched α-glucans it is likely that the difference in G_4-amylase induction is due to either the culture conditions or the source/batch of media constituents. The complex ingredients, trypticase peptone and yeast extract, are likely to vary slightly both in their preparation (both between batches and from different sources) and in their shelf life. Consequently, a study was designed to identify whether an amylase was produced initially or after subculturing in a given medium, and whether G_4 was produced. Amylase activity (U/ml) was determined by the alkaline ferricyanide method except when expressed as Boeh U/ml where activity was determined by the Boehringer assay.

Trypticase II medium was used as a standard and control. Yeast medium used a different batch of yeast extract. Bacto medium used a different source of trypticase peptone. In place of trypticase pep-tone, Soy medium used trypticase soy broth (a mixture of peptones, NaCl, potassium phosphate, and glucose). Ca medium, a defined medium without yeast extract or trypticase peptone, was included in the studies with complex media. A scheme of the subculture sequences is found in the experimental, Section 2.3.4.

P. stutzeri from the NCIB slope (the sample from the National Collection) grew readily in Soy medium producing a turbid, gummy mass (A>3 at 52h) of gram-negative rods and G_4-amylase (>0.4 U/ml). Repeated subculturing in Soy medium produced the highest levels of

activities found in any of the media, a maximum of 1.12 Boeh. U/ml and 0.60 AF U/ml. A fourth generation subculture is shown in Figures 2.6 and 2.7.

Cells from the NCIB slope also grew readily in Yeast medium producing a turbid solution (A = 2.1 at 52h) of 'normal' rods (normal rods being the same as the control NCIB slope) and low levels of G_4-amylase activity ($\approx$0.06 U/ml) which remained constant through 138h. Repeated subculturing produced decreasing levels of maximum turbidity and decreasing levels of G_4-amylase activity. Later subcultures produced an increasing number of filamentous cells (some cells more than 10 times the 'normal' length) and many of the cells clumped together.

Culture in Trypticase II with cells from the NCIB slope grew (A = 1.6 at 52h) as normal rods but produced no G_4-amylase. The supernatant contained maltose but showed no hydrolysis of starch. When Trypticase II medium was inoculated with cells from a Soy culture producing G_4-amylase, the Trypticase II culture continued to produce G_4-amylase albiet at greatly reduced levels (max 0.25 Boeh U/ml, 0.13 AF U/ml) as shown in Fig. 2.7. During growth the rods became irregularly shaped and filamentous and probably accounted for the low turbidity shown in Fig. 2.6.

Bacto medium was also inoculated with cells from the Soy culture, and also produced G_4-amylase at greatly reduced levels (max Boeh 0.12 U/ml, 0.13 AF U/ml) as shown in Fig. 2.7. The cells were initially small gram-negative rods, later becoming large, long rods.

NCIB Growth in Various Media

Figure 2.6
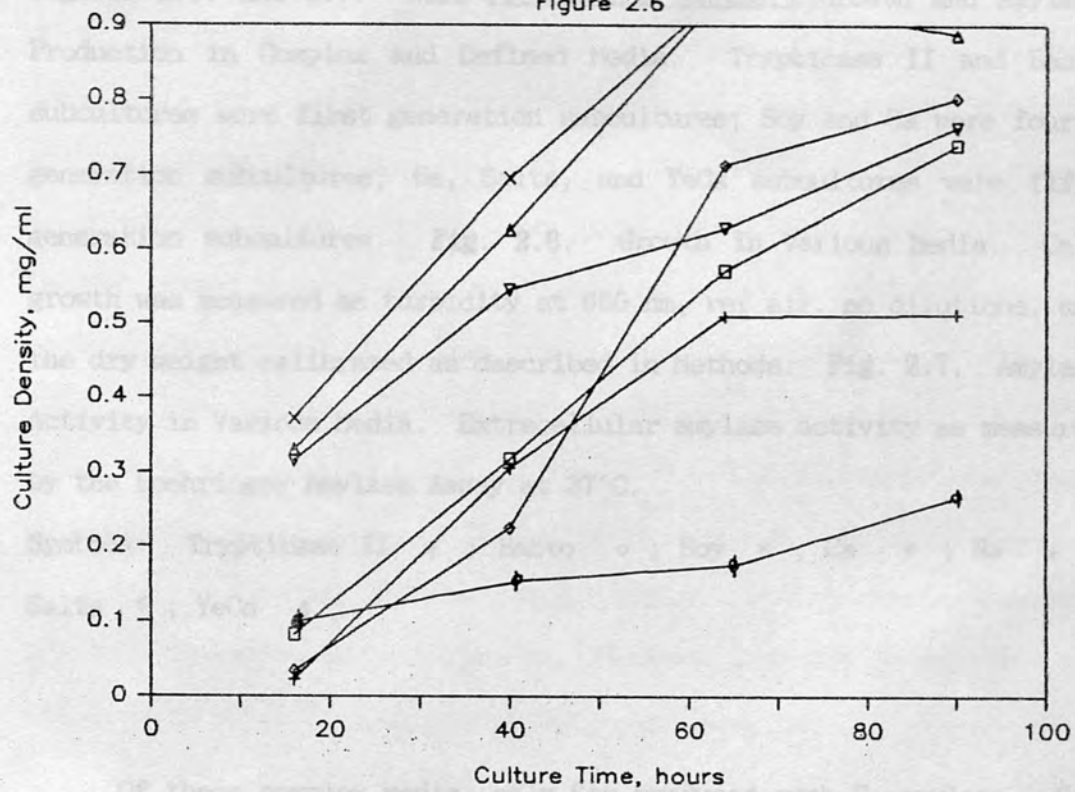
Amylase Activity in Various Media

Figure 2.7
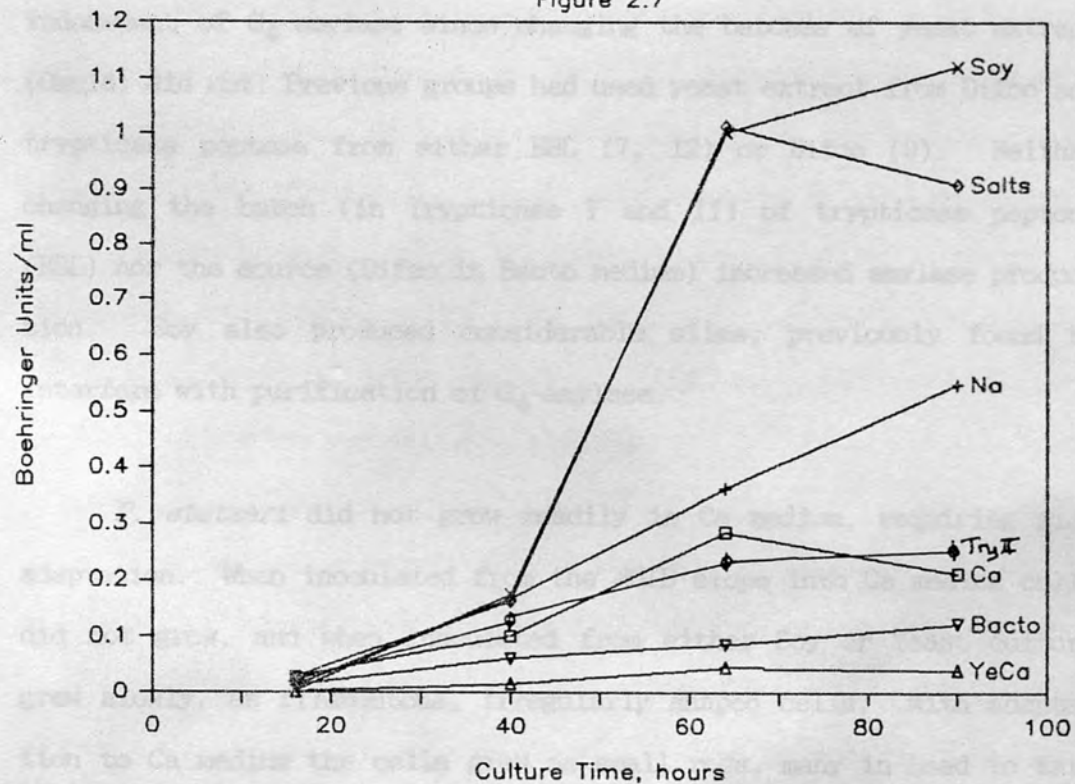
Figures 2.6. and 2.7.   NCIB <u>Pseudomonas stutzeri</u> Growth and Amylase Production in Complex and Defined Media.   Trypticase II and Bacto subcultures were first generation subcultures; Soy and Ca were fourth generation subcultures; Na, Salts, and YeCa subcultures were fifth generation subcultures.   **Fig. 2.6.**   Growth in Various Media.   Cell growth was measured as turbidity at 650 nm, ref air, no dilutions, and the dry weight calibrated as described in Methods.   **Fig. 2.7.**   Amylase Activity in Various Media.   Extracellular amylase activity as measured by the Boehringer Amylase Assay at 37°C.

Symbols: Trypticase II ϕ ; Bacto $\triangledown$; Soy × ; Ca $\bowtie$; Na + ; Salts ◊ ; YeCa   4 .

Of these complex media, only Soy produced much G_4-amylase.   Soy also contained a second yeast extract which was part of the trypticase soy broth (BBL) and this BBL yeast extract may have contributed to the inducement of G_4-amylase since changing the batches of yeast extract (Oxoid) did not. Previous groups had used yeast extract from Difco and trypticase peptone from either BBL (7, 12) or Difco (9).   Neither changing the batch (<u>in</u> Trypticase I and II) of trypticase peptone (BBL) nor the source (Difco in Bacto medium) increased amylase production.   Soy also produced considerable slime, previously found to interfere with purification of G_4-amylase.

P. stutzeri did not grow readily in Ca medium, requiring slow adaptation.   When inoculated from the NCIB slope into Ca medium cells did not grow, and when inoculated from either Soy or Yeast culture grew slowly, as filamentous, irregularly shaped cells.   With adaptation to Ca medium the cells grew as small rods, many in head to tail

chains during early growth and as very tiny rods during later growth. In all of the cultures in this study G_4-amylase continued to be produced. In two separate adaptations in Ca (one originating from a Soy culture and one from a Yeast culture), G_4-amylase activity continued to reach about the same maximum level of activity 0.11-0.14 U/ml through three subcultures in Ca medium. The only oligosaccharide detected by tlc was G_4. Even the fourth Ca subculture (originating from a Yeast subculture) retained G_4-amylase activity (max 0.29 Boeh U/ml, 0.14 AF U/ml) as shown in Fig. 2.7. This was surprising since G_4-amylase was not detected in the batch cultures previously reported in 2.2.2. However, in the batch cultures, the bacteria had been adapted through more subcultures, at more frequent intervals, and resulted in total loss of detectable G_4-amylase.

Besides the Ca, three other media were inoculated with cells from Yeast culture and subsequently adapted to their respective medium. One medium, YeCa, contained yeast extract (a mixture of growth cofactors) in the Ca medium. It supported more rapid cell growth than Ca, and, with adaptation, the growth was comparable to growth in Soy, producing normal gram-negative rods. G_4-amylase activity reached 0.09 U/ml in the first YeCa culture but decreased in subsequent subcultures. The fifth subculture, shown in Figures 2.6 and 2.7, produced the least amylase activity (max 0.04 Boeh U/ml, 0.05 AF U/ml) of any culture in these studies.

Salts medium, a second defined medium, contained NaCl in the Ca medium. Cells grew readily, as small rods during early growth, but were most frequently observed as tiny ellipsoidal cells, sometimes in clumps. G_4-amylase activity reached 0.17 U/ml in the first culture,

rising in subsequent subcultures to a maximum of 1.01 Boeh U/ml, 0.58 AF U/ml.

In a third defined medium, Na medium, NaCl replaced $CaCl_2$ in Ca medium. Cell growth and morphology were very similar to that in Salts medium, except clumping was not observed and cell density was lower during stationary phase as shown in Fig. 2.6. In the first two subcultures in Na, G_4-amylase activity was slightly higher (max >0.21 U/ml) than in Salts cultures, although in the fifth subculture in Na, shown in Fig. 2.7, amylase activity (max 0.55 Boeh U/ml, 0.27 AF U/ml) did not reach the concentration found in the comparable Salts culture.

In summarizing, *Pseudomonas stutzeri* was found to adapt to a variety of media but did not necessarily produce G_4-amylase. Three media (Salts, Na, and Soy) produced G_4-amylase at >0.5 Boeh U/ml, a concentration at least 2 times and as much as 28 times the concentration found in other media. These three media contain 0.5% NaCl whereas the other media contain less than 0.01% NaCl. Sodium ion was added to the defined media because it is known to be involved in active transport in bacteria (33). Consecutive subculturing in these media continued to produce high levels of amylase. Amylase was produced during exponential growth and remained high into the stationary phase of growth. The media containing 0.0015% calcium chloride (Ca, YeCa and Salts) showed a decline in amylase activity during the early stationary phase of cell growth.

Neither protein extract found in Trypticase nor Bacto induces the high levels of amylase activity as found by other groups (7, 9, 12). Using a different yeast extract, as in Yeast medium, or adding

extract to Ca medium, as in YeCa medium, supports cell growth but not the induction of G_4-amylase.

In all of the cultures in which amylase could be detected, maltotetraose was the predominant or only oligosaccharide observed by tlc. At high amylase concentrations, G_2 could occasionally be detected along with G_4. Maltotetraose was observed by tlc in all of the culture supernatants which contained amylase (Trypticase II from the NCIB slope had no amylase and showed only G_2), although some G_2 could also be detected in many of the supernatants.

The highest level of amylase activity, 0.6 U/ml (1 Boeh U/ml) was produced by *P. stutzeri* NCIB 11359 cultured in Soy and Salts media, two media not expected to give the highest levels of G_4-amylase. Soy medium contains glucose, an alternative carbon source which does not induce G_4-amylase (7, 29). Salts medium is a defined medium; defined media do not usually produce high levels of enzymes (59). The maximum G_4-amylase activity in either medium was considerably lower than the activity reported by others (7, 9,12), which was produced by *P. stutzeri* NRRL B-3389. Different strains of *P. stutzeri* are known to produce different levels of G_4-amylase (7). However, it was assumed that *P. stutzeri* NRRL B-3389 and *P. stutzeri* NCIB 11359 are identical since *P. stutzeri* NCIB 11359 has the same reference number, 17686, as in the original classification by Stanier (S4), who also gave it a second strain number, 320, which corresponds to the reference number first used by Robyt and Ackerman (R5)for NRRL B-3889.

2.2.5. Culture of <u>Pseudomonas stutzeri</u> NRRL B-3389.

P. stutzeri NRRL B-3389 was adapted to Soy, Trypticase II, Ca, Salts, and Na media and compared with previous cultures of *P. stutzeri* NCIB 11359.

Culture in Soy medium produced rods which after 21h were found to be increasingly smaller and ellipsoidal, clustered together in sheets. On analyzing the fifth subculture, shown in Figure 2.8, it was found that when the samples taken after 21 h were diluted 7-fold the optical density ranged from 1.45-1.65 (or $7*A = 10.2\text{-}11.6$) and when diluted 26-fold ranged from 0.59-0.64 (or $26*A = 15.3\text{-}16.6$). It is likely that the high turbidity (from which the dry weight shown in Fig. 2.8 was calibrated) resulted at least in part, from slime which caused the cells to cluster together and the higher dilutions permitted greater dispersion of the cells. The figure shows the average of duplicate cultures (absorbances differed by <10%) and assumes the 7x dilution at 21 h ($A = 11.1$, 3.31 mg/ml) would have been 4.80 mg/ml ($A = 16.1$) if diluted 26 times. Stationary growth phase was reached by 21h, at which time G_4-amylase activity was near maximum. Amylase activity, shown in Figure 2.9, decreased during stationary phase to 0.92 Boeh U/ml at 100 h (not shown in the figure) when the pH was found to be 8.4. The overall pattern of growth and amylase production in Soy was similar to that shown in the previous experiment with *P. stutzeri* NCIB 11359, although there the growth was much slower and the maximum amylase activity reached was 1.1 Boeh U/ml.

In Trypticase II medium Gram´s stain showed *P. stutzeri* NRRL B-3389 as clusters of rods in a pale blue film with some irregular, filamentous cells. As with Soy cultures, dilutions also gave an

NRRL B—3389 Growth in Various Media
Figure 2.8
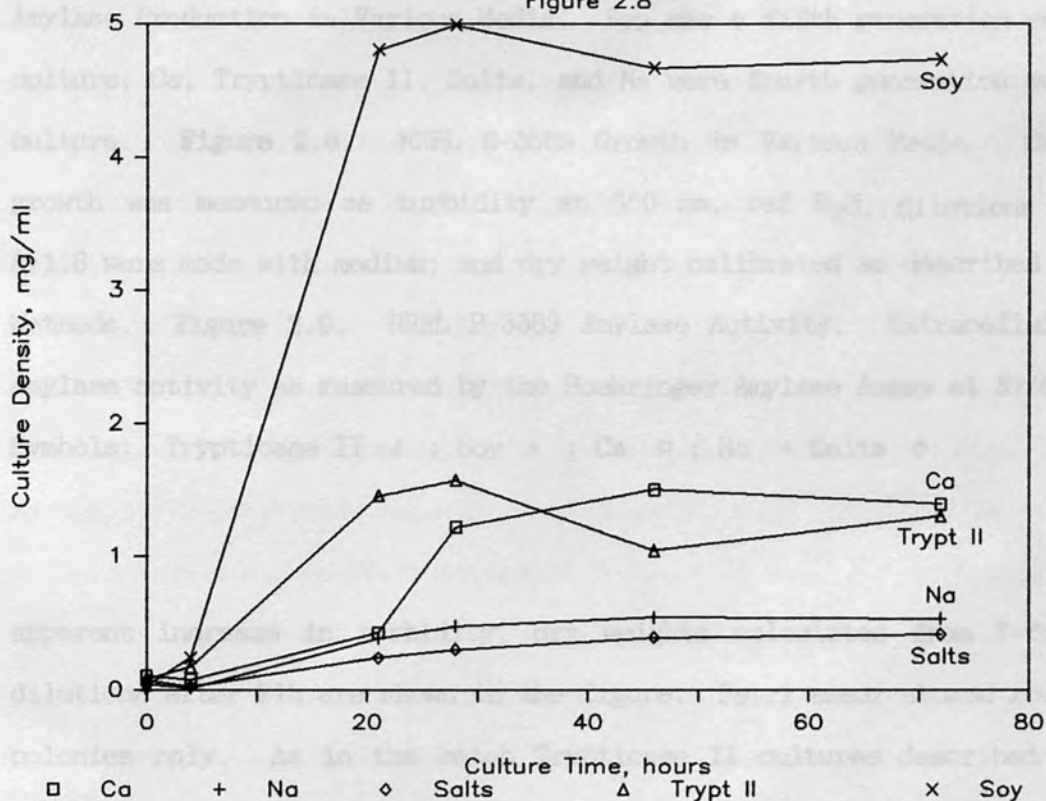
NRRL B—3389 Amylase Activity
Figure 2.9
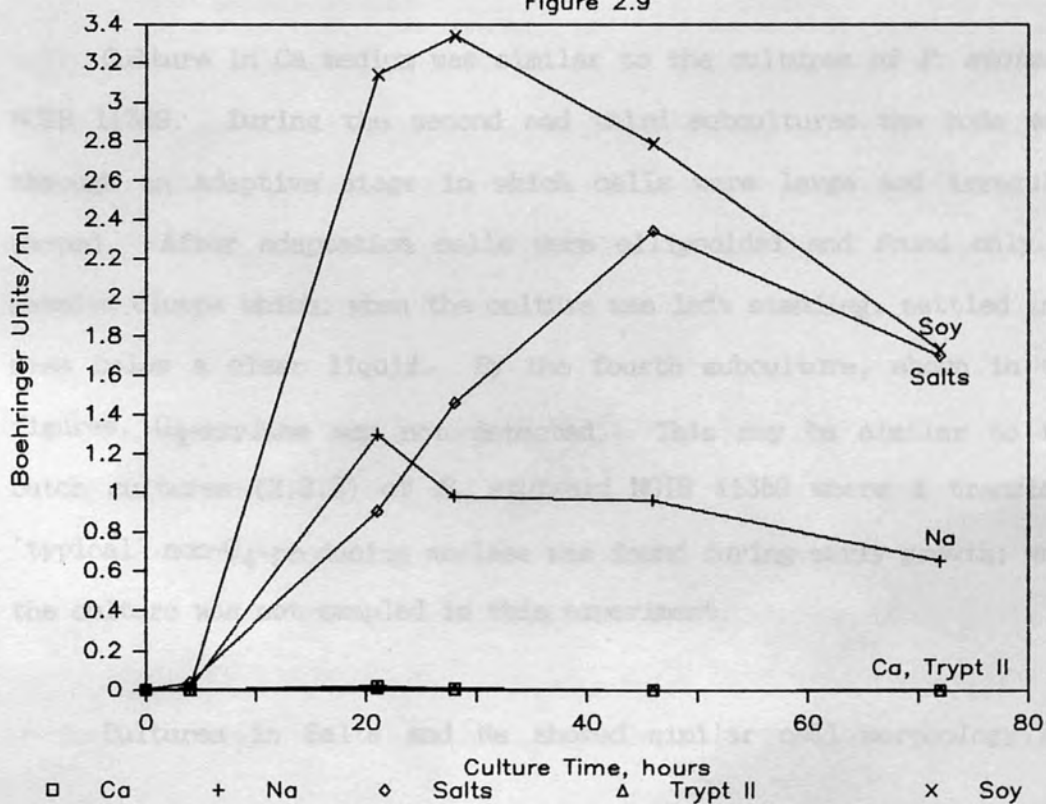
Figures 2.8 and 2.9.   *Pseudomonas stutzeri* NRRL B-3389 Growth and
Amylase Production in Various Media.   Soy was a fifth generation sub-
culture; Ca, Trypticase II, Salts, and Na were fourth generation sub-
culture.   **Figure 2.8.**   NRRL B-3389 Growth in Various Media.   Cell
growth was measured as turbidity at 650 nm, ref H_2O, dilutions of
A>1.8 were made with medium; and dry weight calibrated as described in
Methods.   **Figure 2.9.**   NRRL B-3389 Amylase Activity.   Extracellular
amylase activity as measured by the Boehringer Amylase Assay at 37°C.
Symbols: Trypticase II *◁* ; Soy × ; Ca ¤ ; Na + Salts ◊ .

apparent increase in turbidity, dry weights calculated from 7-fold
dilutions after 21h are shown in the figure.   Petri smear showed rough
colonies only.   As in the batch Trypticase II cultures described in
2.2.3, no G_4-amylase was produced but there was some hydrolysis of the
Boehringer substrate (maximum activity, 0.01 Boeh U/ml at 21h).

Culture in Ca medium was similar to the cultures of *P. stutzeri*
NCIB 11359.   During the second and third subcultures the rods went
through an adaptive stage in which cells were large and irregular
shaped.   After adaptation cells were ellipsoidal and found only in
massive clumps which, when the culture was left standing, settled in a
mass below a clear liquid.   By the fourth subculture, shown in the
figures, G_4-amylase was not detected.   This may be similar to the
batch cultures (2.2.2) of *P. stutzeri* NCIB 11359 where a transient
´typical´ non-G_4-producing amylase was found during early growth; when
the culture was not sampled in this experiment.

Cultures in Salts and Na showed similar cell morphology and

growth, both with each other and with *P. stutzeri* NCIB 11359. Very small ellipsoidal cells were produced, which also looked like the *P. stutzeri* NRRL B-3389 cells in Soy. The cells showed some clumping in Salts medium and possibly some in Na. Figure 2.8 shows the biomass calculated from optical density of the undiluted fourth subcultures in Salts and Na media. A 6-fold dilution of the Salts culture at 46h and 72h gave absorbances of 5.0 and 7.1 respectively (1.49 mg/ml and 2.11 mg/ml) whereas the Na culture diluted 6-fold at 72h gave A = 3.3 (0.98 mg/ml); indicating that both probably formed slime but more was formed in Salts medium than in Na. A petri smear of Salts culture showed rough colonies of *P. stutzeri*.

In both Salts and Na cultures, G_4-amylase was produced at a concentration comparable to that found in *P. stutzeri* NCIB 11359 cultures. In the Salts culture, G_4-amylase was produced during stationary growth phase. The loss in activity in·Salts at 72h was not due to a reversible inhibitor since a further 10-fold dilution of the supernatant gave a similar net activity (1.69 vs 1.71 Boeh U/ml). In Na culture, G_4-amylase reached a maximum concentration (1.3 Boeh U/ml) much earlier in growth and maintained activity through stationary phase. At 100h, not shown on Figure 2.9, G_4-amylase activity in the Na culture was 0.57 Boeh U/ml and pH 6.2.

Cultures of *P. stutzeri* NRRL B-3389 in Soy, Trypticase II, Ca, Salts, and Na media were found to be similar to the previous cultures of *P. stutzeri* NCIB 11359 in the same media. Soy medium produced the most turbid cultures and had the highest concentrations of G_4-amylase. Salts medium also produced high levels of G_4-amylase activity; Na produced intermediate levels of G_4-amylase. In Ca and Trypticase II

media, cells grew in irregular shapes until after adaptation when no G_4-amylase could be detected. Although in Salts and Soy media, G_4-amylase activity was higher (2-3x) in *P. stutzeri* NRRL B-3389 cultures than in the *P. stutzeri* NCIB 11359 cultures, the author feels that this is within the limits due to variation in culture conditions and that the two cell lines are probably the same.

2.2.6. Cultures in Defined 'Na' Medium. Even though Salts medium produced higher G_4-amylase concentrations than Na medium, Na medium was chosen for three batch cultures of *P. stutzeri* NCIB 11359 because Salts medium (Figure 2.9 and also early adaptations in Salts medium) showed a transient level of G_4-amylase activity. The transient level of G_4-amylase may have been related to calcium ion present in the medium and/or proteases but in any case would have required additional considerations as to time of harvest and loss of activity during purification. In addition, the Salts medium appeared to produce more slime than Na medium.

2.2.6.1. <u>Culture A</u>. In preparation for batch culture A, cells which had been adapted to Na medium by three sequential boiling tube subcultures were grown in Soy medium in the orbital shaker. The Soy culture (0.27 mg/ml, 0.013 Boeh U/ml), probably in early exponential growth, was centrifuged and the gram-negative rods added to Na medium for batch culture.

The batch culture grew exponentially (determined from the semi-log plot of dry weight with time) between 1.5h and 7h at a specific growth rate of 0.18 h^{1-}. During this time only rods were observed.
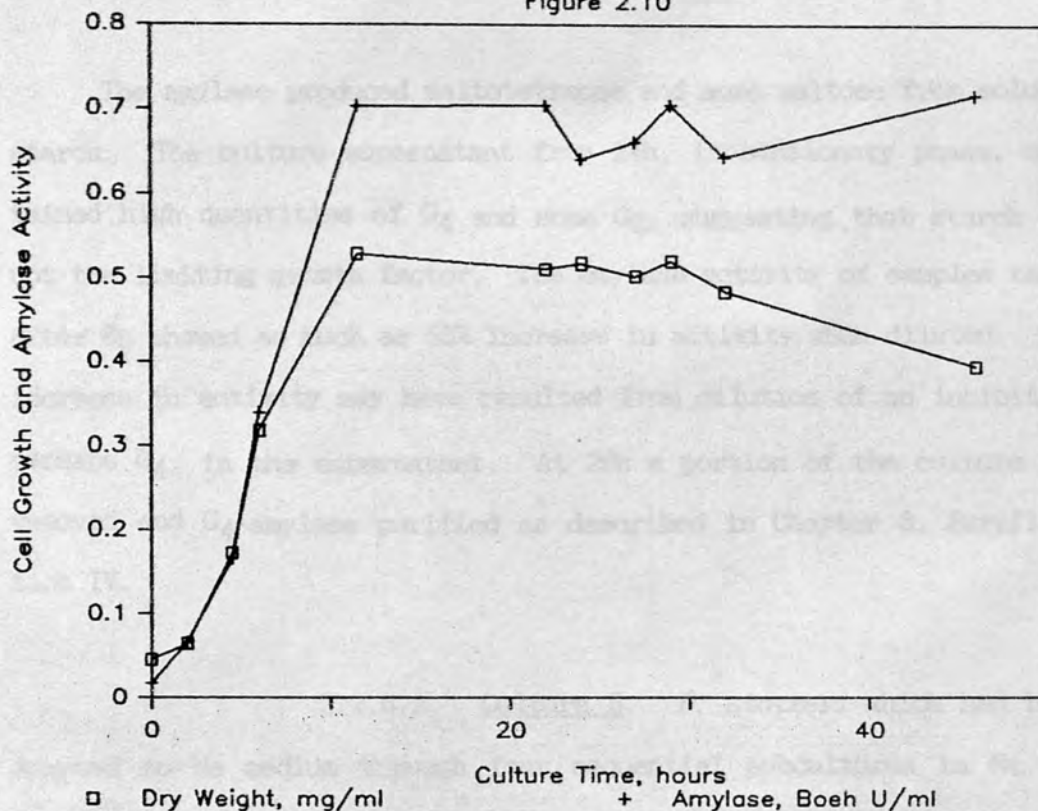
Fig. 2.10. Growth and the amylase activity of *Pseudomonas stutzeri* NCIB 11359 in Na medium, Culture A. Growth was measured turbidimetrically at 650 nm and the dry weight calibrated from the biomass of a Na culture. Amylase activity was determined by the Boehringer Assay. A portion of the culture was removed at 29 h for partial purification of maltotetraohydrolase.

Amylase activity also increased logarithmically at a specific rate of 0.18 h^{1-}. During stationary phase when there was little change in culture density, amylase concentration showed little change, remaining at 0.69 ±0.05 Boeh U/ml for more than 35 hours. During growth, the pH rapidly decreased from pH 7.2 during exponential growth to pH 6.5 in stationary phase; gradually decreasing to pH 6.4 at 46h. The cells in

stationary phase were found to be small and ellipsoidal.

The amylase produced maltotetraose and some maltose from soluble
starch. The culture supernatant from 24h, in stationary phase, con-
tained high quantities of G_4 and some G_2, suggesting that starch was
not the limiting growth factor. The amylase activity of samples taken
after 6h showed as much as 55% increase in activity when diluted. The
increase in activity may have resulted from dilution of an inhibitor,
perhaps G_4, in the supernatant. At 29h a portion of the culture was
removed and G_4-amylase purified as described in Chapter 3, Purifica-
tion IV.

2.2.6.2. Culture B. *P. stutzeri* which had been
adapted to Na medium through four sequential subcultures in Na was
grown in a batch culture, Culture B, and the G_4-amylase purified as
described in Chapter 3, Purification V.

The fourth Na subculture, cultured in the orbital shaker, grew
slowly (Figure 2.11) and was observed as rods in clumps (0.05 mg/ml)
at 18h and as small ellipsoidal cells at 72h (0.72 mg/ml, 0.49 Boeh
U/ml) at which time it was used as inoculum for batch Culture B.

During the first seventeen hours aeration was low in Culture B
and there was little growth, change in pH, or amylase activity.
Gram's stain showed clumps of small rods in a blue-black film. Imme-
diately after aeration was increased at 17 h amylase activity incre-
ased logarithmically for at least five hours at a specific amylase
rate of 0.16 h^{1-} (Table 2.3). After increased aeration Gram's stain
showed small, ellipsoidal cells and a low specific growth rate of

Na Culture B
Figure 2.11
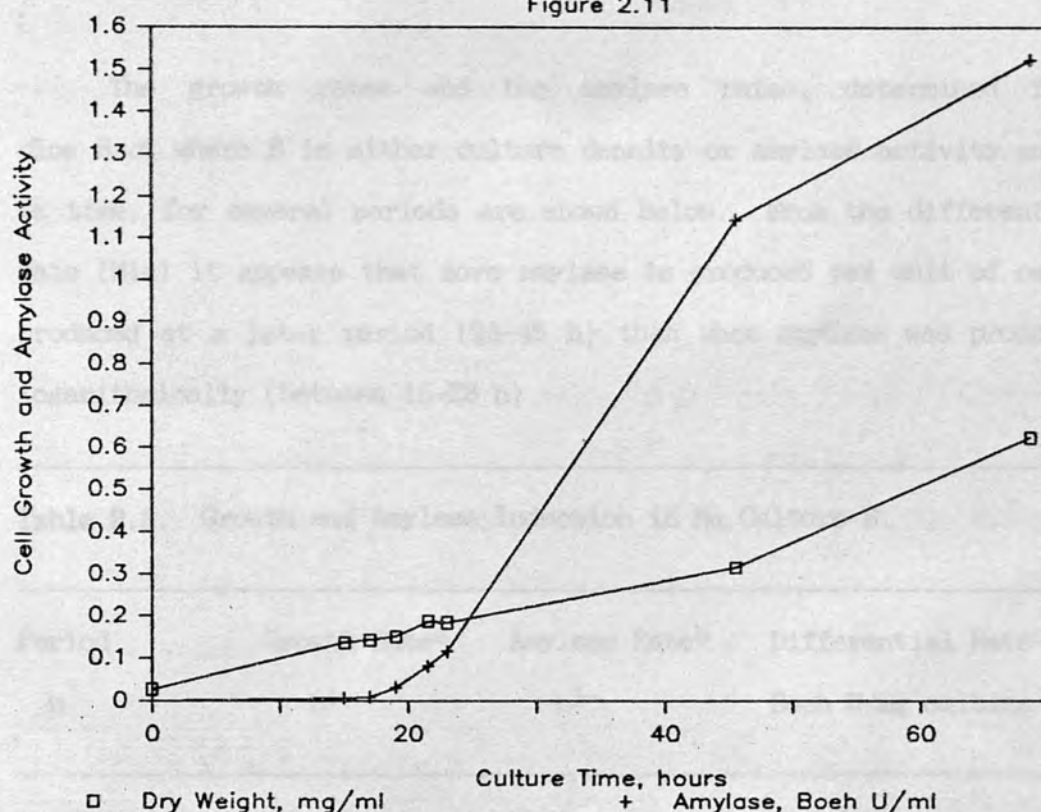
Fig. 2.11. Growth and the amylase activity of *Pseudomonas stutzeri* NCIB 11359 in Na medium, Culture B. During the first 15 hours the air flow became constricted, at 17 hours air flow was increased. Growth was measured turbidimetrically at 650 nm and the dry weight calibrated from the biomass of a Na culture. Amylase activity was determined by the Boehringer Assay.

0.01 h^{1-} which remained constant until harvest of the culture at 69 hours. In this culture amylase activity increased at a high rate although growth remained at a steady low rate, suggesting that amylase induction is only indirectly related to cell growth. The final G_4-amylase concentration in this culture (1.52 Boeh U/ml) was the highest level of activity detected in any of the cultures of *P. stutzeri* NCIB

11359.

The growth rates and the amylase rates, determined from $d$log B/dt where B is either culture density or amylase activity and t is time, for several periods are shown below. From the differential rate (M14) it appears that more amylase is produced per unit of cells produced at a later period (23-45 h) than when amylase was produced logarithmically (between 15-23 h).

Table 2.3. Growth and Amylase Induction in Na Culture B.

Period	Growth Rate[a]	Amylase Rate[b]	Differential Rate[c]
h	h^{1-}	h^{1-}	Boeh U/mg culture
0-15	0.05	0	0
15-23	0.01	0.16	1.9
23-45	0.01	0.04	8.0
45-69	0.01	0.005	1.2

[a]Growth Rate: rate of change in culture density/mg culture.
[b]Amylase Rate: rate of change in amylase concentration/ Boehringer unit of activity.
[c]Differential Rate: Boehringer amylase units produced/ mg culture produced. (Calculated as described by Mandelstam and McQuillen, M14).

During the first fifteen hours when no samples were taken the average growth rate was 0.05 h^{1-} and little amylase was produced. During the first 23 hours the pH remained at 7.1 and then gradually

decreased to a final pH of 6.5. Culture samples stored overnight at 9°C showed a sharp increase in turbidity, e.g. the absorbancy of the sample at 45.5h increased from 1.1 to 1.9. This was in contrast to the samples from Ca cultures which showed a marked decrease in absorbancy.

2.2.6.3. <u>Culture C</u>. *P. stutzeri* NCIB 11359 which had been adapted to Na medium was cultured in batch culture from a 10% inoculum (0.31 mg/ml) of tiny ellipsoidal cells and the acidity, cell growth and amylase activity measured. Petri smear of the inoculum gave shiny, smooth, translucent cream coloured colonies.

Figure 2.12 shows the parallel increase in cell growth and amylase activity during the 20 hours before harvest of the culture for purification of G_4-amylase. Purification of the amylase is described in Chapter 3, Purification VI. In addition, the culture samples were centrifuged and resuspended in saline to determine whether the high turbidity was due to extracellular slime easily removed from the culture suspension. The biomass, calibrated from the turbidity, of the resuspended cells was >85% of the whole culture suspension; therefore determination of the turbidity in this culture reflected cell growth and not slime production.

Both amylase induction and cell growth were logarithmic from 2h probably through 15h, but both still increased through 20h. The specific growth rate was 0.11 h^{1-} and the specific amylase rate was 0.13 h^{1-}. The increase in culture density paralleled the increase in amylase activity, giving a differential rate of synthesis of amylase of 0.9 Boeh U/mg culture produced.

Na Culture C
Figure 2.12
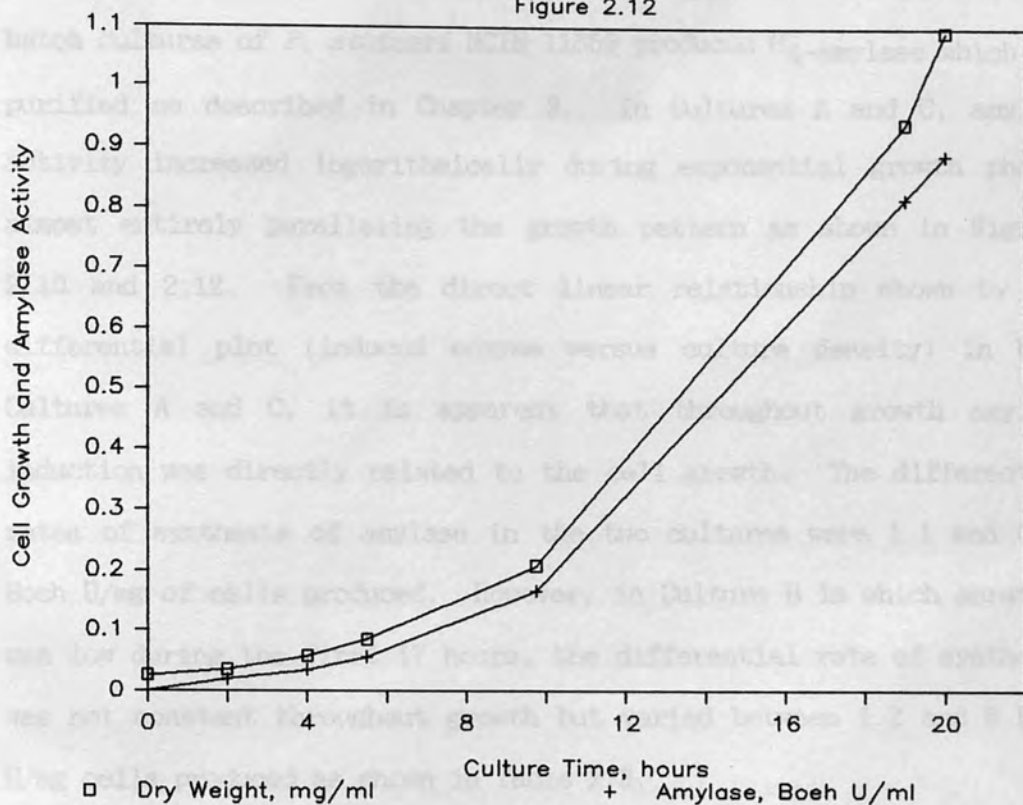
Fig. 2.12. Growth and the amylase activity of *Pseudomonas stutzeri* NCIB 11359 in Na medium, Culture C. Growth was measured turbidimetrically at 650 nm and the dry weight calibrated from the biomass of a Na culture. Amylase activity was determined by the Boehringer Assay.

The pH of the medium before inoculation was 6.8. The pH of the culture remained at 6.8 for 5.5h, was pH 6.6 at 10h, and pH 6.0 at 19h and 20h. The decreases in pH corresponded with decreases in the growth and amylase rates and may have inhibited growth and the induction of amylase.

2.2.6.4. <u>Summary of Cultures in Na Medium</u>.   Three
batch cultures of *P. stutzeri* NCIB 11359 produced G_4-amylase which was
purified as described in Chapter 3.   In Cultures A and C, amylase
activity increased logarithmically during exponential growth phase,
almost entirely paralleling the growth pattern as shown in Figures
2.10 and 2.12.   From the direct linear relationship shown by the
differential plot (induced enzyme versus culture density) in both
Cultures A and C, it is apparent that throughout growth amylase
induction was directly related to the cell growth.   The differential
rates of synthesis of amylase in the two cultures were 1.1 and 0.89
Boeh U/mg of cells produced.   However, in Culture B in which aeration
was low during the first 17 hours, the differential rate of synthesis
was not constant throughout growth but varied between 1.2 and 8 Boeh
U/mg cells produced as shown in Table 2.3.

        The Na subcultures described in 2.2.4 and 2.2.5 (Figures 2.6-
2.9) also produced G_4-amylase during exponential growth; and amylase
activity remained high during stationary phase in the *P. stutzeri* NRRL
B-3389 subculture.   The results from these Na subcultures and the
three batch cultures are summarized in Table 2.4.

        Although G_4-amylase was induced in all of the cultures at a
similar rate (μ_{am}), the maximum amylase concentration reached in the
cultures was very different.   Amylase was produced during cell growth,
however the amylase concentration did not depend directly on growth or
on cell mass, at least not at the final, maximum concentration.   In
all of the cultures amylase induction was logarithmic from the time
that the culture density was <0.15 mg/ml.   Although in most cultures
the specific rates (shown in Table 2.4.) were similar, the maximum

Table 2.4. Growth and Amylase Induction in Na Cultures.

	Cell Growth			Amylase Activity			
Culture	μ h^{1-}	Max. Density mg/ml	At	μ_{am} h^{1-}	Max. Boeh U/ml	At	Culture SA Boeh U/mg cells
Na, A	0.18	0.5	11h, S	0.18	0.72	11h, S	1.4
Na, B	0.01?	0.6	69h, H	0.16	1.52	69h, H	2.4
Na, C	0.11	1.1	20h, H	0.13	0.89	20h, H	0.8
Na, D	0.05	0.5	64h, S	0.06	0.55	90h, H	1.1
Na, E	0.09	0.5	46h, S	0.13	1.30	21h, *	3.4

μ, specific growth rate; μ_{am}, specific amylase rate;

Culture SA (specific activity of the culture), maximum amylase

   activity/mg of cell mass.

S, in stationary growth phase; H, harvest of culture before stationary

   phase; * during late exponential phase (during stationary phase

   activity remained at 1.0 Boeh U/ml); ND, not determined.

A, B, and C were NCIB batch cultures; D was an NCIB subculture

   described in 2.2.4 shown in Figs. 2.6 and 2.7; E was an NRRL

   subculture described in 2.2.5, shown in Figs. 2.8 and 2.9.

specific activities of the cultures were different.

The acidities of the cultures differed considerably. In both

Cultures A and B, growth and amylase induction either stopped or were

slower by the time the pH of the cultures had reached pH 6.5.   In
Culture C the pH also decreased during amylase induction (and concur-
rent growth), however growth and amylase induction continued as the
acidity decreased to pH 6.0, at harvest of the culture. Pseudomonads
do not tolerate acid conditions (B26) so it is unlikely that growth
would have continued even though amylase induction may have continued,
particularly since G_4-amylase is not stable at pH 6.0 (S21).

Culture B produced the highest concentration of G_4-amylase and
the lowest specific growth rate. Growth was slow because aeration was
low during the first seventeen hours of culture and *Pseudomonas stut-*
zeri are obligate aerobes (B17).   Immediately after aeration was
increased, the growth rate remained low but G_4-amylase was induced at
a specific rate comparable to Cultures A and C.

Perhaps the most influential factor influencing the growth and
amylase induction in the cultures was the state of the subcultures
used as inocula. When bacterial cells in exponential growth phase are
transferred to fresh medium the cells will continue to grow logarith-
mically (E5). The cells in the inoculum for Culture A and probably
those for Culture C were in exponential growth and continued to grow
exponentially in the batch cultures. The cells in the inoculum for
Culture B were in stationary phase and also had grown at a very low
rate at low temperature in the orbital shaker. Growth was then slow
in the batch culture, initially augmented by low aeration, but
remaining slow throughout culture.

G_4-amylase induction may also have been related to maltotetraose
concentration in the inocula. Since starch cannot directly induce

amylase since it is too large a molecule to cross the cell wall, maltotetraose is the more likely inducer of G_4-amylase. Maltotetraose was observed in the inoculum from Culture B and probably was present in the inoculum from Culture C. Culture B produced the highest concentration of G_4-amylase. Culture A, prepared from resuspended cells, would have had little G_4 in the inoculum.

2.2.7. General Discussion and Summary of Microbial Cultures. Subtle differences influence bacterial growth and amylase induction even when media and culture conditions are nearly identical. This was apparent not only within the batch cultures in a particular defined media but especially in culture in the complex Trypticase medium. Trypticase I and II and the media used by three other research groups appeared to be identical, the peptone, yeast extract and starch differing only in the source or batch (from a particular source) number. Culture conditions appeared to be similar except for rate of aeration; yet there were significant differences in the induction of G_4-amylase.

Other researchers found high concentrations of G_4-amylase in medium similar to Trypticase. Robyt and Ackerman reported that G_4-amylase was not found during exponential growth but excreted during stationary phase (7) and reached maximum concentration (9.4 U/ml) after 3 days (R13). Dellweg *et al* (9) found a rapid fall in amylase activity (maximum 8.5 U/ml) after the beginning of stationary phase; presumably the amylase was produced during the exponential phase. Sakano *et al* (12) also harvested G_4-amylase (3.6 U/ml) at early stationary phase. Although these groups found G_4-amylase was induced they differed in the cultural stability of the G_4-amylase and probably

in the growth phase of amylase induction.   However all of these differed from Trypticase I and II which produced little or no G_4-amylase in several batch cultures.

Trypticase II produced no extracellular G_4-amylase, results similar to those of Wober (29) who used a different complex medium. Wober (29) found that no extracelluar G_4-amylase was induced by soluble starch.   However he also tested for G_4-amylase in the periplasmic space (located between the cell wall and the cytoplasmic membrane) and found cell-bound G_4-amylase activity was induced during stationary phase.   Wober also identified intracellular amylomaltase and maltodextrin phosphorylase which may be the extracellular glycosidase excreted in Trypticase II batch cultures in the present work.

The apparent discrepancies in G_4-amylase induction may be differences in cell membrane permeability rather than amylase induction. In *Pseudomonas aeruginosa*, the extracellular release of alkaline phosphatase from the periplasmic membrane was dependent on the growth conditions and attributed to the permeability of the outer membrane layers (C11,80).   Wober (29) found no extracellular G_4-amylase induced by soluble starch; however, maltodextrins did induce secretion of G_4-amylase during stationary phase and the induction of cell-bound G_4-amylase during exponential growth.

In all of the cultures of *Pseudomonas stutzeri* studied here, when extracellular G_4-amylase was induced it was found to be secreted during exponential growth phase.   Secretion paralleled an increase in cell mass even in very different media, and in several media the G_4-amylase concentration reached a maximum in early stationary phase.

The level of G_4-amylase activity in one medium was found to remain at maximum concentration throughout stationary growth phase.

P. stutzeri NCIB 11359 and NRRL B-3389 were found to grow in several defined media obviating the need for the complex media used by others and in Trypticase cultures. Pseudomonads do not require organic growth factors and most can grow in mineral media with ammonium ions or nitrate and a single organic compound as the sole carbon and energy source (4). *P. stutzeri* is one of the few pseudomonads that will grow with starch as the single organic compound (2), although not all strains can do so (1). In a complex medium cells could grow by first utilizing peptones and yeast extract (only later requiring G_4-amylase for hydrolysis of starch) and are more likely to produce proteases (for hydrolysis of the peptone) resulting in the rapid loss of G_4-amylase observed in the Soy cultures and by Dellweg *et al* (9).

In three different complex media, G_4-amylase induction was either absent or diminished, even though all three contained starch. One of the media produced a glycosidase which degraded *p*-nitrophenyl-maltoheptaoside but not starch.

Three defined media were found to support growth of *P. stutzeri*. The media differed by the addition or deletion of Ca^{2+} and Na^+ from a defined medium. Cells thoroughly adapted to medium containing Ca^{2+} but not Na^+ (Ca medium in Table 2.5) produced an extracellular amylase during early to mid-exponential growth. The amylase which appeared to be a typical endo-α-amylase could no longer be detected by stationary phase. Amylolysis of starch produced primarily maltose and a mixture of oligosaccharides. However, with the addition of Na^+ to the same

Table 2.5.   Summary of Glycosidase Activities in Batch Cultures.

Medium, Culture	Growth Phase[1], Stability	Glucosidase Activity Boehringer[2] Boeh U/ml	Starch[3] U/ml	Product Specificity[3]
Trypticase I	ND P		0.25	maltotetraose
Ca, A	E T	0.26		ND
Ca, B	E T	0.12		maltose
Ca, C	E T	0.30		maltose
Trypticase II, A	E+S P	0.06	0.00	ND
Trypticase II, B	E+S P	0.04	0.00	maltose?
Na, A	E P	0.72		maltotetraose
Na, B	E ND	1.52		maltotetraose
Na, C	E ND	0.89		maltotetraose

ND: not determined

[1] Growth Phase: E, exponential; S, stationary;

Stability: T, transient; P, plateau or decreases slowly.

[2] Degrades the Boehringer substrate p-nitrophenylmaltoheptaoside.

[3] Determined from the hydrolysis of starch. One unit of activity is that enzyme producing 1 μmole of reducing sugar per minute.

Ca^{2+} medium (Salts medium shown in Figures 2.6-2.9), cultures did not appear to produce this endo-α-amylase but instead produced high concentrations of G_4-amylase. The G_4-amylase was secreted throughout exponential growth. In the G_4-amylase producing cultures,

maltotetraose was observed in the culture supernatants throughout both exponential growth phase and stationary phase.

The third defined medium, containing Na^+ but not Ca^{2+} (Na medium), also produced G_4-amylase. The G_4-amylase concentration remained high through stationary phase. The maximum G_4-amylase concentration may be related to aeration rate of the culture and/or the growth rate of the culture. (Aeration may positively, or negatively (21), affect the growth rate. Other factors may also influence growth rate, particularly pH and metabolic products (18).) The highest concentration of G_4-amylase in Na medium was found shortly after a period of low aeration, in a culture which grew slowly. Welker and Campbell also found an inverse relationship between growth rate and the total α-amylase produced by *Bacillus stearothermophilus* (61). They found that cells grew more slowly on maltose but produced more total amylase than cells grown on starch (62). Although no growth rates were given for *Bacillus licheniformis*, maltotetraose was found to induce much more α-amylase activity than either maltose or soluble starch (63).

One complex medium (Soy medium in Figures 2.6-2.9) was found to produce cultures of high density and high G_4-amylase activity. This medium contained 0.5% NaCl whereas the other complex media contained less than 0.01% NaCl. It also contained a higher phosphate concentration than any of the other media. Phosphates are stimulators of amylase production (64), and with the additional stimulus from Na^+, may have countered the repressive effects from glucose (7), also present in the medium.

In the complex media cell morphology was consistent with that

described (3, 6) for *Pseudomonas*, being straight rods about 3-6 times longer than wide. During adaptation to one defined medium (Ca) cells were found to be irregular in shape and occasionally filamentous; however, after adaptation cells appeared to be of normal rod shape. In two other defined media (Salts and Na) cells were usually observed as tiny ellipsoids.

2.3. Experimental.

General microbiological methods and materials including bacterial strain and its maintenance, media and its preparation, cell culturing, adaptation, and batch culturing techniques are described in Chap. 5.5. *P. stutzeri* NCIB 11359 was used in all experiments except for one (2.3.5, and results in 2.2.5). Also in Chapter 5 are analytical methods for measurement of bacterial growth and some definitions for microbiological terminology. Glycosidase activity and purification techniques are described in Chapter 5.6.

2.3.1. Cultures in Trypticase I Medium. Procedures for culture revival, subculturing, and inoculation were those described by Robyt and Ackerman (7), except that all inocula were 2% and the cultures were grown in a Gallenkamp Orbital Incubator (1H-460). *Pseudomonas stutzeri* cultures originated from NCIB Ampoule I. Trypticase I medium was the same as that described by Robyt and Ackerman except that BDH Analar soluble starch replaced Baker soluble potato starch.

Each inoculum and culture was inspected for contamination by agar smears incubated at least 24 h at 30°C. Amylase activity was measured at room temperature by the turbidimetric assay described in Chapter 5.6 using 2% soluble starch in 10 mM glycerophosphate buffer containing 5 mM calcium chloride, pH 7.0 (Buffer B).

2.3.1.1. <u>Small Scale Culture</u>.   Several small scale cultures were grown in 250 ml Erlenmeyer flasks containing 51 ml each of bacterial culture.   In one study the increase in amylase activity was followed by assaying after 2, 3, 5, 6, and 7 days of culture.   One preparation of 5 flasks was incubated for 6 days, then stored at 9°C for 12 days, before purification of the amylase as described in Chapter 3, Purification I.

2.3.1.2.   <u>Large Scale Culture</u>.   Trypticase I medium (400 ml x 12) was sterilized in twelve 2.0 liter Erlenmeyer flasks and stored on the workbench for 6.5 weeks before inoculation (2%) from 4 subcultures grown 1 day in the orbital shaker as described in Chapter 5.5.4. (Subculture activity averaged 0.024 turbidimetric U/ml). The 2.0 liter flasks were incubated in the orbital shaker for 137 h, at which time amylase activity was measured using the DNS assay as described in *Methods in Enzymology, Vol I* (65). After confirmation of amylase activity in each flask (which ranged from 0.33 to 0.71 U/ml), the culture was harvested for purification of amylase as described in Chapter 3, Purification II.

2.3.2.   Cultures in Defined 'Ca' Medium.   Cells of *P. stutzeri* NCIB 11359 from two different NCIB ampoules were adapted to Ca medium and cell growth and amylase induction followed. The subculture sequences for adaptation to Ca medium are shown in Scheme 2.1.

2.3.2.1.   <u>Culture A</u>. A loopful of *Pseudomonas stutzeri* from an Ampoule I maintainance slope was inoculated directly into Ca medium for adaptation to medium (see Chapter 5.5.) and incubated for three days, then subcultured daily for a further five days

(subcultures 2-6).   Culture procedures are described in Chapter 5.5, except that cells were not examined microscopically by Gram's stain, however agar-petri dishes were smeared and examined for bacterial integrity. Subculture 6 was incubated 2.5 days, subculture 7 for 1 day before a last boiling tube subculture, 8, was made.   After 8 hours, subculture 8 was used to inoculate three flasks for incubation in the orbital shaker. The flasks were incubated for 16 hours at a high incubation temperature, 35°C, due to a control fault in the orbital shaker.

Scheme 2.1. Subculture Sequences for the Three Cultures in Ca Medium.
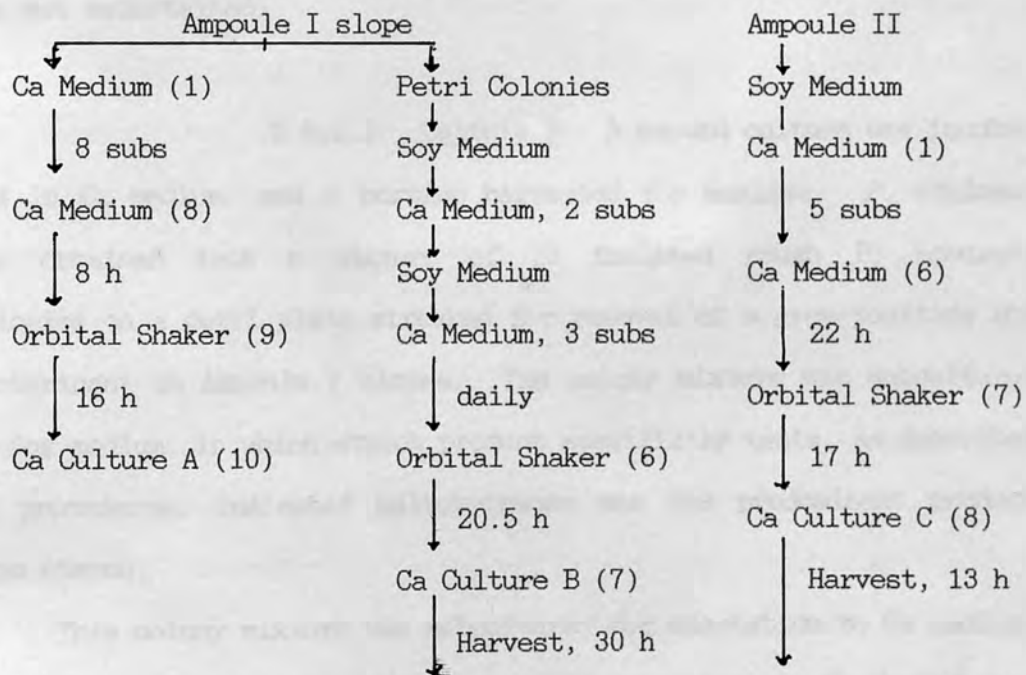
Numbers is parentheses indicate the number of consecutive subcultures that have been made in Ca medium.

The subculture from the orbital shaker, 102 ml, was used to inoculate 1000 ml of Ca medium in the Bio Flo Fermenter; then,

incubated at 30°C, agitated at 200 rpm, and aerated at 1.0 liter/min. After 4 hours, aeration was decreased to 0.5 liter/min, the same rate of aeration/liter of culture used by Robyt and Ackerman (7).

Samples were removed from the fermenter before inoculation, just after inoculation, and at 1h, 2h, 3h, 4h, 6h, 8h, 11h, 23h, 25h, and 28h after inoculation. Cell growth was measured turbidimetrically at 650 nm using culture medium in the reference cell (the absorbance of medium was 0.62) and the biomass calibrated as described in Chapter 5. The same preparation of medium was used for the batch culture as for the subcultures, consequently the medium was 12 days old and the turbidity of the medium high. Amylase activity was determined on the culture samples using the Boehringer assay, but product specificity was not ascertained.

2.3.2.2. Culture B. A second culture was incubated in Ca medium, and a portion harvested for amylase. *P. stutzeri* was obtained from a mixture of 10 isolated rough *P. stutzeri* colonies on a petri plate streaked for removal of a gram-positive rod contaminant on Ampoule I slopes. The colony mixture was subcultured in Soy medium, in which starch product specificity tests, as described in procedures, indicated maltotetraose was the predominant product from starch.

This colony mixture was subcultured for adaptation to Ca medium, then resubcultured in Soy when the second generation in Ca showed poor growth. Revitalized cells from the Soy subculture were then adapted by three, daily subcultures in Ca medium. Each of these subcultures were examined by Gram's stain, by petri smears, and for product specificity. The bacterial integrity of the *P. stutzeri* subcultures was repeatedly checked by Petri smear, Gram's stain, and G_4-amylase speci-

ficity. In subcultures where G_4-amylase did not appear to be present, the subcultures were readapted to Soy (in addition to the sequence described above) and G_4-amylase induced.

The cells adapted to Ca medium were used to inoculate three flasks of Ca medium for incubation in the orbital shaker with agitation at 260 rpm. Other details are as described in Chapter 5.5. After 20.5 hours in the orbital shaker, 150 ml of subculture was added to 1.5 liters Ca medium containing 1 drop of isopropylene glycol in the Bio Flo fermenter. The culture was incubated at 30°C, 200 rpm, and aerated at 0.75 liters/min (= 0.5 lpm/liter culture). After 9 hours the aeration was increased to 1.5 liters/min. Samples were taken hourly from immediately after inoculation, 0h, through 11h. After 11 hours of culture the fermenter was turned off and the culture left at room temperature overnight. At 26 hours after inoculation, the fermenter was turned on and samples collected at 26h, 27h, 28h, 29h, 30h, 31h, 33h, 35h, 50h, 52h, and 56h. Samples were analyzed for cell growth, pH, and amylase activity.

Cell growth was measured turbidimetrically using water in the reference cell, and the biomass calibrated as described in Chapter 5.5 even though biomass calibrated this way would be artificially high due to the turbidity of the starch in the medium. The first five culture samples were also measured turbidimetrically using medium in the reference cell, and cell growth appeared to be negative, probably because amylase was hydrolyzing the starch and thereby decreasing the turbidity of the medium. (The biomass of the subculture was 0.385 mg/ml and its amylase activity 0.003 Boehringer units/ml.) The turbidity of the culture as it matured was difficult to measure as the absorbance varied by up to 10% as white clumps, presumably of cells, settled in the cuvette.

The pH of each culture sample was measured. Gram's stains were
made at 3h, 6h, 28h and 33h.

Amylase activity was monitored by the Boehringer assay. Amylase
specifity of the culture was analyzed at 4h, 10h, 27h, 29h, 31h and
35h by tlc of the culture supernatants.

Amylase was partially purified from culture at 30.5h, removed
just after a decrease in amylase activity was observed. Cells were
removed by centrifugation at 8°C, 2520x*g* on the MSE bench centrifuge.
Two samples of the supernatant were removed; one was stored at 8°C for
1 day, one was stored at -7°C for 1 day and then thawed and the Boeh-
ringer amylase activity determined on both samples. The supernatant,
1001 ml, was concentrated at 4°C to 250 ml by ultrafiltration using a
ChemLab C-400 cell fitted with a G10T membrane. Amylase activity and
specificity were analyzed at each step.

2.3.3.3. Culture C. *P. stutzeri* NCIB 11359 from
Ampoule II were adapted to Ca medium for batch culture and partial
purification of an amylase.

P. stutzeri from Ampoule II were grown in Soy medium, then sequ-
entially subcultured five times in boiling tubes containing Ca medium
(at days 1, 2, 3, 6, and 7). After 22 hours the fifth subculture was
used for a 2% inoculum into flasks containing Ca medium for the orbi-
tal shaker. The flasks were incubated in the Shaker for 17 hours at
200 rpm and 35°C. Three flasks (biomass, 0.59 mg/ml) were used for a
10% inoculum into 1500 ml freshly prepared Ca medium in the Bio Flo
fermenter. The culture was incubated at 30°C, agitated at 200 rpm,
and aerated at 1.6 liters/min. Culture samples were removed at 0h,
2h, 3h, 4h, 5h, 6h, 7h, 8h, 12h, 13h, 24h, 26h, 30h, 33h, 36h, and 52h
after inoculation.

The appearance was noted and cell growth, pH, and Boehringer amylase activity measured. Cell growth was measured turbidimetrically using medium as a reference. The turbidity of the culture samples at 7h and 8h was measured both immediately and again after storage at 8°C for 18 hours. The dry weight equivalence of the medium was 0.04 mg/ml, calibrated as described in Chapter 5.5. Product specificity was determined on the 12h and 13h supernatants using 0.200 ml supernatant, 1.0 ml of 1.5% starch and incubating for 12 h and 1 h respectively. These were compared to the tlc of the culture supernatants from 3h, 6h, 12h, 13h, and 24h. In order to check for the presence of inhibitor the supernatant from 24h was assayed, then diluted 5-fold with 10 mM glycerophosphate buffer, pH 7, and reassayed for amylase activity.

At 13h approximately 1 liter of culture was removed by syphoning cells from the fermenter and cooling promptly to 0-4°C and storing overnight. The harvest was then centrifuged at 2075xg (MSE 6L) for 30 min. The slightly turbid supernatant was assayed, and the loose pellet with remaining supernatant was recentrifuged on the CamLab bench centrifuge. The clear supernatant from the pellet required diluting 10-fold with 10 mM glycerophosphate buffer for determination of Boehringer activity.

The supernatant from centrifugation was then sterilized at 0-4°C by hollow fiber filtration using the 0.1 micron membrane. The filtrate was assayed, frozen and reassayed. During filtration which took 45 min there was no evidence of slime. The hollow fiber concentrate was removed and the fibers washed by back flushing with about 100 ml of 30 mM phosphate buffer; a total of 148 ml was collected and assayed for Boehringer activity.

2.3.3. Cultures in Complex Trypticase II Medium. The two Trypticase media, I and II, were similar except that Trypticase I used soluble starch and Trypticase II used potato starch and, probably more importantly, the two trypticase peptones were from different batch preparations. The trypticase peptone used for Trypticase II was hard, vs free-flowing for Trypticase I, and no date was given for expiring date, which was not noted for Trypticase I.

2.3.3.1. <u>Culture A in Trypticase II Medium</u>.

P. stutzeri NCIB 11359 from the NCIB slope were adapted to Trypticase II medium via four boiling tube subcultures at 37°C instead of the standard 30°C. The fourth subculture (at 8 hours, turbid) was used for a fifth subculture in the orbital shaker as shown in Scheme 2.2.

Scheme 2.2. Subculture Sequences for the Cultures in Trypticase II.
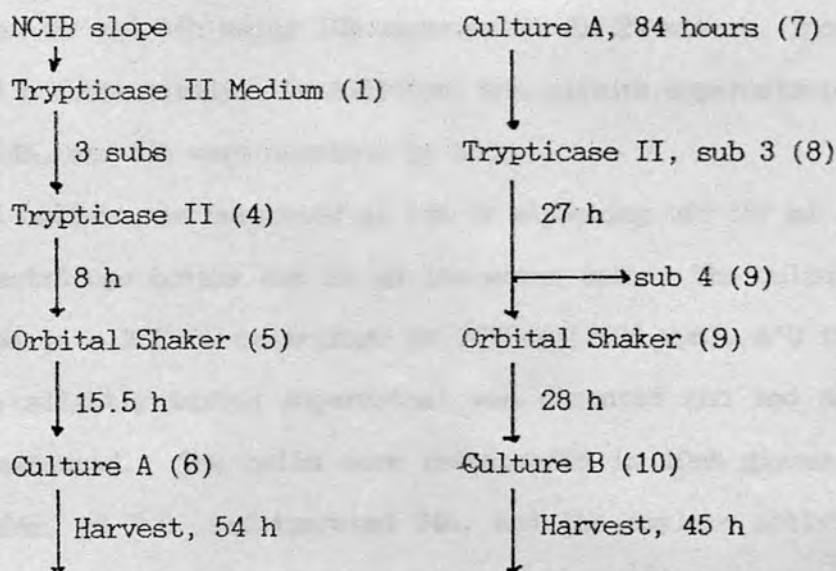
```
NCIB slope                    Culture A, 84 hours (7)
    |                                |
Trypticase II Medium (1)             |
    |                                |
    | 3 subs                         | Trypticase II, sub 3 (8)
    |                                |
Trypticase II (4)                    | 27 h
    |                                |
    | 8 h                            +--------->sub 4 (9)
    |                                |
Orbital Shaker (5)            Orbital Shaker (9)
    |                                |
    | 15.5 h                         | 28 h
    |                                |
Culture A (6)                 Culture B (10)
    |                                |
    | Harvest, 54 h                  | Harvest, 45 h
    v                                v
```

Numbers is parentheses indicate the number of consecutive subcultures that have been made in Trypticase II medium.

After 15.5 hours culture in the orbital shaker, 100 ml of the subculture was added to 900 ml of medium in the Gallenkamp fermenter. Procedures were as described for cultures in the Gallenkamp fermenter except that the stock solution for the medium was neutralized with 2M KOH before sterilization and addition to the sterile 1.5% starch preparation. Aeration was greater than 600 ml/min.

Samples were removed from the fermenter hourly between 0-10h, and 24-26h, then at 28h, 30h, 31h, 48h, 49h, 52h, 56h, 72h, 76h, and 82h. The pH of these samples was taken and the cell growth measured turbidimetrically. At 8h and thereafter, culture samples were diluted with medium before measurement of cell growth. The absorbance of the medium was 0.18, equivalent to an apparent cell mass of 0.04 mg/ml. Cells from samples taken at 25h, 28h, 48h, and 72h were observed by Gram's stain. The amylase activity of each culture sample supernatant was measured using the Boehringer amylase assay. The 25h sample was also assayed by the turbidimetric method. Product specificity was examined at 6h and 54h using 10% supernatant in 2% starch, incubated 18 h and 1 h respectively. In addition, the culture supernatants from 6h, 10h, 24h, and 54h were examined by tlc.

Cell culture was harvested at 54h by siphoning off 587 ml into a sterile centrifuge bottle set in an ice-water bath. The culture was centrifuged in a MSE 6L centrifuge at 2075xg (2500 rpm), 4°C for 100 min. The slightly turbid supernatant was decanted and and amylase activity measured. The cells were resuspended in 10mM glycerophosphate buffer, pH 7.0, refrigerated 24h, and the amylase activity of the supernatant measured. The original culture supernatant was filtered by ultrafiltration through the 0.1 micron hollow fiber membrane (see 5.6.2.4.). The hollow fiber concentrate, about 60 ml, was washed with 30mM phosphate buffer, pH 7.0 containing 1.5 M NaCl by

recirculating in an ice bath for 1 hour and then collecting the filtrate.

                     2.3.3.2. <u>Culture B in Trypticase II Medium</u>. A subculture (subculture 7) from Culture A, 84h, was made in a boiling tube and incubated with aeration over the weekend. This subculture was further subcultured (subculture 8) and a culture smear made as well as the usual Gram's stain. After 27 h, subculture 8 was used for subculture 9 in a boiling tube as well as inoculating a flask (4.5 ml inoculum) containing 100 ml of medium, which was then aerated in a waterbath at 30°C as described for the boiling tube subcultures (see 5.5.4.1). Samples were taken for determination of absorbance/biomass. Because growth in the flask was much slower than in the boiling tube, at 20 h aeration in the flask was increased by incubation in the orbital shaker set at 100 rpm while continuing to bubble filtered air through the culture.

After 28 hours, the culture in the flask was used as inoculum (94 ml, 28h, 0.97 mg/ml) for 900 ml medium containing several drops of sterile antifoam agent (Silicone M.S. Antifoam A, Hopkin & Williams) in the Gallenkamp fermenter. Cell growth was determined by measuring the absorbance, the reference cell containing medium, and calibrated for biomass as described in Methods. Samples were collected at 10.5h, 16h, 19h, 25h, 34.5h, 39h, and 43.5h and required diluting with medium before absorbance could be measured. The pH and Boehringer activity was measured for each sample.

At 45h the Gallenkamp condenser broke and the entire culture was stored at 8°C for 60 h. A Gram's stain was made and the culture centrifuged at 2075xg (MSE 6L) for 60 min. The slightly turbid supernatant, 865 ml, was stored at 5°C for 8 days. Vacuum filtration of this

supernatant through an 0.22 micron membrane filter (Oxoid cellulose acetate ´Nuflow´ N47/22G) was attempted; then a new filter placed in the Amicon ultrafiltration system and the supernatant slowly filtered at <15 psi, 5°C for 3 days. A Gram´s stain was made of the slimy grey coat remaining on the filter. After cold storage for 60 h the filtrate, again slightly cloudy, was concentrated by ultrafiltration using a PM-10 membrane in an Amicon cell. The filtrate, 560 ml, was analyzed for Boehringer activity and found to be a negligible 0.001 Boeh U/ml. The turbid PM-10 concentrate was centrifuged on a MSE bench centrifuge for 20 min at 2500xg (3800 rpm). The clear supernatant, 35.5 ml, was decanted from the pellet and analyzed.

The PM-10 supernatant was analyzed by both the Boehringer and alkaline ferricyanide assays. For the AF assay, 0.5 ml supernatant was added to 2.0 ml of 1% starch in phosphate buffer, pH 7, and after 3 h product specificity was tested by running tlc on the solution. Tlc was also run on the PM-10 supernatant. The same analyses had also been made using the 0.22 micron filtrate but after incubation of the enzyme-starch solution for 22 hours.

2.3.4. Comparison of Amylase Induction in Different Media.

2.3.4.1. Study A. *Pseudomonas stutzeri* NCIB 11359 from the NCIB slope was inoculated into Trypticase II, Soy, Yeast, and Ca media (2.0 ml) and incubated in boiling tubes as described in 5.5.4.1. From each of these, consecutive subcultures (2%) were made into 10.0 ml at intervals of 2 days (except in Ca medium which was subcultured at intervals of 3 days). The subculture sequence is shown in Scheme 2.3.

Scheme 2.3. Subculture Sequences for Studies in Various Media.
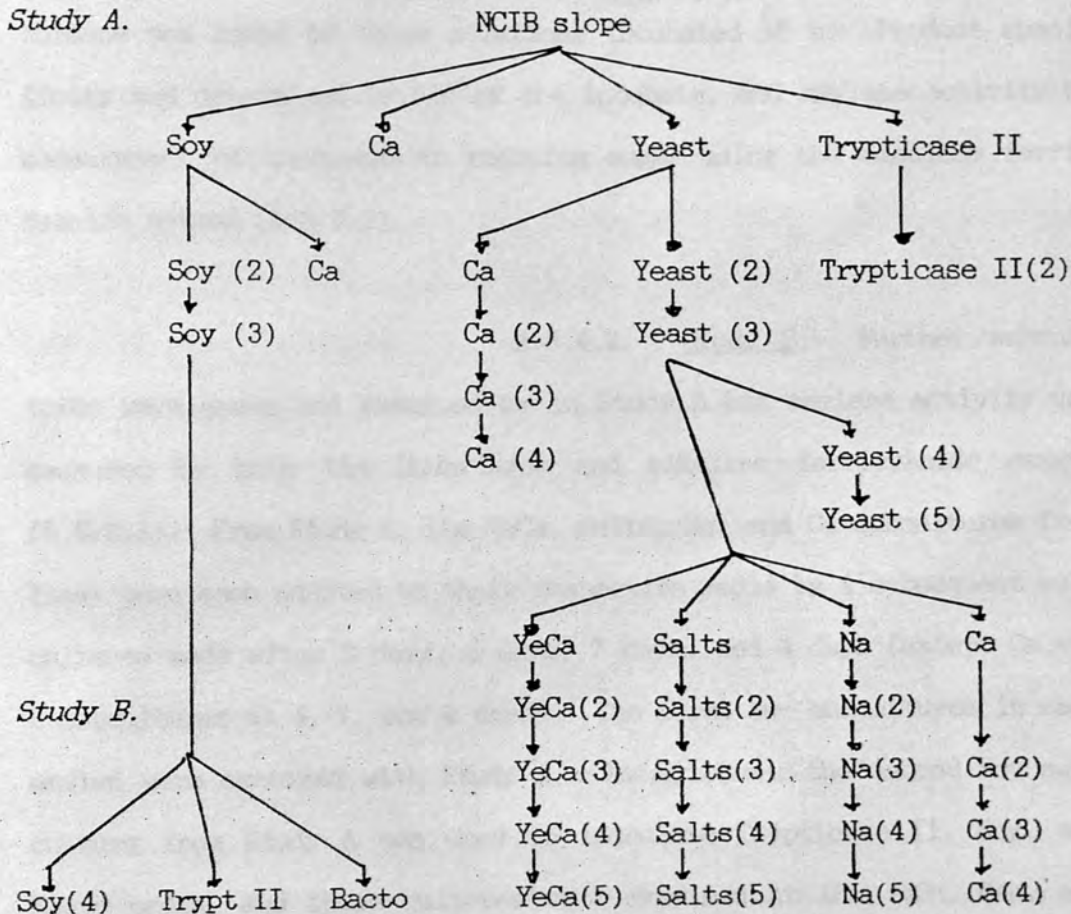
Study A.

```
                            NCIB slope
              ┌───────────┬─────────────┬──────────────┐
             Soy         Ca           Yeast      Trypticase II
          ┌───────┐    ┌──┐          ┌──────┐         │
       Soy (2)  Ca   Ca      Yeast (2)  Trypticase II(2)
          │              │              │
       Soy (3)         Ca (2)       Yeast (3)
                         │              │
                       Ca (3)
                         │
                       Ca (4)                    Yeast (4)
                                                    │
                                                 Yeast (5)

                                  YeCa   Salts    Na      Ca
                                YeCa(2)  Salts(2) Na(2)
Study B.                        YeCa(3)  Salts(3) Na(3)   Ca(2)
                                YeCa(4)  Salts(4) Na(4)   Ca(3)
   Soy(4)  Trypt II  Bacto      YeCa(5)  Salts(5) Na(5)   Ca(4)
```

Numbers is parentheses indicate the number of consecutive subcultures that have been made in a given medium.

Subcultures were analyzed, usually daily, for 2-6 days by removal of 1.0 ml for examination of bacterial integrity (by Gram's stain and sometimes by petri smear) and cell morphology (by Gram's stain). Cell growth was measured turbidimetrically at 650 nm (reference air) and the dry weight calibrated as described in 5.5.5. The 1.0 ml was centrifuged on the CamLab centrifuge and the supernatant

analyzed by tlc and for amylase activity as well as product specifi-
city by incubation at 37°C of 0.50 ml supernatant in 2.0 ml sterile
substrate (1% potato starch in 10 mM phosphate buffer). One drop of
toluene was added to those solutions incubated >8 h. Product speci-
ficity was determined by tlc of the incubate, and amylase activity by
measurement of increases in reducing sugar using the alkaline ferri-
cyanide method (5.8.3.2).

2.3.4.2. Study B. Further subcul-
tures were grown and examined as in Study A but amylase activity was
measured by both the Boehringer and alkaline ferricyanide assays
(5.6.2.3). From Study A, the YeCa, Salts, Na, and Ca subcultures from
Yeast were each adapted to their respective media by 4 subsequent sub-
cultures made after 2 days, 2 days, 7 days, and 4 days (except Ca was
3 subcultures at 4, 7, and 4 days). The first two subcultures in each
medium were examined with Study A. In addition, the second Soy sub-
culture from Study A was used to inoculate Trypticase II, Soy, and
Bacto media, and these cultures were examined at 16h, 40h, 64h, and
90h, along with the fifth subcultures in YeCa, Salts, and Na media and
the fourth subculture in Ca medium. The sequence is shown in Scheme
2.3. Amylase activity was determined by the AF assay using the
initial linear slope from samples taken at 1 hour intervals (for
samples with low amylase activity) or 0.5 hour intervals (for samples
with high activity).

2.3.5. Cultures of Pseudomonas stutzeri NRRL B-3389 in
Various Media. *P. stutzeri* NRRL B-3389 (U.S. Dept. of Agriculture,
Peoria, Il. Dated 3-22-83) was revived Oct 84 in Soy medium at 30°C.
The culture was maintained on slopes as described for *P. stutzeri*

NCIB 11359 and also stored at 5°C in liquid Soy medium. After 1 month a loopful from the inoculum in liquid Soy was added to two boiling tubes containing Soy medium (2 ml each). Procedures were the same as described in the previous experiment (2.3.4) and in 5.5.4.1, except that aeration was not initiated until 1 hour after inoculation. Sub-culture 1, shown in Scheme 2.4, was prepared by inoculating 15 ml of Soy, Trypticase II, Ca, Na, and Salts media with 0.3 ml of the Soy subculture which had been incubated 23 hours. Subsequent subcultures (2%), as shown in Scheme 2.4, were made after incubation of Subculture 1 for 48 h, of Subculture 2 for 28 h, and Subculture 3 for 68 h. All four subcultures were made in duplicate. Subcultures 1, 2, and 3 were boiling tube subcultures containing 15 ml of the respective medium. Subculture 4 contained 100 ml of each of the respective media in 250 ml conical flasks. The flasks were incubated at 30°C in the orbital shaker set at 50 rpm for 1 h, then increased to 150 rpm. Additional aeration was provided after 1 hour by bubbling a thin stream of

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Scheme 2.4. Subculture Sequences for Studies of Pseudomonas stutzeri NRRL B-3389.
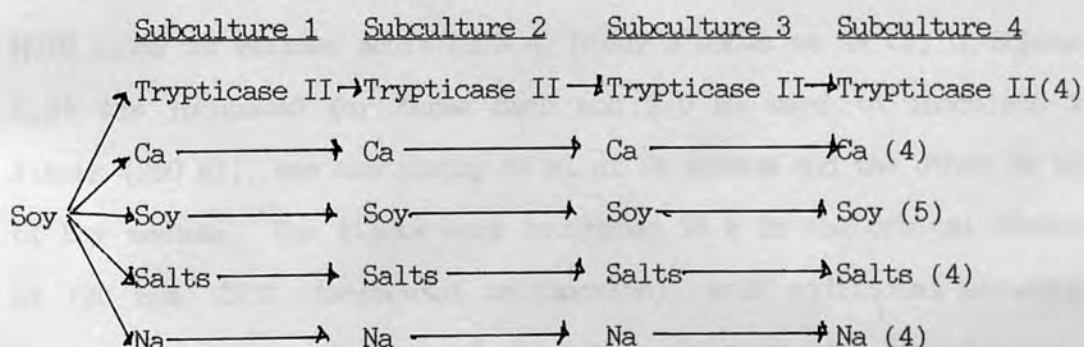
	Subculture 1	Subculture 2	Subculture 3	Subculture 4
	Trypticase II→	Trypticase II →	Trypticase II →	Trypticase II(4)
	Ca ──────→	Ca ──────→	Ca ──────→	Ca (4)
Soy	Soy ──────→	Soy ──────→	Soy ←	Soy (5)
	Salts ──────→	Salts ──────→	Salts ──────→	Salts (4)
	Na ──────→	Na ──────→	Na ──────→	Na (4)

Numbers is parentheses indicate the number of consecutive subcultures that have been made in a given medium.

filtered air through the swirling cultures.

Subculture 4 was analyzed at 0h, 4h, 21h, 28h, 46h, and 72h by removal of 5 ml from each flask. The samples were examined by Gram's stain for cell morphology and bacterial integrity. Cell growth was measured turbidimetrically at 650 nm (reference, water) For A>1.9, cultures were diluted with medium. The sample supernatant was assayed for amylase activity by the Boehringer method and 0.25 ml incubated in 1.0 ml of substrate (1% potato starch in 33 mM phosphate buffer) for analysis of product specificity.

2.3.6. Cultures in Defined 'Na' Medium. All cultures, including subcultures, were examined by Gram's stain. All samples from the three batch cultures (Cultures A, B, and C) were analyzed for acidity, cell growth, and amylase activity. Acidity was determined by measurement of pH. Cell growth was measured turbidimetrically (650 nm, reference water) and dry weight calibrated as described in 5.5.5. Amylase activity was determined by the Boehringer method.

2.3.6.1. <u>Culture A</u>. The third Na subculture from the experiment on the comparison of amylase induction by *P. stutzeri*. NCIB 11359 in various media [2.3.4, Study B shown as Na (3) in Scheme 2.3] was incubated for three days and 1.0 ml used to inoculate 2 flasks (250 ml), one containing 90 ml of Na medium and the other 90 ml of Soy medium. The flasks were incubated 18 h in the orbital shaker at 120 rpm, 22°C (thermostat malfunction), with additional aeration from a thin stream of filtered air bubbled through each swirling culture. The subculture in Soy medium was used for Culture A described below. The subculture in Na medium was used for Culture B described in 2.3.6.2. Subculture sequences are summarized in Scheme 2.5.

The subculture in Soy medium (89 ml--2 ml was used for analysis) was centrifuged 30 min at 2075xg (MSE 6L) and the pellet mixed with Na stock solution (300 ml) and added to 1.5% potato starch (600 ml) in the Gallenkamp fermenter. The culture was incubated as described in 5.5.4.4 and samples were removed at 0h, 2h, 4.5h, 6h, 11.5h, 22h, 24h, 27h, 29h, 32h, and 46h. Each sample was analyzed for acidity, cell growth, and amylase activity. The samples from 6h, 24h, and 46h were also examined by Gram's stain. Product specificity was determined at 24h and 29h. At 29h ≈600 ml was removed for purification of amylase as described in Chapter 3, Purification IV.

Scheme 2.5. Subculture Sequences for the Three Cultures in Na Medium.
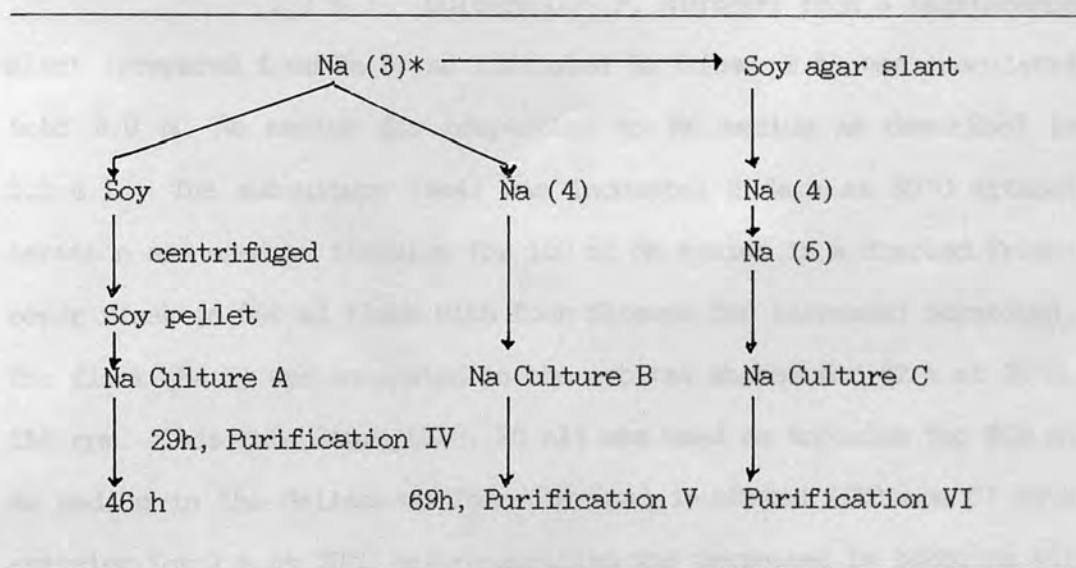
Numbers in parentheses indicate the number of consecutive subcultures made in Na medium. *Na (3) is the third subculture shown in 2.3.4., Scheme 2.3.

2.3.6.2.   Culture B. The 91 ml of Na subculture described above in Culture A was analyzed at 18 h; then incubated in the orbital shaker for a further 54 hours. The temperature in the

orbital shaker fluctuated between 22°C and 30°C. After a total of 72 h incubation, the Na subculture was added to 900 ml of freshly prepared Na medium in the Gallenkamp fermenter. The culture was incubated as described in 5.5.4.4 except that sometime during the first seventeen hours of incubation, aeration from the air pump was restricted (though some aeration from the vigorous mixing would have continued). At 17 h the air pump was reconnected. Samples were removed for analysis at 0h, 15h, 17h, 19h, 21.5h, 23h, 45.5h, and 68.5h. The samples at 15h and 19h were examined by Gram's stain. At 69h the culture was harvested for purification of amylase as described in Chapter 3, Purification V.

2.3.6.3. <u>Culture C</u>. *P. stutzeri* from a maintenance slant (prepared from Na(3) as indicated in Scheme 2.5) was inoculated into 2.0 ml Na medium for adaptation to Na medium as described in 5.5.4.1. The subculture (Na4) was incubated 2 days at 30°C without aeration and used as inoculum for 100 ml Na medium in a dimpled Erlenmeyer flask (a 500 ml flask with four fingers for increased aeration). The flask (Na 5) was incubated in the orbital shaker for 42 h at 30°C, 150 rpm. This subculture (Na5, 90 ml) was used as inoculum for 900 ml Na medium in the Gallenkamp fermenter and incubated (Culture C) with stirring for 1 h at 30°C before aeration was increased by bubbling air through at 0.5 ml/min.

Samples (4-5 ml) were removed at 0h, 2h, 4h, 5.5h, 10h, 19h, and 20h and analyzed for acidity, cell growth, and amylase activity. After measurement of the turbidity each sample (4 ml) was centrifuged (CamLab, 7 min) and the pellet resuspended in 4 ml saline solution. The absorbance (650 nm, reference water) of each supernatant and cell suspension was measured. When absorbancy was >1.5, at 19h and 20h,

the turbidity of diluted samples or cell-saline suspensions (0.5 ml cells in 3.0 ml saline) was measured.

At 20h the fermenter cell with the remaining culture was set in an ice bath and stirring continued until the temperature dropped to 12°C at which time the culture was removed for purification of amylase activity as described in Chapter 3, Purification VI. The samples from 9.5h, 11.5h, 13.5h, and 17h were also examined by Gram's stain and their dry weight calibrated as described in 5.5.5.2.

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CHAPTER 3. PURIFICATION OF MALTOTETRAOHYDROLASE.

3.1. **Introduction.**

Purification of maltotetraohydrolase has previously been repor-
ted by several groups using different procedures. Robyt and Ackerman
(7) were the first to purify and describe some of the properties of
this unusual maltotetraose-producing amylase. They purified G_4-
amylase to a high specific activity (2590 U/mg of protein) and high
purity as shown by disc-gel electrophoresis. The 1036-fold purifica-
tion factor was obtained after only four purification steps, which
included continuous centrifugation, concentration of the culture
supernatant, precipitation by ammonium sulfate, and acetone precipi-
tation. The same four steps were followed in this work, in Purifica-
tions I and II, as reported in 3.2.1.

Other groups subsequently purified G_4-amylase, utilizing several
different purification steps including chromatography on Sephadex G-
100. Dellweg, John and Schmidt (9) included high speed centrifuga-
tion, concentration by lyophilization, ammonium sulfate precipitation,
chromatography on Sephadex G-100 and DEAE-cellulose in the purifica-
tion steps. Schmidt and John (10) later modified this procedure and
replaced chromatography on DEAE-cellulose with chromatography on DNA
grade hydroxyapatite. Sakano, Kashiwagi and Kobayashi (12) purified
G_4-amylase by centrifugation, adsorption to starch, ammonium sulfate
precipitation, and chromatography on Sephadex and DEAE-cellulose
columns. They also replaced chromatography on DEAE-cellulose, but
with chromafocusing on PBE 94 (13). Each of these groups included
chromatography on Sephadex G-100, which specifically binds G_4-amylase

and functions as an affinity column. Several preparations of G_4-amylase discussed in this work, in Purification III (Chapter 3.2.1), were purified by chromatography on Sephadex G-100 and then used for studies described in Chapter 4.

Neither Dellweg et al (9, 10) nor Sakano et al (12, 13) achieved the high specific activity of Robyt and Ackerman (7), although Sakano et al were able to separate and purify two isozymes of G_4-amylase (13). All three groups have reported on various properties of G_4-amylase. This chapter discusses the partial purification of G_4-amylase from several different cultures of Pseudomonas stutzeri in Trypticase I medium and in Na medium.

Maltotetraohydrolase from various stages in purification was used for studies described in Chapter 4. Purification was sometimes hindered by the presence of slime or gel and an inhibitor to which the enzyme was adsorbed. Dellweg et al (9) also found that slime interfered with purification of G_4-amylase and later characterized the polysaccharide found in the slime (24).

3.2. Results and Discussion.

3.2.1. From Cultures in Trypticase I Medium. Maltotetraohydrolase was purified from two cultures of P. stutzeri described in 2.2.1 and 2.3.1. Cell growth and amylase production were not followed in these cultures although at harvest the culture supernatants were shown to contain G_4-producing amylase.

3.2.1.1. Purification I. Purification of Small Scale Culture. Five flasks each containing 51 ml of *P. stutzeri* suspension were cultured in the orbital shaker. After storage for 12 days at 4°C, petri smears indicated that few cells remained viable, however amylase activity (average activity = 0.11 turbid. U/ml) remained at high levels when compared with several other culture studies. The main steps in purification and the results are summarized in Table 3.1.

Table 3.1. Purification I Scheme for Maltotetraohydrolase from
          Pseudomonas stutzeri Cultured in Trypticase I Medium.

Purification Step	Volume (ml)	Activity (turbid. U/ml)	Recovery	Protein (mg/ml)	Purification Factor
1. Culture Supernatant	220.0	0.08	100%	5.3	1
2. Ultraconcentrate	39.5	0.41	90%	12.5	2
3. AmSO$_4$ Ppt, 48% sat.	2.0	3.99	44%	4.0	66
4. 70% Acetone Ppt	8.0	0.59	26%	0.4	98

Amylase activity is expressed as turbidimetric U/ml. One turbidimetric unit is that amount of enzyme which produces a decrease in absorbance of unity in 1 minute. The substrate was 2% starch in 10 mM glycerophosphate buffer containing 5 mM CaCl$_2$, pH 7, at RT.

Purification Factor: Increase in specific activity from Step 1 (specific activity = turbid. U/mg).

Amylase activity was lost during the purification procedures and during storage. There was a 27% loss in activity between the initial analysis of the five cultures (average 0.11 turbid. U/ml) and analysis of the combined supernatants as shown in Table 3.1. The total activity dropped a further 10% during concentration by ultrafiltration; no activity was detected in the ultrafiltrate. However, the greatest loss in activity occurred during ammonium sulfate precipitation (Step 3, Table 3.1) and storage. Storage of the ammonium sulfate precipitate in Buffer B (10 mM glycerophosphate buffer containing 5mM $CaCl_2$) showed a 3-fold loss in activity over 2 days. Precipitate removed at 25% (sat) ammonium sulfate and dissolved in buffer showed total loss in activity after storage for 2 days.

The addition of 25% (saturation) ammonium sulfate precipitated only 1.6% (0.24 turbid. Units, with 14.4 turbid. Units remaining in the supernatant) of the amylase activity. At 41% (sat.) ammonium sulfate, the precipitate and the supernatant had approximately equal amounts of activity - 81% of the activity remaining from the ultraconcentrate. At 48% saturation, no activity remained in the supernatant but only 49% of the activity from the ultraconcentrate was detected in the precipitate (44% recovery from Step 1). Most of the amylase activity was lost during the increase in ammonium sulfate from 41% saturation to 48% saturation. The precipitate removed at 48% sat. was only partially soluble in Buffer B (at 0.4 turbid. U/ml). The mixture was precipitated with acetone (70% v/v).

The acetone precipitate was partially soluble in Buffer B (at 1 turbid. U/ml); however additional G_4-amylase could be extracted into buffer. After repeated extractions 59% of the amylase was recovered

from the previous ammonium sulfate step, leaving the amylase dilute
but with a 98-fold purification factor. One week later the amylase
activity was determined by the DNS assay and found to be 1.8 U/ml,
giving a specific activity of 4.5 U/mg. After storage at 9°C for 3
weeks the activity was remeasured, found to be 1.13 DNS U/ml, and used
for preparation of maltotetraose as described in 4.3.3.2.

During the purification there appeared to be no interference
from gel formation (a problem during purification of other cultures).
Centrifugation and concentration gave clear solutions. The insoluble
precipitate may have been a few isolated cells, denatured enzyme, or,
more likely, polysaccharide. The extracellular polysaccharide repor-
ted by Dellweg et al (24) was precipitated by 2 volumes of acetone.
The absence of gel formation may have been related to the slow growth
conditions with low aeration, the cold storage of the cultures
(possibly removing the gel with cells), and/or the alkaline pH (pH
9.0) of the culture supernatant (first noted in the ultraconcentrate).

3.2.1.2. Purification II. Purification of Large
Scale Culture. Pseudomonas stutzeri was grown in Trypticase I medium
in twelve flasks each containing 408 ml culture. After 5.7 days in
the orbital shaker the flasks were each analyzed for amylase activity
and found to contain 0.33-0.71 U/ml (DNS at 25°C, see 2.3.1) in the
culture supernatants. The twelve cultures were pooled. Centrifuga-
tion at 2075xg only partially removed the cells; the cloudy superna-
tant was recentrifuged at 10000xg to give a clear, golden supernatant.
Amylase activity was lost during centrifugation of the pooled cultures
and the combined culture supernatants contained only 0.25 U/ml as
shown in Step 1, Table 3.2.

Table 3.2. Purification II Scheme for Maltotetraohydrolase from
Pseudomonas stutzeri Cultured in Trypticase I Medium.

Step	Amylase Activity Units/ml	Total Units	Protein mg/ml	Specific Activity Units/mg	Purification Factor
1. Culture					
Supernatant (4425 ml)	0.25	1120	5.7	0.044	1.0
2. Conc. by					
Ultrafiltration (417 ml)	1.87	780	20.0	0.095	2.2
3. Ammonium Sulfate, 48% sat.					
Ppt. in Buffer B (25 ml)	4.0	100	1.0	4.0	91
Ppt. in PIPES (23 ml)	4.2	97	0.75	5.6	127
4. Acetone Precipitation					
from Buffer B (3 ml)	12.7	38	1.5	8.3	189
from PIPES (6 ml)	6.5	39	0.87	7.4	168

Buffer B: 10 mM sodium glycerophosphate buffer, pH 7 with 5 mM $CaCl_2$.
PIPES: 10 mM PIPES buffer, pH 7 containing 5 mM $CaCl_2$.
Amylase activity was determined by the DNS assay at 25°C, pH 7.

Concentration (10.6-fold) by ultrafiltration gave a viscous,
dark golden concentrate which showed a 7.5-fold increase in activity
giving a 70% recovery of the amylase activity in the culture superna-
tant. The ultrafiltrate contained no amylase activity. The pH of the
gel-like ultraconcentrate was pH 8.5 and was brought to pH 7.3 before
the addition of ammonium sulfate.

Ammonium sulfate was added to the UF concentrate in two stages. After addition of ammonium sulfate to 25% saturation a portion of the viscous solution was centrifuged. No precipitate formed but approximately one-third of the solution formed a gel. The supernatant contained 0.50 turbid. U/ml of amylase activity. A slice of the gel was vigorously mixed with Buffer B but did not dissolve completely. The turbidimetric assay indicated that the gel retained 2.3 times more amylase activity/cm^3 than the supernatant as shown in Step a, Table 3.3. The gel and remaining solution were combined and filtered under atmospheric pressure (Step b). The filtrate collected over 2 days contained 0.64 turbid. U/ml and the remaining gel contained 0.15 turbid. U/ml. The amylase was loosely bound to the gel and in effect concentrated by the gel. Additional gel filtrate containing amylase activity was collected over a further 15 days and contained higher amylase activity than the previous solutions.

Table 3.3. Purification II Scheme after Addition of 25% Ammonium Sulfate.

Step		turbid. U/ml
a.	Supernatant (85 ml)	0.50
	Gel	1.1
b.	Gel filtrate and remaining solution (190 ml)	0.64
	Remaining gel	0.15
c.	Filtrate collected from 2-17 days (22 ml, 45 ml)	0.92, 0.68
d.	Combined supernatant and filtrates (333 ml)	0.59

For the second stage of ammonium sulfate precipitation, the 25% saturated supernatant and the gel filtrates were combined (Table 3.3, Step d: 333 ml, 0.6 turbid. U/ml) and ammonium sulfate added to bring the concentration to 48% saturation. The 48% saturated solution was centrifuged at 3600xg and analysis indicated that only a third of the amylase activity had been precipitated and that two-thirds of the activity remained in the supernatant. Centrifugation at higher g force (17500xg) precipitated approximately half of the total amylase activity leaving 77 turbidimetric units in the slightly turbid, dark golden supernatant (350 ml, 0.22 turbid. U/ml, 0.62 U/ml assayed by DNS at 25°C, 21 mg protein/ml). This supernatant was later purified with other samples discussed in Purification III, 3.2.1.3. The precipitate was divided into two equal portions.

Buffer B was added to half of the loose precipitate and 10 mM PIPES buffer was added to the other half. (PIPES buffer was employed because it is does not complex with metal ions. Some amylases are stabilized by Ca^{2+} although Sakano *et al* (13) have reported that G$_4$-amylase shows no enhanced stability between pH 6 and 9.) The precipitates did not totally dissolve in either buffer. The mixtures were centrifuged and extractions with the respective buffers repeated three times. The extractions into Buffer B were pooled, as were the extractions into PIPES buffer. Extraction removed inhibitor, giving a net increase in amylase activity of 161% extracted into Buffer B and and 155% extracted into PIPES buffer. (A net increase was shown for example when analysis of the original PIPES suspension gave 21 turbid. U/ml but after centrifugation and removal of insoluble material the supernatant contained 30 turbid. U/ml of activity). The combined extraction into Buffer B contained 1.56 turbid. U/ml (net 39 turbid.

Units) and into PIPES buffer 1.43 turbid. U/ml (net 33 turbid. Units). Further analyses are shown in Table 3.2, Step 3. Storage for 10 days at 9°C showed no loss of amylase activity in PIPES buffer and after 1 month, analysis at 37°C by the DNS assay gave 13.8 U/ml.

The amylase in Buffer B and PIPES buffer were each precipitated with acetone (70% v/v, 2°C). As with the ammonium sulfate precipitation, a portion of the precipitate was not soluble in buffer and extraction with buffer yielded more total units of activity than was in the precipitate-buffer suspension. The three combined extractions (3.0 ml) in Buffer B contained 4.4 turbid. U/ml, 42 U/ml when assayed by the DNS assay at 37°C, or 13 U/ml at 25°C as shown in Step 4, Table 3.2. The amylase in Buffer B was later used for preparation of malto-tetraose as described in 4.3.3.3. The acetone precipitate from PIPES buffer was extracted into Buffer A (Buffer B without calcium chloride) and contained 2.2 turbid. U/ml, or 14.7 U/ml when assayed by DNS at 37°C. The amylase in Buffer A was used for preparation of maltotetra-ose as described in 4.3.3.4.

PIPES buffer neither extracted nor stabilized G_4-amylase more than glycerophosphate buffer (Buffer B). The total amylase activity recovered in Step 3 (Table 3.2.) was about the same in each buffer; more protein was however found in the Buffer B portion (1.0 mg/ml vs. 0.75 mg/ml), giving a lower specific activity and purification factor. This advantage was negated in Step 4 after extraction of the PIPES precipitate into glycerophosphate buffer.

Further analysis of the gel (after 1 month of filtration) indicated that it still retained high protein concentration (more than 100

mg/ml) and that the total carbohydrate concentration was more than 30 mg/ml. An 8% (v/v) of gel in water did not dissolve at 37°C but was soluble at 60°C. The gel was fluid above 60°C but remained turbid and viscous even when heated to 98°C.

The gel formed on concentration of culture supernatant and at high salt concentration. Amylase was adsorbed to the gel, reversibly, and could be removed by gravimetric filtration. The gel was found to inhibit amylase activity and may have been the same insoluble substance found on precipitation of the amylase. Delleweg *et al* (9) found that a slimy material was excreted in *Pseudomonas stutzeri* cultures which "sometimes" interfered with purification. They were able to separate the "main part" of the slime by concentration, formation of a gel, dissolution in boiling water and acetone precipitation. Characterization of the polysaccharide showed it to be a polysaccharide composed of glucose and mannose in nearly equal amounts, and of rhamnose and an unknown sugar in lesser amounts (24).

The purification step with ammonium sulfate not only produced a gel which interfered with precipitation but also yielded only 18% of the activity from the original culture supernatant or 25% from the UF concentrate (or 36% and 50% if the amylase remaining the the 48% ammonium sulfate supernatant is included). The advantage of this step however was the 100-fold purification of G_4-amylase. Precipitation with acetone produced about 180-fold purification but yielded only 7% of the original amylase activity and only 39% recovery from the previous step. PIPES, which does not complex with cations, did not improve the yield of amylase activity. Amylase could be extracted from an inhibitor, present in both the ammonium sulfate and the

acetone precipitates.

3.2.1.3.   Purification III.   Purification of Pooled Trypticase I Culture Samples. *Step 1*. Amylase from several of the steps in Purification II were pooled.   A major portion of the amylase was from the supernatant in 3.2.1.2, Step 3 (48% ammonium sulfate precipitation).   The pooled samples contained a total of 156 turbidimetric Units of amylase activity (Table 3.4) as well as 6.22 g of carbohydrate (13.6 mg/ml).

Table 3.4. Purification III Scheme for Maltotetraohydrolase
from Pooled Samples.

Step		Vol. ml	Turbid. U/ml	Protein mg/ml	Boehringer U/ml
1.	Pooled Samples	457	0.34	10.8	
2.	UF XM100 concentrate	24.5	3.30	22.5	>10*
	UF XM100 filtrate	417	0.04	10.2	
3a.	UF PM-10 concentrate	150	0.21	21.8	1.4*
3b.	Further PM10 Concentration	17.0			11.6
4.	Centrifugation at 2520x*g*	16.5		24.3	14.3
5.	570 mg in 5 ml, filtered	5		0.29	9.7
6.	691 mg in 5 ml, conc.	3			24.5
9.	Affinity chromatography	116		≈ 0.0002	0.41

Step numbers correspond to Table 3.5; * After storage for 1.8 y.

Step 2. The pooled samples were concentrated by ultrafiltration (Amicon XM100 membrane, 100,000 dalton retention) and most of the amylase found to be retained in the turbid, viscous concentrate. Other groups (10, 12) have reported the molecular weight of the G_4-amylase subunits to be between 48000-58000 and the native enzyme to consist primarily as dimers. The XM100 concentrate had no gel which could be removed by gravimetric filtration (as in Purification II). The concentrate retained 4.8 times as much activity as found in the ultrafiltrate (Table 3.4, Step 2) and contained 46.0 mg/ml carbohydrate (3.4-fold concentration) and 16.5 U/ml when assayed by the DNS assay at 37°C. After storage at 5-9°C for 1.8 y the gel-like concentrate was found to have >10 Boeh U/ml. Centrifugation at 38000xg left 8.9 Boeh U/ml in the supernatant. No further work was done with this amylase, although freezing and filtration may remove the substance which interferes with purification.

Step 3a. The XM100 filtrate (417 ml) contained 10.0 mg/ml carbohydrate as well as amylase as shown in Table 3.4. The filtrate was concentrated 3-fold by ultrafiltration (Amicon PM-10, 10,000 dalton retention). An inhibitor (possibly G_4, reported (10) to be a competitive inhibitor of G_4-amylase) was apparently removed since the recovery was 182%. (A total of 17.0 turbidimetric Units was found in the XM100 filtrate before concentration and 31.0 turbid. Units after concentration.) The PM-10 filtrate was found to contain maltotetraose and perhaps some maltotriose and maltose. After storage for 1.8 y the concentrate was found to retain 1.40 Boeh U/ml. *Step 3b.* After further concentration (PM-10) and washing with phosphate buffer 94% of the activity was recovered in the PM-10 ultraconcentrate as shown in Table 3.5. The concentrate was then frozen at -20°C.

Table 3.5. Sequence for Purification III of Maltotetraohydrolase from Pooled Samples from Large Scale Trypticase I Culture.
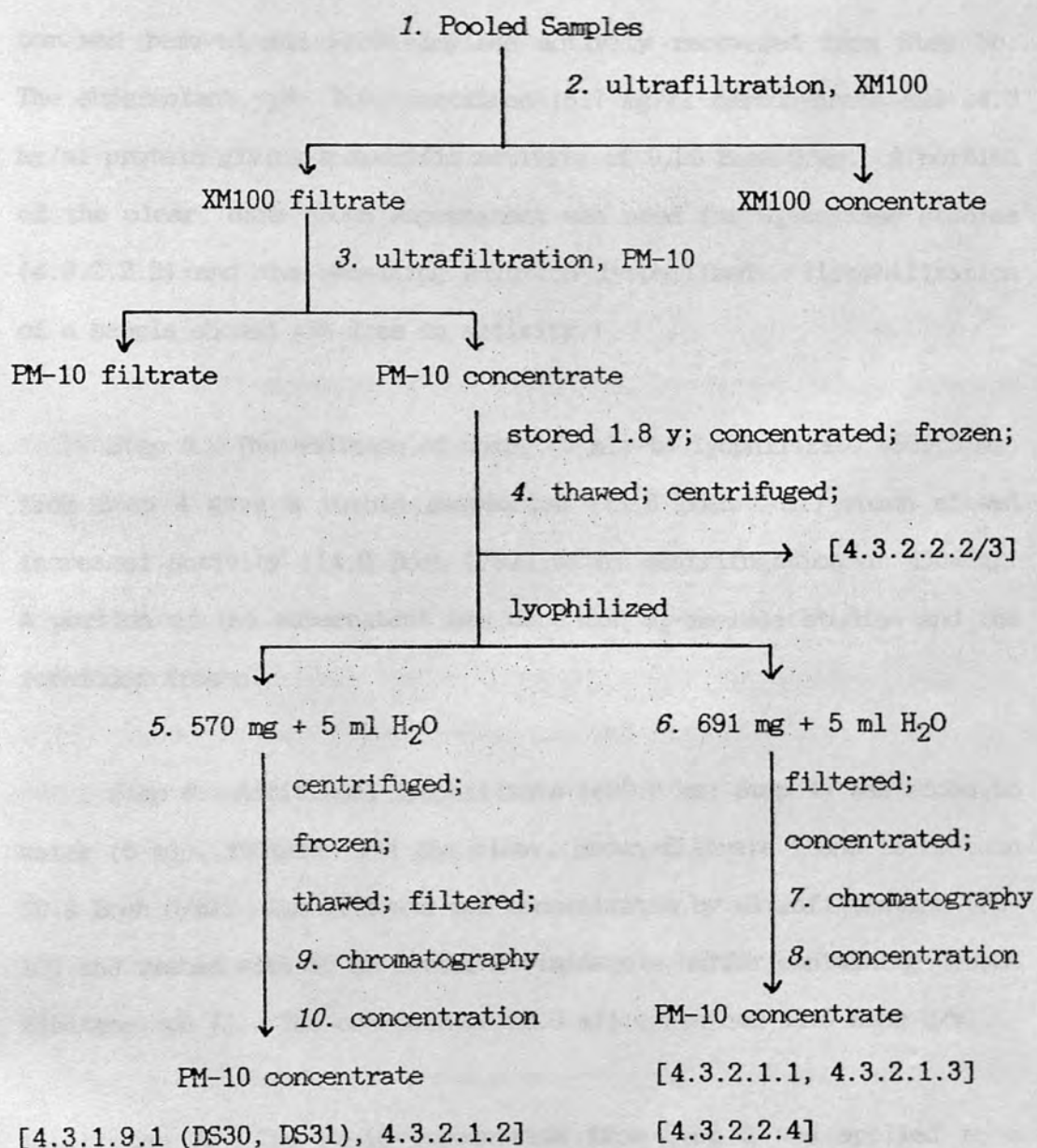
1. Pooled Samples

2. ultrafiltration, XM100

XM100 filtrate   XM100 concentrate

3. ultrafiltration, PM-10

PM-10 filtrate   PM-10 concentrate

stored 1.8 y; concentrated; frozen;

4. thawed; centrifuged;

→ [4.3.2.2.2/3]

lyophilized

5. 570 mg + 5 ml H_2O *6.* 691 mg + 5 ml H_2O

centrifuged;   filtered;

frozen;   concentrated;

thawed; filtered;   *7.* chromatography

9. chromatography   *8.* concentration

10. concentration   PM-10 concentrate

PM-10 concentrate   [4.3.2.1.1, 4.3.2.1.3]

[4.3.1.9.1 (DS30, DS31), 4.3.2.1.2]   [4.3.2.2.4]

Numbers in italics refer to steps in the purification procedures.
Numbers in brackets refer to G_4-amylase studies discussed in Chap. 4.

Step 4. After slowly thawing the concentrate from Step 3b and centrifuging at 2520xg the gel-like precipitate (<0.5 ml) was found to contain about 6 mg/ml of both protein and carbohydrate. Analysis of the supernatant (16.5 ml, Step 4, Table 3.4) showed that again inhibitor was removed and >120% amylase activity recovered from Step 3b. The supernatant, pH 7.0, contained 15.7 mg/ml carbohydrate and 24.3 mg/ml protein giving a specific activity of 0.59 Boeh U/mg. A portion of the clear, dark brown supernatant was used for G_4-amylase studies (4.3.2.2.2) and the remaining solution lyophilized. (Lyophilization of a sample showed <3% loss in activity.)

Step 5. The addition of water (5 ml) to lyophilizate (569.5 mg) from Step 4 gave a turbid suspension (11.8 Boeh U/ml) which showed increased activity (14.0 Boeh U/ml) after centrifugation at 45000xg. A portion of the supernatant was used for G_4-amylase studies and the remainder frozen.

Step 6. Additional lyophilizate (690.8 mg, Step 4) was added to water (5 ml), filtered and the clear, brown filtrate found to contain 10.9 Boeh U/ml. The filtrate was concentrated by ultrafiltration (PM-10) and washed with 50 mM Buffer E (imidazole buffer containing 0.002% Hibitane, pH 7). The concentrate (3.0 ml) contained 24.5 Boeh U/ml.

Step 7. The PM-10 concentrate from Step 6 was applied to a Sephadex G-100 column (2.6 cm x 6.9 cm) and washed first with 3 column volumes of 50 mM Buffer E, followed by 1 column volume of Buffer E containing 1M NaCl, and finally by 30 mM phosphate buffer containing 2M NaCl. Approximately 11% of the G_4-amylase eluted with other uv absorbing substances in a dark brown effluent (fractions 11-26 in Fig.

3.1). The amylase in fractions 11-26 hydrolyzed soluble starch to produce primarily maltotetraose and some maltose (similar to the concentrate from Step 8). G_4-amylase was slowly desorbed from the Sephadex through 5 column volumes. The first description of adsorption of G_4-amylase by Sephadex G-100 (Dellweg *et al* (9)) reported no desorption of amylase when eluting with 30 mM phosphate buffer for several column volumes but desorption of G_4-amylase in a 0-2M NaCl gradient. Sakano *et al* (12) found that G_4-amylase was slowly desorbed (between 3-6.5 column volumes after application) in Tris buffer, pH 8.5.

Step 8. Sephadex G-100 fractions 31-80 (fractions after 5 column volumes) from Step 7 were pooled and the 116 ml found to contain 0.406 Boeh U/ml and <1 µg/ml protein (estimated to be 0.2 µg/ml protein, a specific activity of 2000 Boeh U/mg). These fractions contained 64% of the amylase applied to the column. Concentration to 4.7 ml yielded 7.0 Boeh U/ml (73% recovery from the pooled fractions) which hydrolyzed soluble starch to produce primarily G_4 and some G_2. The concentrate was used in G_4-amylase studies described in 4.3.2.1.1 and in 4.2.2.1.3 (Preparation of Limit Dextrins).

Step 9. The frozen solution from Step 5 was thawed, filtered and the 6.6 ml found to contain 7.4 Boeh U/ml. The solution was applied to the Sephadex G-100 column and chromatographed as shown in Figure 3.2. Some G_4-amylase was eluted with other dark brown substance in the second uv absorbing peak. Most of the G_4-amylase was eluted (73% of the activity applied to the column) in fractions collected after desorption by oligosaccharide. Two peaks were eluted, results similar to those reported by Dellweg *et al* (9) when desorbing with starch. Maltotetraose was first detected by tlc in fraction 47

Chromatography of Maltotetraohydrolase
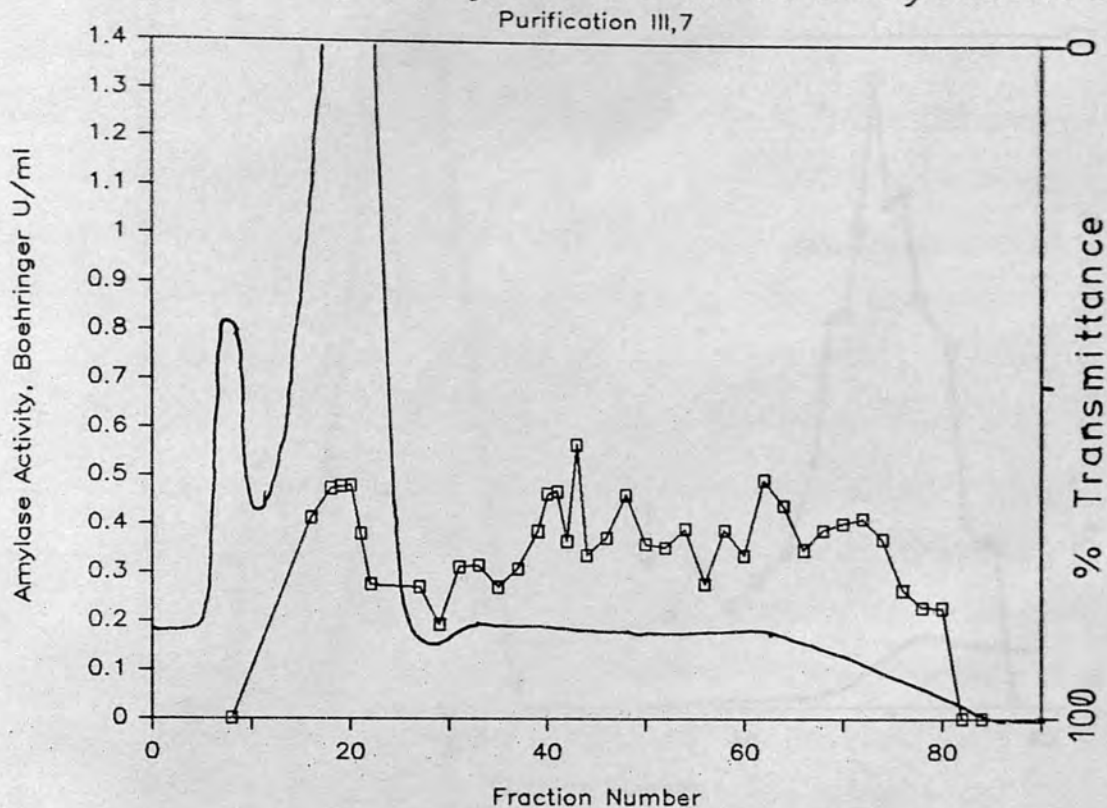
Purification III,7

Fig. 3.1. Chromatography on Sephadex G-100 of Maltotetraohydrolase. Filtered lyophilizate in 50 mM imidazole buffer containing 0.002% Hibitane, pH 7 from Purification III, Step 6 was applied to a Sephadex G-100 column (2.6 x 6.9 cm) and eluted at a flow rate of 28 ml/h with fractions of 40 drops (2.4 ml) collected. After fraction 49 the buffer was changed to 50 mM Buffer E containing 1M NaCl and to 30 mM phosphate buffer, pH 7, containing 2M NaCl after fraction 64.

% transmittance at 280 nm (————); amylase activity determined by the Boehringer assay (—☐—).

along with some G_3, G_2, and material at the origin. The major portion of the G_4-amylase was eluted between 24 and 54 ml after the buffer change and was at a maximum at 36 ml (1 column volume) after the buffer change.

Chromatography of Maltotetraohydrolase
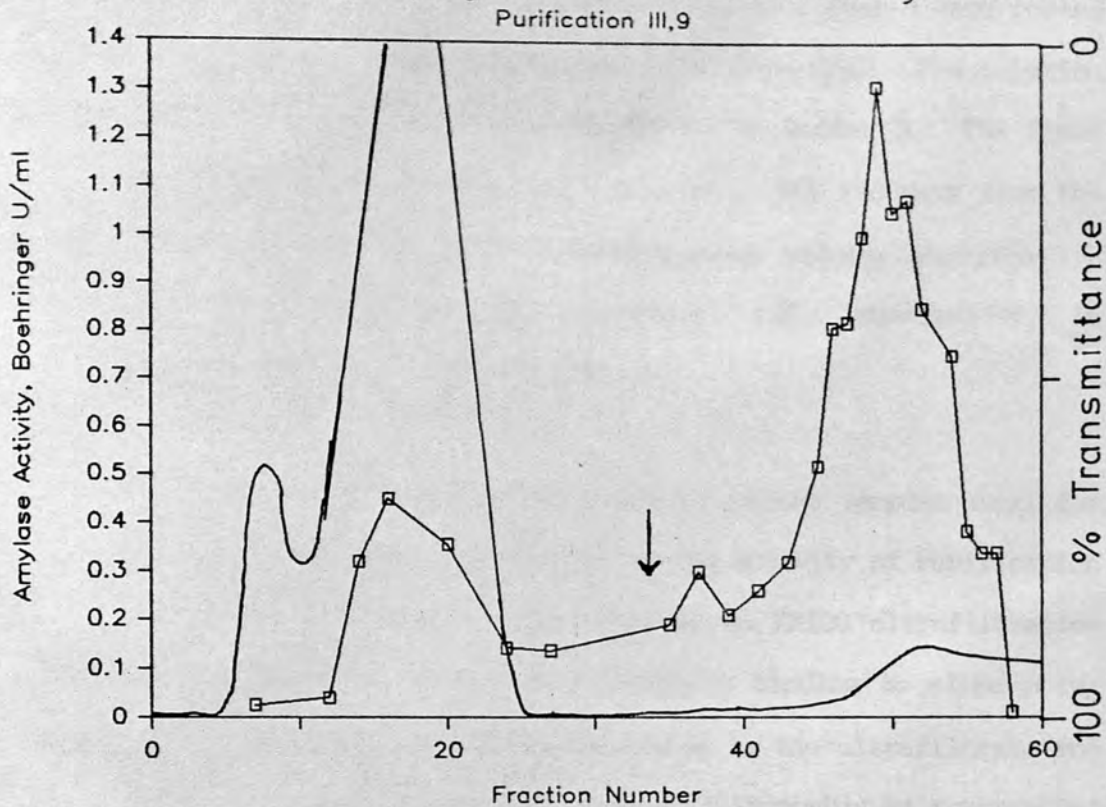
Purification III,9

Fig. 3.2. Second Chromatography on Sephadex G-100 of Maltotetrao-
hydrolase. Filtered lyophilizate in 20 mM Buffer D (imidazole buffer
containing 0.002% chlorhexidene, pH 7) from Purification III, Step 5
was applied to the same Sephadex G-100 column shown in Fig. 3.1 which
had been previously equilibrated with 20 mM Buffer D. The flow rate
was 28 ml/h; fractions of 40 drops (2.4 ml) were collected. The
eluent was changed after fraction 34 (↓) to 50 mM Buffer D containing 10.6
mg/ml of oligosaccharide from Experiment III, Chapter 4.3.3.4
(estimated to be 9 mM G_4).

% transmittance at 280 nm (————); amylase activity determined by the
Boehringer assay (—□—).

Step 10. Sephadex G-100 fractions 31-58 from Step 9 were pooled and the combined 63 ml found to contain 0.565 Boeh U/ml. The solution was concentrated (PM-10) and washed with 50 mM Buffer D. The final 1.6 ml of concentrate contained 20.1 Boeh U/ml (90% recovery from the pooled fractions) and was used for G_4-amylase studies described in 4.3.1 (nmr Hydrolyses X and XI), 4.3.2.1.2 (HPLC studies) and in 4.3.2.1.1 (Preparation of Limit Dextrins).

The G_4-amylase found in the original pooled samples used for Purification III had approximately 40% of the activity of Purification II. Most of the activity was retained by an XM100 ultrafiltration membrane (100,000 dalton retention), probably binding to slime poly-saccharide. Some G_4-amylase (17%) was found in the ultrafiltrate and this was further purified. Amylase inhibitor (probably maltotetraose) was removed when G_4-amylase was concentrated by ultrafiltration (PM-10 membrane). Additional inhibitor (probably polysaccharide) was removed upon freezing or lyophilization and centrifugation of the amylase solution. Most of the G_4-amylase applied to a Sephadex G-100 column was adsorbed to the Sephadex gel and slowly desorbed in 50 mM imida-zole buffer, pH 7. The addition of 1 M NaCl to the buffer did not increase desorption; however phosphate buffer containing 2 M NaCl desorbed the remaining G_4-amylase. After concentration the specific activity of the desorbed G_4-amylase was estimated to be 2000 Boeh U/mg, a specific activity comparable to that reported (2590 U/mg) by Robyt and Ackerman (7). In a second application of G_4-amylase the Sephadex column was used as an affinity column and the amylase was desorbed by a 1% oligosaccharide (primarily G_4) solution.

3.2.2. From Cultures in Na Medium. Maltotetraohydrolase produced in the three batch cultures described in 2.2.6 was partially purified from each culture.

3.2.2.1. _Purification IV_. Approximately two-thirds of Na Culture A (Figure 2.10, 2.2.6.1) was removed after 29 h of culture at which time the cells had been in stationary growth phase for at least 20 hours and during which time G_4-amylase activity remained at an average maximum concentration of 0.7 Boeh U/ml.

Table 3.6 briefly summarizes the purification sequence for maltotetraohydrolase from Na Culture A. Centrifugation at 2075xg removed some cells and gave a turbid supernatant (595 ml) containing 0.71 Boeh U/ml. The remaining cells were removed from the supernatant by hollow fiber filtration (0.1 micron membrane). The hollow fiber filtrate was found to contain 0.53 Boeh U/ml or 69% recovery of amylase activity from the supernatant (Table 3.7). The cells in the hollow fiber concentrate were washed with phosphate buffer and the 146 ml wash collected found to contain only 10% of the original amylase activity. There appeared to be no interference with slime and little or no G_4-amylase loosely bound to the cells.

The hollow fiber filtrate was stored at -20°C and after thawing, the amylase activity was found to be 0.38 Boeh U/ml. The loss in activity (29%) was probably due to the acidity of the solution (pH 6.0-6.5); the solution was neutralized. The cell culture after 29 h growth was at pH 6.5 (see 2.2.6.1) and may have contributed to the loss in activity during hollow fiber filtration. As discussed in 2.2.6.1, the culture supernatant was shown by tlc to contain

maltotetraose and some maltose. Carbohydrate analysis of the filtrate showed it to have a DP of 5.0 (48 μmole/ml carbohydrate, 9.5 μmole reducing sugar/ml).

Table 3.6. Sequence for Purification IV of Maltotetraohydrolase from Na Culture A.
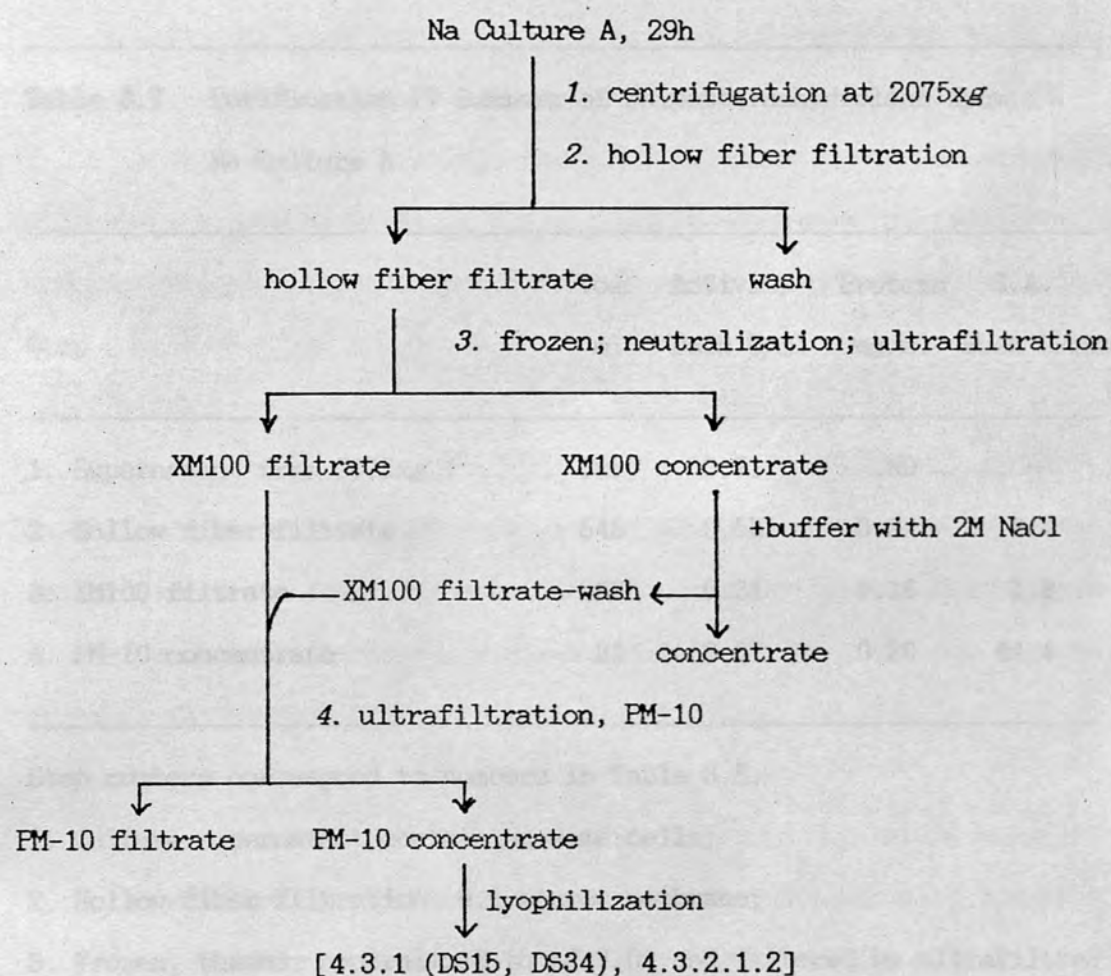
Na Culture A, 29h

| 1. centrifugation at 2075xg
| 2. hollow fiber filtration

hollow fiber filtrate   wash

| 3. frozen; neutralization; ultrafiltration

XM100 filtrate   XM100 concentrate

+buffer with 2M NaCl

XM100 filtrate-wash ⟵

concentrate

4. ultrafiltration, PM-10

PM-10 filtrate   PM-10 concentrate

| lyophilization

[4.3.1 (DS15, DS34), 4.3.2.1.2]

Numbers in italics refer to steps in the purification procedures.

Numbers in brackets refer to G_4-amylase studies discussed in Chap. 4.

Ultrafiltration using an XM100 membrane (100,000 dalton reten-
tion) recovered 81% of the amylase in the XM100 filtrate. The fil-
trate contained 86% of the carbohydrate and had a DP of 3.8. The
XM100 concentrate retained 18 units of amylase activity, of which 9
units were removed by washing the concentrate with a 2M NaCl solution.
Surprisingly the carbohydrate retained by the membrane appeared to
have a DP of 9.4; the XM100 membrane would not be expected to retain
such a small molecule.

Table 3.7. Purification IV Summary of Maltotetraohydrolase from
Na Culture A.

Step	Vol ml	Activity Boeh U/ml	Protein mg/ml	S.A. Boeh U/mg
1. Supernatant from 2075xg	595	0.71	ND	--
2. Hollow fiber filtrate	545	0.53	0.22	2.4
3. XM100 filtrate	530	0.31	0.16	1.9
4. PM-10 concentrate	21	8.87	0.20	44.4

Step numbers correspond to numbers in Table 3.6.

1. Culture supernatant containing some cells;

2. Hollow fiber filtration, 0.1 micron membrane;

3. Frozen, thawed, neutralized to pH 7.0, and filtered by ultrafiltra-
tion (XM100A membrane);

4. Concentration and wash by ultrafiltration (PM-10 membrane); ND, not
determined.

The XM100 filtrate and wash were concentrated by ultrafiltration (PM-10 membrane) and the concentrate washed with phosphate buffer. The PM-10 concentrate showed an apparent increase in the total units of amylase activity (186 Boeh Units versus 162 Boeh Units in the XM100 filtrate, 115% recovery) probably due to the removal of 64.3 mmole of reducing sugar found in the PM-10 filtrate. The concentrate was freeze-dried for use in G_4-amylase studies described in Chapter 4.

During purification of G_4-amylase from Na Culture A, there was no interference from slime polysaccharide, and G_4-amylase was readily filtered through an 0.1 micron and 100,000 dalton membranes although only 46% of the amylase in the culture supernatant was recovered. Concentration by ultrafiltration removed an inhibitor, probably malto-tetraose.

3.2.2.2. Purification V. Na Culture B was harvested after 69 hours of slow but continuous growth and high G_4-amylase induction (Figure 2.11, 2.2.6.2). At the time of harvest the rate of amylase induction had decreased but was at a maximum concentration of 1.52 Boeh U/ml.

Centrifugation at 2075xg gave a slightly turbid supernatant which on concentration by hollow fiber filtration (Amicon, 0.1 micron retention membrane) formed a thick slime, eventually clogging the membrane fibers. Both the filtrate and the concentrate contained amylase which, as shown in Table 3.8, were each partially purified.

Slime and cells concentrated in the hollow fiber filtration apparatus were removed by reversing the flow across the membranes

Table 3.8.   Sequence for Purification V of Maltotetraohydrolase from
             Na Culture B.

```
              1. Na Culture B, 69h; centrifuged at 2075xg
                                 |
                              2. hollow fiber filtration
         ┌───────────────────────┴───────────────────────┐
hollow fiber filtrate                          hollow fiber concentrate
         3. concentrated (G10T);                   +buffer with NaCl
            + buffer; frozen;                    5. centrifuged, 2520xg
            thawed;                                      |
            centrifuged, 38000xg               'salt-conc' supernatant
G10T concentrate                                    frozen;
         4a. + AmSO₄ (45% sat);                     thawed;
             centrifuged, 35000xg                6. centrifuged, 35000xg
no precipitate                                   supernatant
            concentrated (PM-10);               7. concentrated (PM10);
            centrifuged, 30000xg                   +AmSO₄ (45% sat);
                                                   centrifuged, 35000xg
no precipitate                                  AmSO₄ precipitate
         4b. diluted with 45% AmSO₄;            8. +buffer; centrifuged
             centrifuged, 28000xg               supernatant
supernatant                                     microfiltration (T30);
   [4.3.2.2.2]                                   supernatant
                                                  microfiltration; repeat
                                          supernatant [4.3.1.(DS16,DS18)]
```

Numbers in italics refer to steps in the purification procedures.

Numbers in brackets refer to G_4-amylase studies discussed in Chapter 4.

A

using 30mM phosphate buffer containing 1.5 M NaCl. After centrifuga-
tion at 2520xg (Step 5, Tables 3.8 & 3.9), this 'salt-conc' superna-
tant was found to have higher G_4-amylase activity (1.28 Boeh U/ml)
than the hollow fiber filtrate (0.89 Boeh U/ml). Analysis for product
specificity indicated only G_4-producing amylase. As in Purification
II, where slime was evident the G_4-amylase appeared to be retained
with the slime.

The cloudy salt-conc supernatant was frozen; after thawing the
activity was found to be 0.86 Boeh U/ml, a 30% loss in activity.
Centrifugation (35000xg) of the remaining solution gave a clear super-
natant containing 0.79 Boeh U/ml (Step 6, Table 3.9), 92% of the
amylase activity. Resuspension of the pellet and analysis of the
pellet solution indicated 8% of the activity had been precipitated
with the pellet.

The clear supernatant from centrifugation at 35000xg (Step 6,
Tables 3.8 & 3.9) was concentrated by ultrafiltration (PM-10 membrane,
Step 7) to dryness. Phosphate buffer was added and the concentrate
(15.0 ml) found to contain 4.30 Boeh U/ml, 43% recovery from the clear
supernatant. Ammonium sulfate (45% sat) was added and precipitated
G_4-amylase leaving 3% of the activity in the supernatant. The preci-
pitate had a gel-like appearance and was insoluble at 20°C.

Ammonium sulfate was removed from the gel-precipitate by the
addition of phosphate buffer, centrifugation to remove gel ($\approx$25% v/v),
concentration of the supernatant by microfiltration (Centricon 30T
membrane), and washing with phosphate buffer. The initial clear
microconcentrate (0.9 ml) contained 21.4 Boeh U/ml, giving only 30%

recovery from the PM-10 concentrate (Step 7, Table 3.8). The micro-filtrates were found to contain <1% of the amylase activity. The microconcentrate was used for nmr studies with G_4-amylase, discussed in Chapter 4.

Table 3.9. Purification V Summary of Maltotetraohydrolase from Na Culture B.

Step		Vol ml	Activity Boeh U/ml	Protein mg/ml	S.A. Boeh U/mg
1.	Culture supernatant	695	1.52	0.89	1.7
2.	Hollow fiber filtrate	680	0.89	0.37	2.4
3.	UF (G10T) concentrate	36	14.66	0.67	21.8
4b.	AmSO$_4$ supernatant	48	1.63		
5.	´Salt-conc´ supernatant	205	1.28	0.26	4.9
6.	Supernatant from 35000xg	192	0.79	0.21	3.8
8.	Supernatant, microconc.	2	8.3		

Step numbers correspond to Table 3.8: 1. Culture supernatant after centrifugation at 2075xg; 2. Hollow fiber (0.1 micron) filtrate including 50 ml buffer wash; 3. Hollow fiber filtrate after concentration by ultrafiltration, freezing, thawing, and centrifugation at 38000xg; 4b. 45% AmSO$_4$ solution, diluted, and centrifuged.
5. Hollow fiber concentrate + buffer-NaCl solution after centrifugation at 2520xg; 6. Salt-conc supernatant after freezing, thawing, and centrifugation at 35000xg; 8. Supernatant from gel-concentrate, after microfiltration.

The hollow fiber filtrate (660 ml) from Step 2 (Tables 3.8 &
3.9) was concentrated by ultrafiltration (G10T) and the slightly
turbid concentrate (38 ml) found to contain 11.04 Boeh U/ml, 71%
recovery.   After freezing and thawing, the concentrate (36 ml) was
centrifuged at 38000xg and the clear supernatant (36 ml) found to
contain 14.66 Boeh U/ml (Step 3, Tables 3.8 & 3.9), 90% recovery from
the hollow fiber filtrate.   The freeze-thaw-centrifugation process
apparently removed G_4-amylase inhibitor.

The addition of ammonium sulfate (45% sat) gave a turbid
solution from which precipitate was not removed by centrifugation at
35000xg.   Further concentration by ultrafiltration (PM-10) and centri-
fugation of the concentrate (8.5 ml) at 30000xg still would not preci-
pitate flocculent material found in the viscous solution.   The PM-10
filtrate (26 ml) was acidic (pH 6.0), had little amylase activity
(0.02 Boeh U/ml), and 14 mg/ml carbohydrate.

Dilution of the ammonium sulfate concentrate (8.5 ml) with
ammonium sulfate solution (45% sat, Step 4b) gave a mixture in which
precipitate began to settle but was incompletely removed by centrifu-
gation. (The high viscosity, perhaps due to polysaccharide, probably
interfered with sedimentation of the amylase; dilution could permit
removal of the G_4-amylase.)   Ammonium sulfate solution was added to
give a final 6-fold dilution of the concentrate however little amylase
activity was removed by centrifugation at 28000xg.   The precipitate
did not dissolve in buffer nor did it appear to inhibit amylase acti-
vity.   The supernatant contained 4.3 mg/ml carbohydrate, some floccu-
lent material, and most of the amylase activity (1.63 Boeh U/ml, 79
Boeh Units of amylase).   This was, however, only 15% recovery from the

G10T concentrate (before addition of ammonium sulfate) or 7% of the activity found in the original culture supernatant. The pH of the supernatant, pH 6.5, and storage for 2 months may have contributed to the loss of activity. The supernatant was used for G_4-amylase studies discussed in 4.3.2.2 (Hydrolysis of Blue Amylose).

Maltotetraohydrolase was produced in Na Culture B along with considerable slime which interfered with purification of the G_4-amylase. Most of the amylase was retained in the hollow fiber concentrate with the slime and cells. Amylase was dissociated from the slime and cells by NaCl (>0.5 M) and by a freeze-thaw process; centrifugation removed insoluble materials. The clear supernatant was readily concentrated by ultrafiltration and precipitated by 45% ammonium sulfate. The G_4-amylase had to be extracted from the gel-like ammonium sulfate precipitate, giving only 30% recovery from the ammonium sulfate step.

Slime appeared to also interfere with purification of the maltotetraohydrolase found in the hollow fiber filtrate. The freeze-thaw-centrifugation process removed G_4-amylase inhibitor; however, addition of ammonium sulfate to 45% saturation gave a viscous solution from which little G_4-amylase could be removed by centrifugation.

3.2.2.3.   <u>Purification VI</u>. Na Culture C was harvested after 20 h of growth, at which time the differential rate of amylase synthesis had just decreased and the culture was probably in early stationary growth phase. Amylase activity was at maximum concentration in the culture (2.2.6.3, Fig. 2.12) and there appeared to be little slime. A sample from the culture supernatant showed it

to contain 32 mg/ml carbohydrate and 0.89 Boeh U/ml of amylase activity. The pH of the culture was 6.0, a pH at which G_4-amylase is reported to be unstable in the absence of calcium ion (13).

The culture was rapidly cooled and brought to pH 6.9 by the addition of saturated calcium hydroxide solution. In addition to neutralizing the culture solution and possibly stabilizing the amylase, the calcium hydroxide could act as a flocculent for cell precipitation (the usual method is to use calcium chloride (25)) as well as remove slime by forming a matrix between acid polysaccharides and the divalent calcium ion. The capsular polysaccharides found in slime are usually high molecular weight acidic polysaccharides (31) although none were identified by Dellweg *et al* (24) in the extracellular polysaccharide of *Pseudomonas stutzeri*.

The calcium hydroxide sedimented considerable white solid in the culture and after centrifugation at 24000x*g* (Step 2, Table 3.10) gave a clear supernatant of high specific amylase activity (Table 3.11). The supernatant (pH 6.5), contained 28 mg/ml carbohydrate; a sample showed 45% loss of amylase activity on freezing. The neutralization step gave a loss of only 16% of the amylase activity. Little carbohydrate was removed from the solution (total carbohydrate: Step 1. Harvest, 27 g; Step 2. Neutralization, 26 g). Analysis of the hard, sticky pellet indicated that only 2% of the amylase was precipitated with the pellet.

During concentration of approximately 550 ml of the supernatant (Step 3, Table 3.10) a clear gel formed on the ultrafiltration membrane. The pH of the concentrate was 7.0 --although neutral, there

Table 3.10. Sequence for Purification VI of Maltotetraohydrolase from Na Culture C.
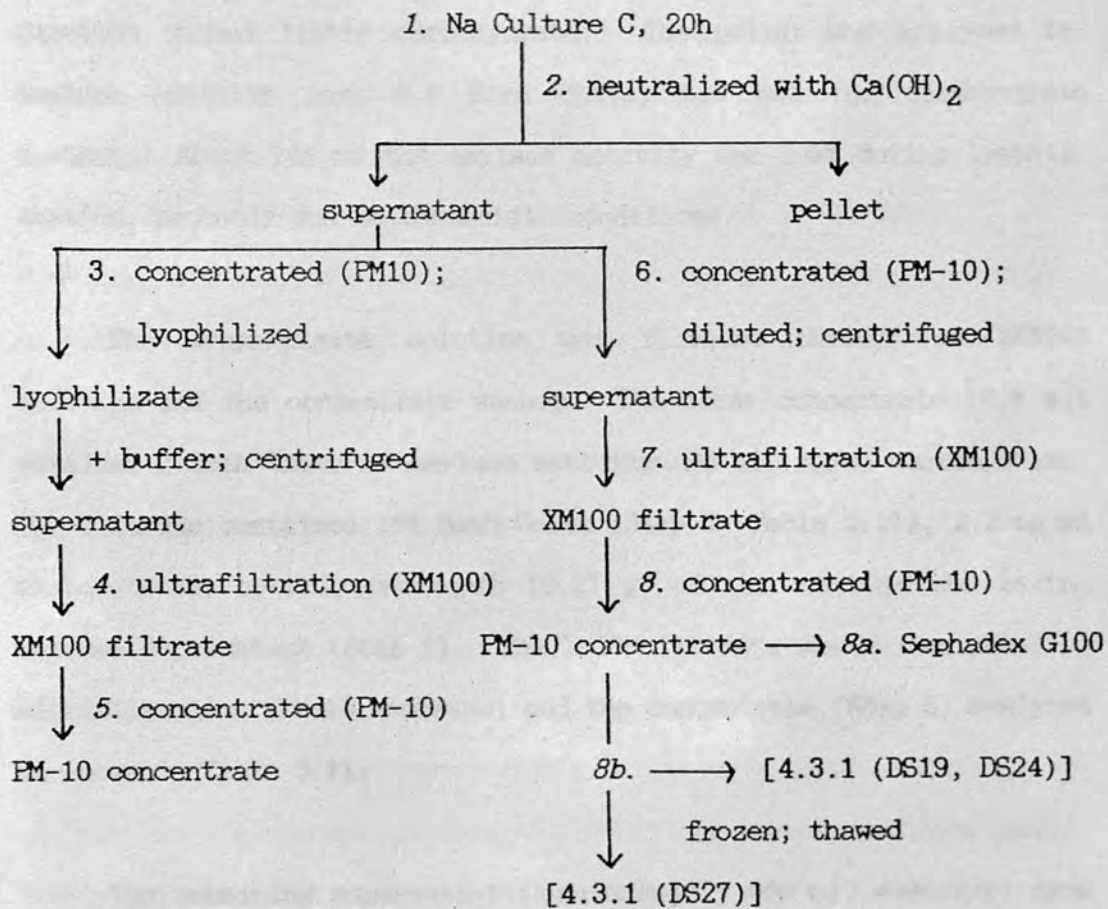
1. Na Culture C, 20h

2. neutralized with $Ca(OH)_2$

supernatant   pellet

3. concentrated (PM10);   *6.* concentrated (PM-10);
lyophilized   diluted; centrifuged

lyophilizate   supernatant

+ buffer; centrifuged   *7.* ultrafiltration (XM100)

supernatant   XM100 filtrate

4. ultrafiltration (XM100)   *8.* concentrated (PM-10)

XM100 filtrate   PM-10 concentrate ⟶ *8a.* Sephadex G100

5. concentrated (PM-10)

PM-10 concentrate   *8b.* ⟶ [4.3.1 (DS19, DS24)]

                               frozen; thawed

                               [4.3.1 (DS27)]

Numbers in italics refer to steps in the purification procedures.
Numbers in brackets refer to G_4-amylase studies discussed in Chap. 4.

was probably little buffering capacity since most of the phosphate buffer remaining from the culture medium would have precipitated as calcium phosphate. The ultraconcentrate was further concentrated by freeze-drying. The pH of the lyophilizate in glycerophosphate buffer was pH 5; 100 mM phosphate buffer was immediately added to bring the

pH to 7.0.   The lyophilizate solution (Step 3, Tables 3.10 and 3.11) contained flocculent solid which was removed by centrifugation.   The flocculent solid may have been calcium phosphate and/or an insoluble slime polysaccharide since analysis of the supernatant after centri- fugation showed little carbohydrate.   The pellet was analyzed for amylase activity (net 0.6 Boeh Units) but not for carbohydrate content.   About 74% of the amylase activity was lost during lyophil- ization, probably due to the acidic conditions.

The lyophilizate solution was filtered through an XM100A membrane and the concentrate washed.   The clear concentrate (8.8 ml) retained 2 Boeh Units of amylase activity and >10 mg/ml carbohydrate. The filtrate contained 104 Boeh Units (Step 4, Table 3.11), 2.2 mg/ml carbohydrate, or only one-tenth (0.27 g) of the carbohydrate in the culture supernatant (Step 1).   The XM100 filtrate was concentrated by ultrafiltration (PM-10 membrane) and the concentrate (Step 5) analyzed as shown in Table 3.11.

The remaining supernatant (approximately 350 ml, ≈39% v/v) from Step 2 (neutralization with calcium hydroxide) was also partially con- centrated by ultrafiltration (Step 6, Table 3.10).   The concentrate formed a gel on the UF membrane; the concentrate was removed with the gel and the apparatus washed with phosphate buffer, subsequently a flocculent suspension formed.   Centrifugation at 27000xg did not remove the flocculent substance and the mixture was analyzed with the suspension (Step 6, Table 3.11).   The mixture retained 36% of the amylase activity from the neutralization Step 2.

Table 3.11. Purification VI Summary of Maltotetraohydrolase from Na Culture C.

Step	Vol ml	Activity Boeh U/ml	Protein mg/ml	S.A. Boeh U/mg
1. Culture, supernatant sample	867	0.89	0.0095	93
2. Neutralization with Ca(OH)$_2$	907	0.71	0.0042	169
3. Lyophilizate solution	93	1.06	0.0090	118
4. XM100 filtrate	122	0.85	0.0077	111
5. PM-10 concentrate	10.5	9.37	ND	--
6. Step 2, concentrated, diluted	478	0.48	0.0044	109
7. XM100 filtrate	457	0.49	0.0038	129
8b.PM-10 concentrate	8.1	16.50	0.0475	347

Steps correspond to numbers in Table 3.10: 1. Sample from Culture C at 20h; 2. After neutralization and centrifugation; 3. After concentration by ultrafiltration and lyophilization, addition of buffer, and centrifugation at 27000x*g*; 4. Ultrafiltration using a XM100A membrane; 5. Concentration by ultrafiltration using a PM-10 membrane; ND, not determined; 6. Solution from neutralization Step 2 was partially concentrated, the resulting gel and solution were diluted with phosphate buffer with the formation of a suspension; 7. Ultrafiltrate from Step 6; 8. Concentration by ultrafiltration of 65% v/v of XM100 filtrate from Step 7.

Ultrafiltration through an XM100 membrane (very slow when compared with ultrafiltration of the lyophilizate solution, Step 4) increased the specific activity of the amylase in the filtrate (Step 7, Table 3.11). The filtrate contained 19.4 mg/ml carbohydrate (8.9 g), 34% of the carbohydrate found in the supernatant after neutralization, indicating that little carbohydrate had been retained by the XM100 membrane.

The XM100 filtrate was divided into two portions and each portion concentrated by ultrafiltration (Step 8, Table 3.10). After concentration of one portion (160 ml), the concentrate (4.5 ml, 7.4 Boeh U/ml) was applied to a Sephadex G-100 column (Step 8a). No uv absorbing peaks were detected in the initial 50 fractions collected, however the pooled 120 ml contained 0.18 Boeh U/ml, 63% of the activity applied to the column. The remaining XM100 filtrate (295 ml) was concentrated by ultrafiltration (PM-10 membrane) and washed with phosphate buffer. The concentrate (Step 8b, Tables 3.10 & 3.11, 90% recovery) was used for amylase studies (4.3.1, nmr Hydrolyses IV and IX) and the remainder frozen. After thawing the concentrate retained 13.8 Boeh U/ml and was used for further amylase studies (4.3.1, nmr Hydrolysis V).

Maltotetraohydrolase was produced in Na Culture C along with a gel-forming substance. The addition of calcium hydroxide to the acidic culture solution precipitated the cells and neutralized the solution, but removed little of the carbohydrate. The carbohydrate was not retained by the XM100 membrane (Step 7, 87% found in the ultrafiltrate) unless the solution had been lyophilized, then the carbohydrate was retained and little carbohydrate was observed in the

ultrafiltrate (Step 4). Little amylase activity was lost during the calcium hydroxide neutralization step (84% recovery). Lyophilization of the solution, which would have had little/no buffer capacity, gave only 25% recovery in an acidic lyophilizate. Once lyophilized 100% of the amylase activity was recovered in the XM100 and PM-10 ultrafiltration steps, the solution which had not been frozen showed 10% loss in activity during each ultrafiltration step.

3.2.3. Summary.

Maltotetraohydrolase from two types of media was partially purified for use in G_4-amylase studies described in Chapter 4. The G_4-amylase from Trypticase I cultures showed considerable loss in activity (>50%) during some of the first stages in purification, with the greatest losses in those steps which took longer. It is probable that loss in activity was due to the presence of protease. In Purification I where slime was not evident, a substance, perhaps protease, was coprecipitated with G_4-amylase which caused a rapid loss in activity upon dissolution of the precipitate. Once amylase solutions had been filtered through an XM100 ultrafiltration membrane, there was little loss in activity (III, IV, VI) during storage or in subsequent purification steps. If protease or other G_4-amylase inactivating substance was retained by the XM100 membrane it did not inactivate G_4-amylase stored 1.8 years in slime concentrate (Purification III). It has been suggested that slime layers may protect enzymes from proteolytic hydrolysis (27).

More G_4-amylase was recovered from Na cultures, as shown in Table 3.12 (Total Units recovered; some, for example in Purification VI, were divided into two parts and the net recovery would be VI,5 + VI,8). The highest specific activity was obtained in III,8 and

Table 3.12. Summary of Yields and Activities of Maltotetraohydrolase from Six Batch Cultures.

Purification, Step	Initial[1] Units/ml	Recovery Total Units	Recovery %	Specific Activity Units/mg
I,4	0.08[t]	5.5[t]	26%	1.5
II,4av	0.25[D]	77.[D]	7%	7.8
III,4	0.34[t]	236.[B]	42%	0.6
,8 & 10*		82.[B]	14%	> 2000.
IV,4	0.71[B]	186.[B]	44%	44.4
V,3	1.52[B]	528.[B]	50%	21.8
,4b		79.[B]	7%	
,6		151.[B]	14%	3.8
,8		17.[B]	2%	
VI,4	0.89[B]	104.[B]	14%	111
,5		98.[B]	13%	
,8		134.[B]	17%	347

This summary includes the last step for which the specific activity was measured and, where reasonable, the last step in G4-amylase preparations for studies in Chapter 4.

Purification I-III were from Trypticase I cultures, Purifications IV-VI were from Na cultures.

Purifications II, III, V, and VI were divided into two parts and recovery in V, for example, would include V,4b + V,8; av, average;

* is an average of Steps 8 and 10, recovery calculated from 1.6 g lyophilizate in III,4 and S.A. is estimated from the previous step.

[1] Initial activity in culture supernatant.

[t] turbid. U/ml, turbidimetric assay; [D] DNS U/ml, dinitrosalicylate assay; [B] Boeh U/ml, Boehringer assay. Relationship between assays (Chapter 5.6.2.): 2.9 turbid U : 1 DNS U : 3.8 Boeh U.

Recovery calculated on total Units in the culture supernatant.

III,10, after affinity chromatography. Only Robyt and Ackerman (7) have reported a higher specific activity although other groups (9-13) also attempted to purify G_4-amylase. In Purifications IV and VI, high specific activities were obtained although the Na culture supernatants had only been concentrated and filtered. (The lower specific activity in Purification IV is believed to partly result from denaturation due to acidic conditions.) The specific activity in Purification VI was higher than that reported by Dellweg *et al* (9, 10) and Sakano *et al* (12).

The greatest loss in activity occurred during the hollow fiber filtration purification step (<31%), when the acidic conditions may have contributed to the loss in activity. The most effective method for removal of cells, with little loss in amylase activity, was by addition of calcium hydroxide. This step also gave a 1.8-fold increase in the specific activity of amylase.

As also reported by others (9, 24), purification was frequently hindered by the presence of slime. The slime polysaccharide (24) formed a gel upon concentration, reversibly binding and in effect concentrating and purifying G_4-amylase. Gelation depends on many factors (66) and in purifications here, did not always occur in a predictable pattern. Most of the slime carbohydrate was retained by an XM100 membrane (III, IV and VI-Step 4), but was also found in some XM100 ultrafiltrates (III, VI-Step 7). A freezing-thawing-centrifugation process removed much but not all of the carbohydrate. After lyophilization, all of the interfering carbohydrate could be removed, however it was important to have suitable buffer during lyophilization in order to maintain the pH and avoid loss in amylase activity.

Insoluble material removed after freezing or lyophilization did not bind G_4-amylase but appeared to contain amylase inhibitor. Insoluble material precipitated by acetone and believed to be slime polysaccharide did bind G_4-amylase and required repeated extractions to remove the amylase.

Precipitation by ammonium sulfate purified G_4-amylase by as much as 100 times but gave only 15-50% recovery of the amylase. The low recovery was similar to that reported by others (7, 9, 12) but a higher purification factor was obtained here. The greatest loss in activity occurred when the ammonium sulfate concentration was increased above 41% (Purification I). When slime polysaccharide was present during ammonium sulfate precipitation, then G_4-amylase either did not precipitate (VI) or was trapped in a gel. Acetone precipitation also gave low recovery of amylase activity (34-39%), and less than a 2-fold purification.

G_4-amylase was stable to freezing and lyophilization when at neutral pH. After thawing, amylase activity was frequently found to be greater and retrograded polysaccharide could be removed by centrifugation.

Sephadex G-100 specifically binds G_4-amylase (9, 12) and can be utilized for affinity chromatography. Amylase solutions which had been lyophilized and from which most of the slime carbohydrate had probably been removed were purified to a high specific activity (Purification III) by chromatography on Sephadex G-100. Chromatography of other amylase solutions was unsuccessful and, similar to the one described in Step 8a, Purification VI, were not adsorbed by the Sephadex.

In these solutions carbohydrate was probably present with the G_4-amylase applied to the column and eluted with other uv absorbing material, as did 25-36% of the G_4-amylase as shown in Figures 3.1 and 3.2.

Several Sephadex column chromatographies were not reported because bacterial growth was later observed in the fractions and/or on the column.   Throughout the purifications, contamination from *P. stutzeri* was a recurring problem (a problem alluded to by Robyt and Ackerman (7)) and suspect solutions, including one purification not reported, were discarded.   As a precaution many solutions were stored frozen or as lyophilizates, although some solutions (e.g., in Purifications II and III) were stored for extended periods with no indication of bacterial growth.   Buffer containing the antimicrobial agent, chlorhexidene (Buffers D and E), inhibited growth of *P. stutzeri* and was used as eluent for the chromatographies shown in Figures 3.1 and 3.2.

3.3.   Experimental. Unless otherwise specified, purification procedures were manipulated in the cold room at 4-9°C, or else in an ice bath.   Methods and materials are detailed in Chapter 5.

3.3.1. From Cultures in Trypticase I Medium.

3.3.1.1.   Purification I.   Purification of Small Scale Culture.   Each culture in the five flasks from the small scale culture (described in 2.3.1.) was analyzed for bacterial integrity by petri smear and for amylase activity by the turbidimetric assay. Amylase activity was measured by the turbidimetric assay using the substrate soluble starch in 10 mM Buffer B.   Protein concentration was

determined by the Folin Lowry Assay. Samples were centrifuged on the MSE bench centrifuge set in the cold room (4°C).

Step I,1. The five cultures were pooled (220 ml) and centrifuged for 1 h at 2520x*g*, 1-13°C.

Step I,2. Four drops of n-octanol were added as antifoam agent to the clear supernatant which was then concentrated by ultrafiltration using a PM-10 (Amicon) membrane. The pH of the UF concentrate was adjusted from 9.0 to 7.3 with 3 M HCl.

Step I,3. During the addition of ammonium sulfate to 25% saturation the pH was maintained between pH 6.9 and 7.7. The precipitate was removed by centrifugation at 2500x*g* (MSE bench, 3800 rpm, 25 min) and and the process was repeated two times with the addition of ammonium sulfate to 25% w/v (41% sat.) and 30% w/v (48% sat.). Buffer B (10 mM glycerophosphate containing 5 mM calcium chloride, pH 7) was added to each precipitate for analysis of amylase activity.

Step I,4. The precipitate-solutions were combined and precipitated with acetone (70% v/v), stored 3 days, then centrifuged at 4600x*g* (MSE bench, 5200 rpm) for 20 min. Amylase was extracted four times by centrifuging the insoluble acetone precipitate in 2.0 ml Buffer B. The amylase solution was stored at 8°C, the amylase activity measured by the DNS assay after 1 week storage and again after 3 weeks (when it was used for the preparation of maltotetraose as described in 4.3.3.2.)

3.3.1.2. Purification II. Purification of Large Scale Culture. Amylase activity was determined by the DNS assay at 25°C unless otherwise specified (expressed as U/ml) and/or the turbidimetric assay (expressed as turbid. U/ml). Protein concentration was determined by the Folin Lowry Assay.

Step II,1. A large scale culture grown in twelve flasks containing Trypticase I medium (described in 2.3.1.) was pooled. The combined 4540 ml of culture was centrifuged in the MSE 6L centrifuge for 1.5 h at 2075x*g*. The cloudy supernatant and loose sediment were stored 24 h, then centrifuged on the MSE 18 centrifuge for 1 h at 8000 rpm (10000 x*g*), 4°C.

Step II,2. The supernatant was concentrated by ultrafiltration using a ChemLab GT-10 membrane. The 10.6-fold concentration took 5 days and required the addition of several ml of n-octanol to eliminate foaming during concentration. The pH of the concentrate was adjusted from 8.5 to 7.3 with 3.3 ml of 2M HCl.

Step II,3a. 25% Saturation. Ammonium sulfate was added slowly to the UF concentrate at 2°C; the pH was maintained at pH 7.0 ± 0.2 with dilute HCl. At 25% saturation a portion of the dark, viscous solution was centrifuged on the MSE bench centrifuge for 25 min at 3500x*g* (4500 rpm), 2°C. Clear supernatant (85 ml) was decanted from gel (some gel was floating but most was in the bottom half of centrifuge tubes) and both the gel and supernatant were analyzed by the turbidimetric assay. Filtration of the gel under pressure clogged the filter paper, however liquid could be filtered by mechanically disrupting the gel. The gel and the remaining 25% saturated solution were placed in a ChemLab C400 ultrafiltration cell fitted with Whatmann glass fiber filter paper and filtered at atmospheric pressure with constant stirring for 2 days. The gel continued to filter without stirring, and the filtrate was collected for 7 weeks.

Step II,3b. 48% Saturation. The gel supernatant (85 ml) and 248 ml of gel filtrate (collected over 17 days) were combined and ammonium sulfate added to give approximately 48% saturation. A portion of the turbid solution (355 ml) was centrifuged on the MSE

bench centrifuge at 3600xg(4600 rpm) for 25 min at 2°C. The precipitate from one of the centrifuge tubes was dissolved in 3.0 ml Buffer B for analysis by the turbidimetric assay. The supernatant was added to the remaining solution, then divided into two bottles for centrifugation in the MSE 18 centrifuge at 17500 xg (10500 rpm, 20 min, 5°C). To the precipitate in one bottle 15 ml Buffer B was added and then the bottle rinsed with an additional 2 ml Buffer B. To the precipitate in the other bottle was added 15 ml PIPES buffer (10 mM PIPES, containing 5 mM $CaCl_2$, pH 7.0). Each precipitate-solution was vigorously mixed and centrifuged in the CamLab centrifuge. This extraction was repeated two more times, with 8.0 ml of the respective buffer added in the second extraction, and 1.0 ml added in the third extraction. The first two PIPES supernatants (8 ml and 15 ml) were combined, as were the first two Buffer B supernatants and the amylase activity of both determined by the DNS assay at 25°C and at 37°C. At each step solutions were analyzed for amylase activity using the turbidimetric assay.

Step II,4. The amylase in Buffer B was precipitated by 70% v/v acetone at 2°C. Amylase was extracted from the acetone precipitate by adding 1.0 ml of Buffer B, vortexing to mix, and centrifuging in the CamLab bench centrifuge. This was repeated two more times and the three supernatants pooled and used for preparation of maltotetraose described in 4.3.3.3. The amylase in PIPES buffer was stored for 1 month before it was precipitated by 70% acetone, and extracted into 10 mM Buffer A (Buffer B without $CaCl_2$) three times using 3.5, 1.0, and 1.6 ml buffer. The combined supernatants were used for preparation of maltotetraose as described in 4.3.3.4.

3.3.1.3. <u>Purification III</u>. Amylase activity was assayed in Steps 1 and 2 by the turbidimetric assay, or DNS assay at 37°C and by the Boehringer assay in Steps 3-9. Protein concentration was determined by the Folin Lowry assay in Steps 1-4 and by the Bradford Extramicro Assay in Steps 5-10. Total carbohydrate was measured by the phenol sulfuric acid method (5.8.2).

Step III,1. Amylase from cell cultures and purification stages of the above Purification II were pooled. Included were the remaining inocula and two subculture flasks used for the large scale culture (2.3.1.2). Bacterial integrity was checked by petri smear, and 78 ml of pooled culture was centrifuged in the MSE 18 at 23000x*g* (12000 rpm) for 15 min. Amylase samples from Steps 3 and 4 of Purification II, including 337 ml of supernatant from the 48% ammonium sulfate precipitation, were added to the culture supernatant to give a total volume of 457 ml.

Step III,2. The solution was concentrated by ultrafiltration using an Amicon XM100 membrane and analyzed by the turbidimetric assay. The viscous concentrate (24.5 ml) was filtered through a Whatmann No. 1 glass fiber filter and stored at 5-9°C for 1.8 y, then analyzed by the Boehringer assay. The concentrate was then centrifuged in the MSE 18 centrifuge at 38000x*g* (18000 rpm, 4°C, 1 h).

Step III,3a. The XM100 filtrate was concentrated by ultrafiltration using an Amicon PM-10 membrane. The filtrate was examined by tlc. The concentrate was stored at 5-9°C for 1.8 y; then further concentrated (*Step 3b*), again with the PM-10 membrane, and washed with 30 mM phosphate buffer, pH 7.2. After analysis the concentrate was frozen and stored at -20°C.

Step III,4. The concentrate from Step 3b was thawed overnight at 4°C, and centrifuged on the MSE bench centrifuge at 2520x*g*

(3850 rpm) for 15 min at 5°C. The supernatant was analyzed; then stored at 9°C and a portion used in 4.3.2.2.2. The remaining solution was freeze-dried.

Step III,5. Sterile water (5.0 ml) was added to 569.5 mg of the lyophilizate and the pH measured (7.0). The solution was centrifuged on an Europa 24M centrifuge for 30 min at 45000xg, 4°C. The clear, dark brown supernatant was frozen.

Step III,6. Water (5.0 ml) was added to another 690.8 mg of lyophilizate from Step 4 and the solution filtered (Oxoid ´Nuflow´ cellulose acetate filter, 0.22 micron) and washed with 3.0 ml 50 mM Buffer E (imidazole-HCl buffer containing 0.002% Hibitane, pH 7.0). The 8.0 ml was concentrated by ultrafiltration (PM-10 membrane) and washed with an additional 3.0 ml Buffer E.

Step III,7. The concentrate (3.0 ml) was applied to the Sephadex G-100 column described in 5.6.3.4 (2.6 cm x 6.9 cm, eluent 50 mM Buffer E). The effluent was monitored by 280 nm, fractions of 40 drops/testtube were collected and every eighth (or more) fraction assayed by the Boehringer method. The eluent was changed to Buffer E containing 1 M NaCl after fraction 49, and then to 30 mM phosphate buffer containing 2 M NaCl after fraction 64.

Step III,8. Fractions 31-80 were pooled, concentrated to 4.7 ml by ultrafiltration using a PM-10 membrane, and the product specificity determined as described in 5.6.1 (as well as amylase activity and protein concentration).

Step III,9. The solution from Step 5 was thawed and filtered through a Millipore 0.22 micron membrane. The filtrate was applied to the Sephadex G-100 affinity column from Step 7 which had been previously washed with 500 ml of 20 mM Buffer D (imidazole-HCl buffer containing 0.002% chlorhexidene, pH 7.0). Fractions (2.4 ml) were

collected at a flow rate of 28 ml/h. After fraction 34, 66 ml of 50 mM Buffer D containing 700.0 mg lyophilizate containing oligosaccharide from Experiment III in 4.3.3.4 (estimated to be 9 mM maltotetraose) was applied to the column. Fractions were analyzed by tlc as well as the Boehringer assay.

Step III,10. Fractions 31-58 were pooled, and concentrated (by ultrafiltration using a PM-10 membrane) and washed with water; then with 50 mM Buffer D.

3.3.2. From Cultures in Na Medium. Amylase activity was determined by the Boehringer assay (5.6.2.4). Reducing sugar was determined by the alkaline ferricyanide method (5.8.3.2). Protein was determined by the Folin Lowry method (5.8.1.1) in Purification IV and Purification V and by the Bradford Extramicro method (5.8.1.2) in Purification VI.

3.3.2.1. Purification IV. *Step IV,1.* A portion of Na Culture A (2.2.6.1, shown in Fig. 2.10) was removed at 29 h by siphoning culture into a sterile centrifuge bottle cooled in an icebath. The culture was centrifuged at 4°C, 2075x*g* (Mistral 6L) for 30 min.

Step IV,2. The supernatant was sterilized by hollow fiber filtration (Amicon HIMP-01, 2 h at 11-15 psi), the filtrate analyzed for amylase activity and frozen. The cells (determined by Gram's stain) in the filtration apparatus were washed with 150 ml of 30 mM phosphate buffer, pH 7.0 and the wash analyzed for amylase activity.

Step IV,3. The hollow fiber filtrate was thawed and 2M KOH added to bring the pH to pH 7.0. Total carbohydrate, reducing sugar, protein and amylase activity were measured. The solution was filtered

by ultrafiltration Amicon XM100A membrane), the XM100 concentrate and filtrate analyzed and the concentrate washed with 30 ml of 30 mM phosphate buffer, pH 7.2, containing 2M NaCl.

Step IV,4. The XM100 filtrate and the wash were combined and the amylase concentrated by ultrafiltration (Amicon PM-10 membrane). The PM-10 concentrate ($\approx$20 ml) was washed four times with a total of 105 ml of 30 mM phosphate buffer. The concentrate (1.5 ml) was freeze-dried and used for G_4-amylase studies in 4.3.1 (DS15); the remaining 19.5 ml was lyophilized, found to weigh 183 mg, and used in studies in 4.3.2.1 (Experiment II) and in 4.3.1 (DS34).

3.3.2.2. <u>Purification V</u>. *Step V,1.* The Na Culture B described in 2.2.6.2 (Figure 2.11) was harvested at 69h and centrifuged at 2075xg, 4°C (Mistral 6L) for 30 min.

Step V,2. The slightly turbid supernatant was filtered through 0.1 micron hollow fibers (Amicon, HIMP-01) at 10-15 psi for 1.5h until no additional supernatant was filtered although some supernatant remained in the holding flask. Phosphate buffer (100 ml, 30 mM) was added to the concentrate and 50 ml filtrate was slowly added to the first filtrate; finally 30 mM phosphate buffer containing 1.5M NaCl was backflushed across the membrane in order to remove the cells and slime from the hollow fibers. The hollow fiber concentrate with salt-wash from the backflush was stored at 9°C. On disassembling the hollow fiber apparatus was found to be clogged with a golden slime.

Step V,3. The hollow fiber filtrate was concentrated to 22.5 ml by ultrafiltration using a ChemLab G10T membrane and the UF cell rinsed with 15.5 ml of 100mM phosphate buffer which was then added to the concentrate. After analysis for amylase activity the concentrate was frozen. After thawing the concentrate was centrifuged for 15 min

at 38000xg, 4°C (in the MSE 18 at 18000 rpm). The amylase activity and the protein concentration of the clear supernatant (36 ml) was determined.

Step V,4a. Ammonium sulfate (9.4 g) was added to 35.5 ml of G10T concentrate from Step V,3 and left stirring overnight. The turbid solution was centrifuged at 35000xg, 10°C (17000 rpm, MSE 18) for 1 h. No precipitate was removed. The mixture was slowly (6h at 60 psi) concentrated by ultrafiltration (PM-10 membrane) to 8.5 ml. The filtrate (26.0 ml) was analyzed for amylase activity (0.020 Boeh U/ml), total carbohydrate (87.5 μmole ge/ml) and pH (=6, pH paper pH 6-8 range). The PM-10 concentrate was centrifuged at 30000xg, 4°C (19500 rpm, Sorvall RC-5B) for 40 min, however no precipitate was removed.

Step V,4b. The ammonium sulfate solution (11.5 ml) from Step 4a was diluted by the addition of 23 ml of 45% (saturated) ammonium sulfate in 30 mM phosphate buffer. Flocculent material remained suspended after centrifugation at 28000xg for 40 min; another 20 ml of 45% ammonium sulfate in buffer was added and the mixture centrifuged a second time. A loose precipitate (about 3 cm^3) formed which did not appear to dissolve when 10 μl of suspension was added to 3 ml of 100 mM phosphate buffer. The precipitate was analyzed for the presence of inhibitor by measurement of amylase activity, 10-fold dilution and reassaying. The two activities were within experimental error (7%) and inhibitor was judged not to be present. The supernatant was analyzed and used for G$_4$-amylase studies. After storage for five weeks at 9°C the supernatant was examined for amylase activity and by Gram's stain and by petri smear.

Step V,5. The hollow fiber concentrate with salt-wash (buffer containing 1.5M NaCl) from Step V,2 was stored at 9°C for 5 days, a

sample removed and examined by Gram's stain, centrifuged in the CamLab bench centrifuge and assayed for amylase activity. The remaining solution was centrifuged at 2520x*g*, 4°C (MSE bench centrifuge, 3850 rpm) for 20 min. The cloudy supernatant (205 ml) was frozen, thawed, analyzed for amylase activity, amylase specificity, and protein concentration, and a portion (7 ml) used for amylase studies.

Step V,6. The supernatant from Step V,5 was centrifuged at higher *g* force, at 35000x*g*, 4°C (MSE 18, 17000 rpm) for 80 min, and the clear supernatant decanted. The pellet was mixed with 13.5 ml of 100 mM phosphate buffer and analyzed for amylase activity.

Step V,7. The supernatant from Step V,6 was concentrated by ultrafiltration (PM-10) to dryness (accidentally), 30 mM phosphate buffer was added to the UF cell and the solution (concentrate) analyzed for amylase activity. Ammonium sulfate (3.87 g) was added to the 15 ml of PM-10 concentrate, left overnight, then centrifuged at 35000x*g*, 10°C (MSE 18, 17000 rpm) for 1 h. The pellet was removed and suspended in a minimal amount of 50% (sat) ammonium sulfate.

Step V,8. All centrifugation was done in the CamLab bench centrifuge set in the cold room (4°C). The suspension was diluted with approximately 2 volumes of 30 mM phosphate buffer, mixed, and stored at 9°C overnight. The solution was centrifuged and supernatant removed from a gel. The supernatant was centrifuged for >30 min in a Centricon T30 (Amicon) microfiltration apparatus. The T30 concentrate formed a gel upon centrifugation from which supernatant could be removed by pipette. The process was repeated 4 times: the supernatant was washed with buffer and reconcentrated in the microfiltration apparatus; the concentrate centrifuged; supernatant removed from gel by pipette. The fourth time the supernatant was divided in two, the process repeated and the final supernatant freeze-dried. The samples

were used in nmr studies described in 4.3.1 (DS16 and DS18).

                          3.3.2.3. <u>Purification VI</u>. The pH was measured in Steps 1 and 2 using a Pye Unicam pH meter (Model 290) and in Steps 3-8 using narrow range pH paper. *Step VI,1*. The Na Culture C described in 2.2.6.3 (Figure 2.12) was harvested at 20h by setting the fermenter cell in an ice bath placed over a magnetic stirrer. Stirring was continued until the temperature dropped to 12°C.

Step VI,2. The culture solution was brought to pH 6.91 by the dropwise addition of saturated calcium hydroxide solution. The pH of the stirred culture sitting in an icebath was monitored continuously. The mixture was centrifuged at 24000xg, 4°C (MSE 18, 13000 rpm) for 30 min. The pH of the supernatant was estimated with pH paper to be pH 6.5. The firm pellet was broken up into 50 ml of 100 mM phosphate buffer and a portion analyzed for amylase activity.

Step VI,3. During concentration of the supernatant from Step VI,2 the ultrafiltration membrane (PM-10) broke. Approximately 550 ml of the supernatant was further concentrated by lyophilization and the remaining supernatant (approximately 350 ml) purified as described in Steps VI,6-8. To the damp lyophilizate 10 ml of 10mM Buffer A (glycerophosphate buffer, pH 7.0) was added, the pH was estimated with pH paper (pH ≈5), 20 ml of 100 mM phosphate buffer was added and the pH again estimated with pH paper (pH 6.5-7.0). A sample (2.0 ml) was removed and centrifuged (CamLab) and the supernatant and pellet analyzed (pellet + 2.0 ml phosphate buffer, centrifuged) for amylase activity. The remaining lyophilizate solution (43.5 ml) was further diluted with 50 ml of 30 mM phosphate buffer and stored 3 days at 9°C before centrifugation for 20 min at 27000xg, 4°C (MSE 18, 13000 rpm).

Step VI,4. The (lyophilizate) supernatant from Step VI,3 was

rapidly filtered by ultrafiltration (XM100A), the concentrate washed with 30 ml of 20 mM Buffer B (glycerophosphate buffer containing 5mM $CaCl_2$), and the filtrate and the concentrate analyzed for amylase activity.

Step VI,5. The XM100A filtrate was rapidly concentrated by ultrafiltration (<25 psi) using a PM-10 membrane, and the PM-10 concentrate washed with 20 ml of 20mM Buffer B. The filtrate (107 ml) and the concentrate (2.6 ml) in 7.9 ml Buffer B were analyzed.

Step VI,6. The remaining supernatant ($\approx$350 ml) from Step VI,2 was partially concentrated by ultrafiltration until a gel formed on the PM-10 membrane. The flask and UF cell were washed with 119 ml of water and the solution storage for 3 days after which time there was some flocculent material which was not removed by centrifugation at 27000xg 20 min, MSE 18, 13000 rpm).

Step VI,7. The supernatant from Step VI,6 was slowly (about 7 hours at 40 psi) filtered through a XM100A ultrafiltration membrane and the concentrate washed with 35 ml.

Step VI,8. The XM100 filtrate was concentrated by ultrafiltration (PM-10 membrane, <30 psi) in two portions. *Step VI,8a.* Filtrate (160 ml) was concentrated and washed with buffer in the same UF cell used in Step VI,5 (and stored 1 week at 9°C with phosphate buffer). The turbid concentrate was centrifuged on the CamLab bench centrifuge and the supernatant (4.5 ml) assayed (7.4 Boeh U/ml) and found to have lost 65% of the amylase activity. The concentrate was applied to a Sephadex G-100 column prepared as described in 5.6.3.4 except that the eluent was 30 mM phosphate buffer and the uv detector was not used. Fractions 17-100 were measured spectrophotometrically at 280 nm (Perkin Elmer 555), however all absorbances except fraction 94 were <0.01. The amylase activity measured on fractions 40 and 94 was 0.009 Boeh

U/ml, and 0.176 Boeh U/ml on pooled fractions 1-50 (120 ml). The solutions were discarded.

Step VI,8b. The remaining XM100 filtrate (300 ml) was concentrated and washed with 30 mM phosphate buffer in a sterilized UF cell. The pH of the filtrate and of the concentrate was measured with pH paper (both pH 7.0). The concentrate (8.1 ml) was centrifuged (CamLab) with part (43% v/v) of the concentrate lost in the centrifuge (copper lined centrifuge tube holder, 3% activity remaining). The supernatant (4.6 ml) from the remaining centrifuge tube was divided. Part of the supernatant (1.83 ml) was concentrated and exchanged for nmr studies as described in 4.3.1 (DS19 and DS24). The remainder was frozen (3.0 ml) and used for nmr studies described in 4.3.1 (DS27).

Chapter 4. Studies of the Action of Maltotetraohydrolase.

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CHAPTER 4. STUDIES OF THE ACTION OF MALTOTETRAOHYDROLASE.

4.1. Introduction.

Maltotetraohydrolase [E.C. 3.2.1.60] hydrolyzes starch to produce maltotetraose and high molecular weight limit dextrin (7, 9, 10), an action similar to that of the exoamylase β-amylase which produces β-maltose and a high molecular weight limit dextrin. An exomechanism was suggested and reaffirmed when radioactively labeled maltodextrins were consistently hydrolyzed at the fourth glycosidic bond from the non-reducing terminal (7, 11). At high concentrations of G_4-amylase or after extended hydrolysis, however, maltotetraose is reported (7, 12, 13) to be degraded to glucose and maltotriose or to maltose. During the initial stages of starch hydrolysis only G_4 is detected, but after extended hydrolysis G_3-G_1 are also found and, at least in one instance, their amount exceeded that of G_4 (10). In studies reported here of hydrolysis of starch at high concentrations of G_4-amylase, two stages of hydrolysis were also observed. However, G_4 was always the major product from hydrolysis of starch with G_3 and G_2 as minor products, even though G_4-amylase remained active in the hydrolysis mixtures.

The limit dextrins from G_4-amylolysis (G_4-limit dextrin) of amylopectin and shellfish glycogen were prepared by Robyt and Ackerman (7) and found to be 48% and 62%, respectively, of the polysaccharide weight. These percentage weights are similar to the percentage weight generally reported for β-limit dextrins from amylopectin (44-48%) and glycogen (58%-62%) (46). The G_4-limit dextrin from wheat amylopectin has been prepared here (as in ref 7, precipitated by 2 volumes of

ethanol) and the G_4-limit dextrin then examined by nmr and by HPLC. In addition, the G_4-amylase hydrolysis mixture from potato amylopectin was examined although the limit dextrin was not purified. Schmidt and John (10) that found 75% of soluble starch was converted to G_4, G_3, G_2, and G_1. Chromatography of the soluble starch hydrolyzate on Bio-Gel P-2 (fractionation range 100-1800 daltons) showed only dextrin at the void volume and no other oligomers $>G_4$. They concluded that G_4-amylase must have some endo-activity because of the high conversion of soluble starch to oligosaccharide.

Maltotetraohydrolase has been shown to display other characteristics of endo-activity. Cross-linked blue starch, reported to be specific for endo-activity, is hydrolyzed by G_4-amylase (9, 10, 14) and was first used to follow the purification of G_4-amylase. In addition, G_4-amylase shows limited hydrolysis of pullulan (an α-glucan composed of maltotriosyl units and some 5% maltotetraosyl units linked by α-1,6-bonds), the hydrolysis occurring at the maltotetraosyl units (10). The properties of the endo-activity have been further investigated in studies described in this chapter, by examination of the extent of G_4-amylolysis and of the products formed from hydrolysis of α-1,4-glucans. The possibility that endo-activity arises from a contaminating α-amylase has been examined by measuring the activities of different preparation of G_4-amylase towards blue amylose and β-limit dextrin. The results from endo-amylolysis of β-limit dextrin and of amylopectin, and the properties of G_4-limit dextrins are then discussed in terms of the cluster models of amylopectin.

This unique amylase appears to have properties of both exo-amylases and of endo-amylases. As discussed in Chapter 1.3.1, the

majority of other exo-amylases, β-amylase (found mainly in plants) and glucoamylase (found mainly in fungal extracts), hydrolyze starch to yield products in the β-anomeric configuration. The hydrolysis products from G_4-amylolysis, however, apparently contain an excess of the α-anomer, as determined from the small change in optical rotation after treatment with alkali (12, 14). Two other exo-amylases, maltotriohydrolase and maltohexaohydrolase (like G_4-amylase, also from bacterial sources) have similarly been shown to produce α-maltodextrins (14). All three have been described as exo-α-amylases. In order to investigate the anomeric specificity of G_4-amylase, the hydrolysis of amylopectin and β-limit dextrin was followed by ^{1}H-nmr spectroscopy. Nmr has also been used to investigate the linkage specificity of G_4-amylase.

4.2. Results and Discussion.

4.2.1. High Field Proton NMR Studies. The G_4-amylolysis of soluble starch and of several amylopectins and their β-limit dextrins was followed by ^{1}H-nmr spectroscopy and the results discussed in terms of the specificity of G_4-amylase and the structure of amylopectins.

4.2.1.1. <u>Mutarotation Measurements</u>. In order to determine the initial anomeric configuration of amylolysis product it is necessary to have appropriate experimental conditions. In aqueous solutions, mutarotation of reducing sugar is a dynamic process in which the anomers are in constant interconversion. For D-glucose and its oligomers, the predominant species are the α-and β-pyranose forms (67). Mutarotation is initiated as soon as product is formed by

amylolysis; hence, in order to determine the initial anomer produced by amylolysis the rate of amylolysis must exceed the rate of mutarotation. An additional constraint, that of time, is imposed in nmr studies; so that the conditions affecting mutarotation have to be sufficiently controlled to overcome the constraints imposed by the amylase and by the time taken to record an nmr spectrum.

A reasonable nmr spectrum of polysaccharide was obtained after 32 scans of temperature equilibrated solution. A minimum of 3 min was required to reach equilibrium temperature. The 32 scans were accumulated over a minimum of 1.82 min (see 4.2.1.3). For amylolysis studies, additional time was required for addition of the amylase to the substrate solution, mixing and insertion into the spectrometer. The hydrolysis reaction began on the addition of amylase and it was at least 5 min before the first reasonable spectrum could be recorded. It was therefore important to have conditions in which the mutarotation rate was sufficiently slow that the hydrolysis products would not be at anomeric equilibrium by the time in the first nmr spectrum was recorded.

The limitations of nmr spectroscopy required at least 4% conversion of a 2% w/v polysaccharide solution (about 0.8 mg/ml G_4) in order for a measurable proton signal to be recorded in the first spectrum. This required an enzyme concentration of at least 1.5 Units/ml, although concentrations up to 10-fold higher were generally used.

If a mixture of anomers is present in the first recorded spectrum, but not at equilibrium concentration, then the anomer present at zero time can be calculated if the rate of mutarotation is known.

Although the rate of mutarotation of maltotetraose was not determined, the mutarotation constants for maltose and maltotriose in several buffers were measured, as well as the the equilibrium concentrations of the α- and β-anomers. It was thus possible to choose conditions in which mutarotation was slow and to determine the initial anomeric configuration of the amylolysis product.

The rate of mutarotation (anomerization) reflects deuterium isotope effects as well as acid-base catalysis, with the heavier isotope producing lower reaction rates and acid-base mixtures increasing reaction rates (68, 69). The mutarotation constant, k ($k = k_1 + k_2$, $\alpha \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \beta$), reflects the sum of the forward and reverse reaction rates of anomerization and does not depend on the anomer used for determination. From the mutarotation constant the half-life for equilibration of the anomers in solution can be determined. Optical rotations were measured in a jacketed cell using sugar concentrations of 4% w/v (see Experimental, 4.3.1.1). The mutarotation data are presented in Table 4.1.

The results in Table 4.1 are consistent with the results of others (68-71). Nicolle and Weisbuch (70) reported that the mutarotation rate of maltose was lower than that of glucose (1.5-1.8 times) and that the rate in D_2O was 3-fold lower (at 19°C). As shown in Table 4.1, the mutarotation rates were lower in the deuterium oxide solvent systems. And in D_2O, the rate for the longer oligomer, maltotriose, was considerably lower than the rate for maltose. In H_2O (with phosphate buffer), the rate for maltose was lower than the rate for glucose. These results suggest that the longer the oligomer, the lower the mutarotation rate will be, particularly in D_2O. (The rates

Table 4.1. Specific Rotations and Mutarotation Constants for 4%
            Solutions of Sugars at 37°C.

β-D-maltose monohydrate

Solvent	$[\alpha]_e$	$[\alpha]_o$	k (min^{1-})	$t_{1/2}$ (min)
H_2O	+129[a]	+112[a]	0.062	11.2
D_2O	+131[a]	+115[a]	0.037	18.5
H_2O, phosphate buffer[1], pH 7.0	+129[a]	+104[a]	0.493	1.41
H_2O, phosphate buffer[1], pH 7.0	+244[b]	+225[b]	0.499	1.39
D_2O, phosphate buffer[2], pH 7.0	+247[b]	+194[b]	0.217	3.18
H_2O, phosphate buffer[1], pH 6.6	+254[b]	+210[b]	0.487	1.42
D_2O, phosphate buffer[2], pH 6.6	+244[b]	+214[b]	0.193	3.58

α-D-glucose

Solvent	$[\alpha]_e$	$[\alpha]_o$	k (min^{1-})	$t_{1/2}$ (min)
H_2O, phosphate buffer[1], pH 7.0	+52[a]	+126[a]	0.529	1.31

α-D-maltotriose

Solvent	$[\alpha]_e$	$[\alpha]_o$	k (min^{1-})	$t_{1/2}$ (min)
H_2O	+160[a]	+164[a]	0.061	11.2
D_2O	+159[a]	+163[a]	0.021	33.0
D_2O, 18mM Imidazole-DCl, pD 7.2	+162[a]	+167[a]	0.030	22.9

$[\alpha]_e$, specific rotation at equilibrium;
$[\alpha]_o$, specific rotation of anomer;
mutarotation constant, $k = (k_1 + k_2) = 1/t \ln [(r_0 - r_e) / (r_t - r_e)]$;
half-life, $t_{1/2} = \ln 2/k$;
[a] , at 589 nm (Na, D line); [b] , at 436 nm (Hg).
[1] 56 mM phosphate buffer;
[2] 56 mM phosphate buffer at the given pH before being twice exchanged
with D_2O and diluted with D_2O.
pD = pH meter reading + 0.4.

of maltose and maltotriose in H_2O were similar; however it is probable that the rate for maltotriose is slightly high because the H_2O solvent was not degassed prior to determination of its k_{H_2O}.)

Acid-base mixtures catalyze anomerization (68, 69), increasing the mutarotation rate. For glucose, the rate of mutarotation is at a minimum between pH 3-7 and the rate increases rapidly outside this range (69). Optimum conditions for enzyme hydrolysis would, therefore, have minimal buffer, at slightly acidic pH, however G_4-amylase is not stable below pH 6.5 (12) (and its pH optimum is pH 8.0). Phosphate buffer at pH 7 was used in the G_4-amylase purification procedures and for determination of the mutarotation rate of maltose at pH 7.0 and 6.6. The mutarotation rate was slightly lower in the more acidic buffer but in both acid-base mixtures the rate was >5 times faster than in aqueous solvent. The mutarotation rates in phosphate buffer were sufficiently high that the half-life of the anomer was <4 min; consequently both anomers would be present in the first reasonable nmr spectrum which could be recorded. The conditions for determination of the anomeric configuration from amylolysis were optimized when buffer was minimal. In most of the nmr studies, only the buffers (phosphate and imidazole) already in the G_4-amylase preparations were present in the amylolysis solutions. The buffer concentrations used in the nmr studies are given in Table 4.2.

The specific rotations of glucose and maltodextrins up to G_8 have been reported (71). A comparison of chain lengths and the specific rotations of the equilibrium mixtures shows that as the length of the chain increases, the differences in the specific rotations at equilibrium decreases, such that oligomers longer than G_5

have specific rotations which differ by less than 5 degrees ($[\alpha]_D$ = +180° ± 5°). These oligomers have not been crystalized, but from the specific rotations of the α- and β-pyranoses of glucose and maltose, it appears that the difference between the specific rotation of the anomers and the specific rotation at equilibrium decreases as the chain length increases. The specific rotations, $[\alpha]_D$, in H_2O, reported by other groups (temperature gives minor differences in $[\alpha]_D$ (68)) are:

<u>   α-pyranose ----- equilibrium ---- β-pyranose:</u>   <u>Ref</u>.

D-glucose, +112° ------ +52.7° -------- +18.7°   (69)

D-maltose, +173° --- +129-136° ----- +109-118°   (71, 72)

D-maltotriose   --- +155-160° -----   (71, 73, 74)

The specific rotations for maltose and maltotriose, reported in Table 4.1, are similar to the rotations reported by others. The difference in specific rotations between, for example, the α-pyranose and the equilibrium mixture, decreases from a difference of 59.3° for glucose to about 40° for maltose. In addition the specific rotation of α-maltotriose was determined, as shown in Table 4.1, and found to be 164°, a difference of only 4° from the equilibrium rotation. This follows the pattern of a sharp decrease in the difference (of both the anomeric and the equilibrium specific rotations) in specific rotation between oligomers as the chain length increases.

Because there is only a small difference in specific rotation between the anomers of α-1,4-glucooligomers, the determination of the anomeric configuration from amylolysis is consequently difficult when determined by changes in optical rotation. Hence the anomeric rotation of α-maltotetraose probably differs little from the equilibrium rotation of maltotetraose. In addition, equilibration would

be expected to occur rapidly in the aqueous buffered solutions (glycerophosphate-calcium chloride, pH 6.5 and Tris-HCl, pH 7) which were used by others (12, 14) for determination of the anomeric configuration of the product from G_4-amylolysis. Changes in optical rotation after alkalinization of the G_4-amylolysis solution were small; however the changes were in a negative sense, indicating that the α-anomer was produced by G_4-amylase. ^{1}H-nmr spectroscopy was used here as an alternative method for determination of the anomeric configuration of the products from amylolysis.

The anomerizations of β-maltose and α-maltotriose in 56 mM phosphate buffer and in 81 mM imidazole-DCl buffer (4.3.1.3) were too rapid to determine the mutarotation rates from nmr studies. However, it was possible to establish the equilibrium concentration of the α-anomers (see 4.2.1.2). At 37°C, in D_2O exchanged buffers, the following were found:

α-maltose in 56 mM phosphate buffer, p´H 7.2   39%

α-maltotriose in 200 mM imidazole-DCl, p´H 7.35   42%

α-maltotriose in 81 mM Buffer D, p´H 6.2   40%

Buffer D: imidazole-DCl containing chlorhexidene

An equilibrium concentration of 38% has been reported for α-D-glucopyranose in D_2O at 31°C (69). It is the β-anomer which predominates, at 62%, making it particularly apparent in the nmr spectrum if the α-anomer is produced during amylolysis.

4.2.1.2. Chemical Shifts of Standards. ^{1}H-nmr spectroscopy differentiates between the two types of glycosidic linkages found in starch. The chemical shift of the anomeric protons of intrachain α-1,4-linkages is located at 5.4 ppm, and those of the

branch α-1,6-linkages at 5.0 ppm (75-78). The ratio of the α-1,4-linkages to α-1,6-linkages has been used to estimate the average chain length in amylopectin (76) and agrees well with the average chain lengths determined by other methods (46, 79). Because of the low solubility of starch and the broadness of the ^{1}H signals, spectra have usually been obtained at high temperatures and/or in DMSO. Under the conditions employed here and using high field (400 MHz) [1]H-nmr it has been possible to resolve the H-1 glycosidic signals in aqueous solutions at 37°C. Typical spectra are shown in Figure 4.2.

De Bruyn *et al* (77) have identified the chemical shifts for all of the proton resonances in nine glucobioses, including maltose and isomaltose; and Morris and Hall (78) have assigned the resonances of glucose, maltotriose, and Dextran T-10 as well as maltose. The following chemical shifts were used for identification of resonances distinguished in the spectra of the standards (maltose, maltotriose and starch) used for amylolysis studies:

			Ref.
α-1,4-glycosidic protons	(α-H1,4)	5.39-5.41 ppm	(1,2,3)
α-anomeric proton	(α-H1 red)	5.23-5.27 ppm	(1,2,3)
α-1,6-glycosidic protons	(α-H1,6)	4.98-5.00 ppm	(1,2,3)
β-anomeric proton	(β-H1 red)	4.64-4.68 ppm	(1,2,3)
H-4 terminal proton	(H4 term)	3.42 ppm	(1,2)
H-2 of the β-anomer	(β-H2)	3.25-3.28 ppm	(1,2) *

* except maltose, reported at 3.20 ppm (78); 1= ref 77; 2= ref 78; 3= ref 76.

Figure 4.1 shows the spectrum of maltotriose. Four signals were sufficiently resolved to permit integration. These were the proton

^{1}H-NMR spectrum (400MHz)
Maltotriose in
81 mM   imidazole buffer, pD 6.6
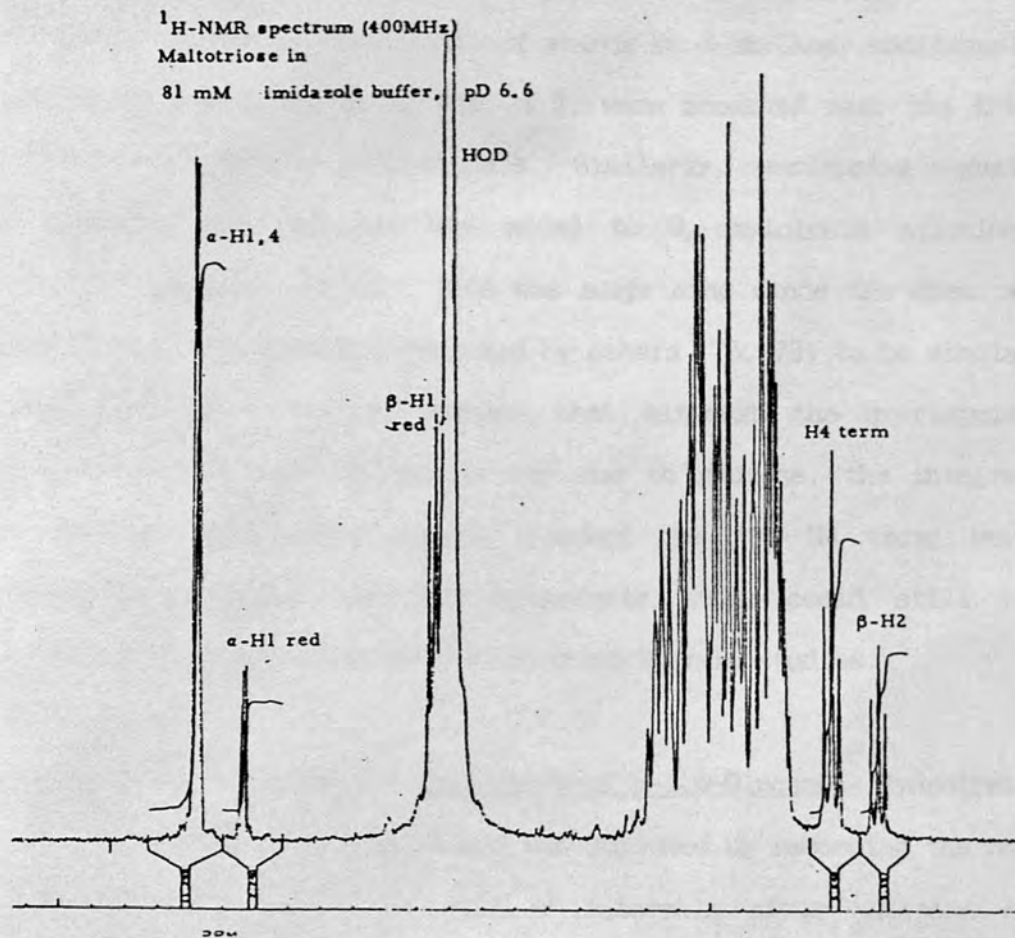
Fig. 4.1.   400 MHz ^{1}H-nmr spectrum of 2% maltotriose in 81 mM imidazole-DCl buffer, p´H 6.2, 37°C, after 24 h equilibration.

resonances from the α-1,4-glycosidic linkage (α-H1,4), the reducing α-anomer (α-H1 red), the H-4 on the non-reducing terminal glucosyl residue (H4 term), and the H-2 of the β-anomer (β-H2); present in the ratio of 2.00 : 0.40 : 1.01 : 0.59. As in most of the spectra, the β-anomeric proton was partially obscured by the HOD peak; however, since the H-2 proton of the β-anomer is shifted upfield from the other proton resonances, the β-anomer could be quantified from integration of the β-H2 signal.

In nmr studies of hydrolysis of starch by α-amylase, additional, overlapping signals, shown in Fig. 4.3, were observed near the β-H1 red, the H4 term and the β-H2 signals. Similarly, overlapping signals were observed when glucose was added to G_4-amylolysis solutions (4.3.1.4.2.c and 4.3.1.5.c). This was surprising since the chemical shifts for glucose have been reported by others (75, 78) to be similar to maltodextrins. It was decided that although the overlapping signals in α-amylolysis solutions may due to glucose, the integral from the two overlapping signals (except for the H4 term) were sufficiently resolved that the hydrolysis study could still be followed and is discussed with the other amylolysis studies.

4.2.1.3. <u>Amylolysis of α-1,4-Glucans</u>. Hydrolysis of the glycosidic linkages in starch was followed by recording the nmr spectra and ^{1}H integrals at regular intervals after addition of amylase to the polysaccharide solution. The nmr solutions and the amylolysis limits are summarized in Tables 4.2-4.4. In each study (Hydrolyses I-XII, described in 4.3.1.5-4.3.1.10) the spectrum of the substrate was first recorded (2-2.5% glucan in D_2O) at 37°C (except Hydrolysis I, at 40°C). Amylase was added directly into the substrate solution and spectra accumulated for 32 scans and recorded at regular intervals. The minimum pulse interval between scans gave a total accumulation time of 1.81 min (see 5.9.4.1. for correction due to partial saturation of the nmr signal). Longer pulse intervals gave accumulation times of 2.72 min (Hydrolyses V and VIII) and 3.79 min (Hydrolyses VII, X-XII) between spectra. No differences in the nmr results were attributed to differences in the length of the pulse interval.

Table 4.2. Summary of Solutions Used in NMR Studies.

Amylase, Source[1]	Activity[2]	Substrate[3]	Buffer[4]
I G_4– IV,4	8.2	1.6% soluble starch	77 mM[a], p´H 7.2
II G_4– V,8	14.8	2.0% soluble starch	30 mM[a], p´H 6.2
III G_4– V,8	15.5	<2.5% potato amylopectin	44 mM[a]
IV G_4– VI,8b	10.0	1.4% potato amylopectin	88 mM[b], p´H 7.4
V G_4– VI,8b	10.5	2.0% potato amylopectin	18 mM[c], p´H 6.8
+G_4– at 202 min	17.8	1.7% potato amylopectin	31 mM[c], p´H 6.7
VI β–	16.5	2.3% potato amylopectin	14 mM[b], p´H 7.4
VII β–	>10.	2.3% potato amylopectin	<7 mM[d], p´H 7.5
VIII α–	31	2.0% potato amylopectin	2 mM[b], p´H 8.2
+α– at 234 min	52	1.7% potato amylopectin	15 mM[b], p´H 6.9
IX G_4– VI,8b	6.4	1.8% wheat amylopectin	56 mM[b], p´H 7.4
X G_4– III,10	3.8	1.7% p.a. β–limit dextrin	15 mM[c], p´H 6.2
XI G_4– IV,4	8.7	2.5% p.a. β–limit dextrin	38 mM[e], p´H 6.2
XII G_4– III,10	2.1	2.1% w.a. β–limit dextrin	8 mM[c]

[1] G_4-amylase from purifications described in Chapter 3; β-amylase from sweet potato; α-amylase from *Bacillus amyloliquefaciens*.

[2] Boeh U/ml

[3] Substrate: p.a., potato amylopectin; w.a., wheat amylopectin.

[4] Buffer: D_2O exchanged buffer (Methods 5.9): p´H = pH meter reading; [a] phosphate buffer; [b] imidazole-DCl; [c] imidazole-DCl containing chlorhexidene diacetate; [d] <5 mM imidazole-DCl with <2 mM sodium acetate; [e] 38 mM phosphate buffer and 25 mM imidazole-DCl containing chlorhexidene.

Table 4.3. Summary I of Amylolysis Limits Observed by NMR.

	Amylase Boeh U/ml	Initial Stage (min), i	P_a / α-H1,6 i	ii
soluble starch				
I	G_4- 8.2	16-40	2.3	2.9
II	G_4- 14.8	30-140	6.2	ND
potato amylopectin				
III	G_4- 15.5	32-44	4.5	4.7
IV	G_4- 10.0	C, 200-770	b	b
V	G_4- 17.8	80-216	4.9	6.3
VI	β- 16.5	NL, (65)	>4.6	6.3
VII	β- >10	NL, (27)	>7.6	7.7
VIII	α- 52	NL, (268)	>6.7	12.4
wheat amylopectin				
IX	G_4- 6.4	NL, (250)	b	b
potato amylopectin β-limit dextrin				
X	G_4- 3.8	38-227	b	b
XI	G_4- 8.7	NL, (140)	>1.0	1.5
wheat amylopectin β-limit dextrin				
XII	G_4- 2.1	C, 444-864		1.0

b, the HOD partially obscured the α-H1,6 signal which was not integrated, calculations exclude the α-H1,6 integral; ND, not determined.

Total H1 ±Std. Dev: average sum of H1 integrals and standard. deviation relative to internal standard, see 5.9.4.4 for description.

i, Initial Stage in min: the time after hydrolysis when an amylolysis limit appeared to be reached; NL, no limit reached by () min;

C, continuous assessment, time during which a limit was observed.

ii, final hydrolysis limit: measured after 1000 min or as C limit.

P_a /α-H1,6 = reducing sugar produced/branch: P_a = α-anomer + β-anomer = α-H1 red + β-H2; α-H1,6 = branch glycosidic linkage proton.

Table 4.4. Summary II of Limits Observed in NMR Hydrolysis Studies.

Amylase		% Hydrolysis		Dextrin CL		Additional Analysis	
		i	ii	i	ii	tlc	HPLC
soluble starch							
I	G_4-	15	17	6	5		
II	G_4-	21	ND	4	ND		
potato amylopectin							
III	G_4-	19	24	6	1		
IV	G_4-	a	22	a	a	$\mathbf{G_4},G_3,G_2$	
V	G_4-	17	24	9	1	$\mathbf{G_4},G_3,G_2$	
VI	β-	>19	26	<16	11	$\mathbf{G_2}$	
VII	β-	>24	26	<16	15	$\mathbf{G_2}$	$\mathbf{G_2}$
VIII	α-	>34	45	<7	3	$G_3,\mathbf{G_2},G_1$	
wheat amylopectin							
IX	G_4-	>12	18	b	b	$\mathbf{G_4},G_3,G_2$	
potato amylopectin β-limit dextrin							
X	G_4-	14	20	b	b	$G_3,\mathbf{G_2},G_1$	$G_4?,G_3,\mathbf{G_2},G_1$
XI	G_4-	>9	14	<9	7.7	$G_3,\mathbf{G_2},G_1$	$G_4?,G_3,\mathbf{G_2},G_1$
wheat amylopectin β-limit dextrin							
XII	G_4-		11		6.4	$\mathbf{G_4},G_3,G_2,G_1$	$\mathbf{G_4},G_3,G_2,G_1$

Symbols as described in Tables 4.2 and 4.3: i, initial hydrolysis
  ´limit´; ii, final hydrolysis limit; b, the α-H1,6 signal was not
  integrated.

% Hydrolysis, percent all glycosidic bonds hydrolyzed:
  $100 * P_a/(\text{Total H1})$; Total H1 = P_a + α-H1,4 + α-H1,6; except for
  IV, IX & X, calculated as % α-1,4 bonds hydrolyzed.

CL, chain length of dextrin: $\{(\text{α-H1,4}) - nP_a + \text{α-H1,6}\}/\text{α-H1,6}$; where
  n= 1 for Hydrolyses VI-VIII, X & XI, n= 1.5 for XII, and n= 3 for
  Hydrolyses I-V and IX.

Additional analysis, oligosaccharides detected: bold, major product;
  normal type, minor products or approximately equal proportions.

In each study, the integrals for all H-1 signals (the β-H2 signal representing the β-H1 red signal) in a spectrum were added together to give the Total H1 (the calculation for Total H1, relative to internal standard, is defined in 5.9.4.4). Comparison of the Total H1s in different spectra within a specific hydrolysis study permitted evaluation of two parameters. (The Total H1 in different studies could not be compared since the amount of internal standard was different in each study.) First, the Total H1 should remain constant throughout amylolysis. If there was aggregation of carbohydrate, retrogradation, or if additional carbohydrate went into solution there would be a change in the Total H1 value. In each of the hydrolysis studies the Total H1 was found to remain constant. Second, the standard deviation of the Total H1 in a study could be calculated to give an indication of the quality of the spectra in the study. The Total H1 and standard deviation for each study were as follows:

Hydrolysis I, 17.2 $\pm$1.9	Hydrolysis VII, 4.7 $\pm$0.2
Hydrolysis II, 29.6 $\pm$4.2	Hydrolysis VIII, 2.0 $\pm$0.1
Hydrolysis III, 24.3 $\pm$2.6	Hydrolysis IX, 1.2 $\pm$0.0
Hydrolysis IV, 2.0 $\pm$0.1	Hydrolysis X, 4.0 $\pm$0.2
Hydrolysis V, 2.6 $\pm$0.1	Hydrolysis XI, 7.2 $\pm$0.7
Hydrolysis VI, 2.1 $\pm$0.1	Hydrolysis XII, 2.9 $\pm$0.1

From the kinetic data collected from changes in the proton integrals during amylolysis, it was possible to determine which glycosidic bonds were hydrolyzed (the anomeric specificity), the anomeric configuration of the major product (the product specificity), the extent of hydrolysis (amylolysis limit), and the average number of product molecules per branch. Each of these is discussed in Sections 4.2.1.3.1-4.2.1.3.6.

Examples of the spectra are shown in Figures 4.2-4.4. Figure 4.2 shows several spectra from G_4-amylolysis of potato amylopectin, and Figures 4.3 and 4.4 of α- and β-amylolysis of the same potato amylopectin preparation.

The spectra recorded at different times during an hydrolysis study could be compared to each other by relating the integrals to the constant integral of an internal standard. Any changes of the integrals between spectra could then be quantified. Tables 4.2-4.4 summarize the hydrolysis mixtures and the results from the hydrolysis studies. In Hydrolyses IV-XII, CH_3CN was included in the substrate solution as internal standard; in Hydrolyses I-III, the α-H1,6 signal was used as an internal standard.

4.2.1.3.1.   <u>Anomeric Configuration of the Product</u>. The anomeric configuration of the principal product was shown in most of the hydrolyses. An example is shown in Figure 4.2 in which G_4-amylase clearly produces the α-anomer. In the spectrum recorded at 11 min after the addition of G_4-amylase, no β-H2 signal could be detected. However the integrals showed that there were almost as many α-anomer protons as branch (α-H1,6) protons. By 125 min not only was the β-anomer present but it was the predominant anomer, representing 56% of the reducing sugar.

As shown in Fig. 4.5 the change in the amount of each of the anomers could be followed with time. In Hydrolysis V, the α-anomer was initially the major reducing sugar but when the rate of mutarotation exceeded the rate of amylolysis the proportion of anomers approached equilibrium concentration.

^{1}H-NMR spectrum (400MHz)

2.5% potato amylopectin in D_2O at 37C

a.

HOD

α-H1,4

α-H1,6

H4 term
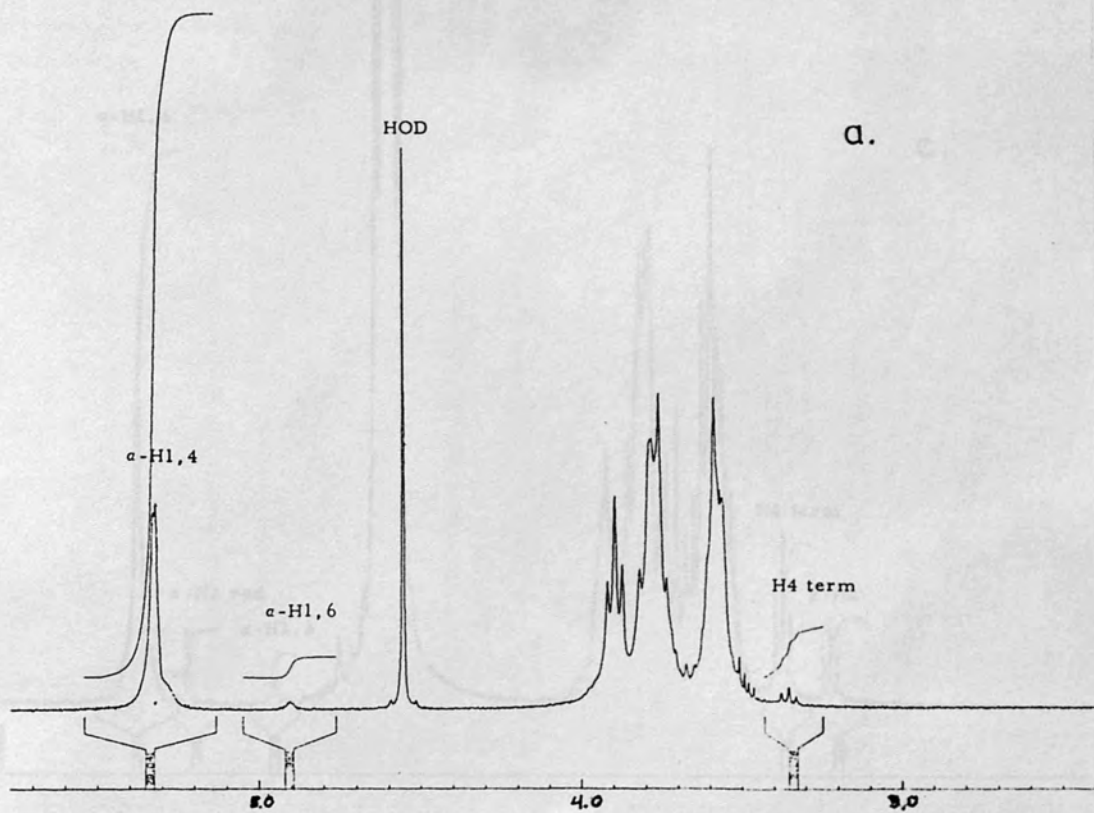
G_4-amylase

potato amylopectin

18 mM d-imidazole buffer, pD 7.2

11 min after mixing, 37C

b.

α-H1,4

α-H1 red

α-H1,6

H4 term
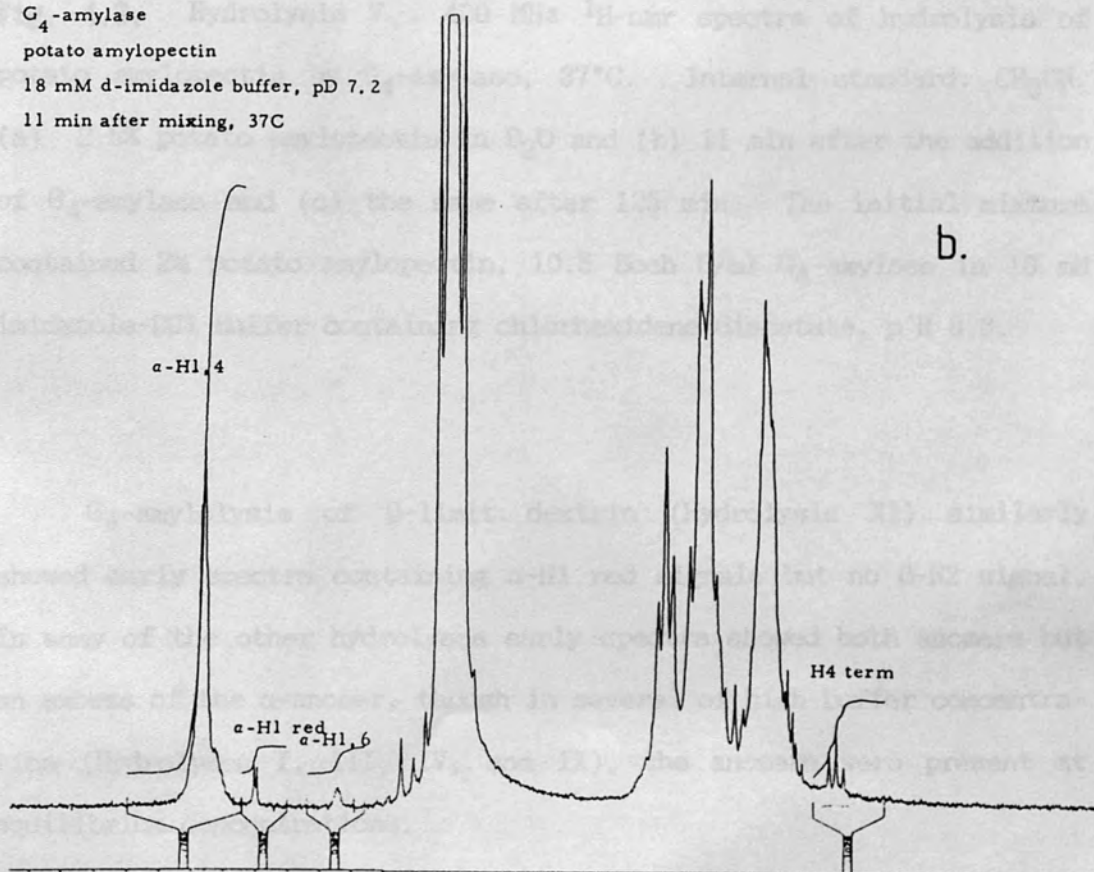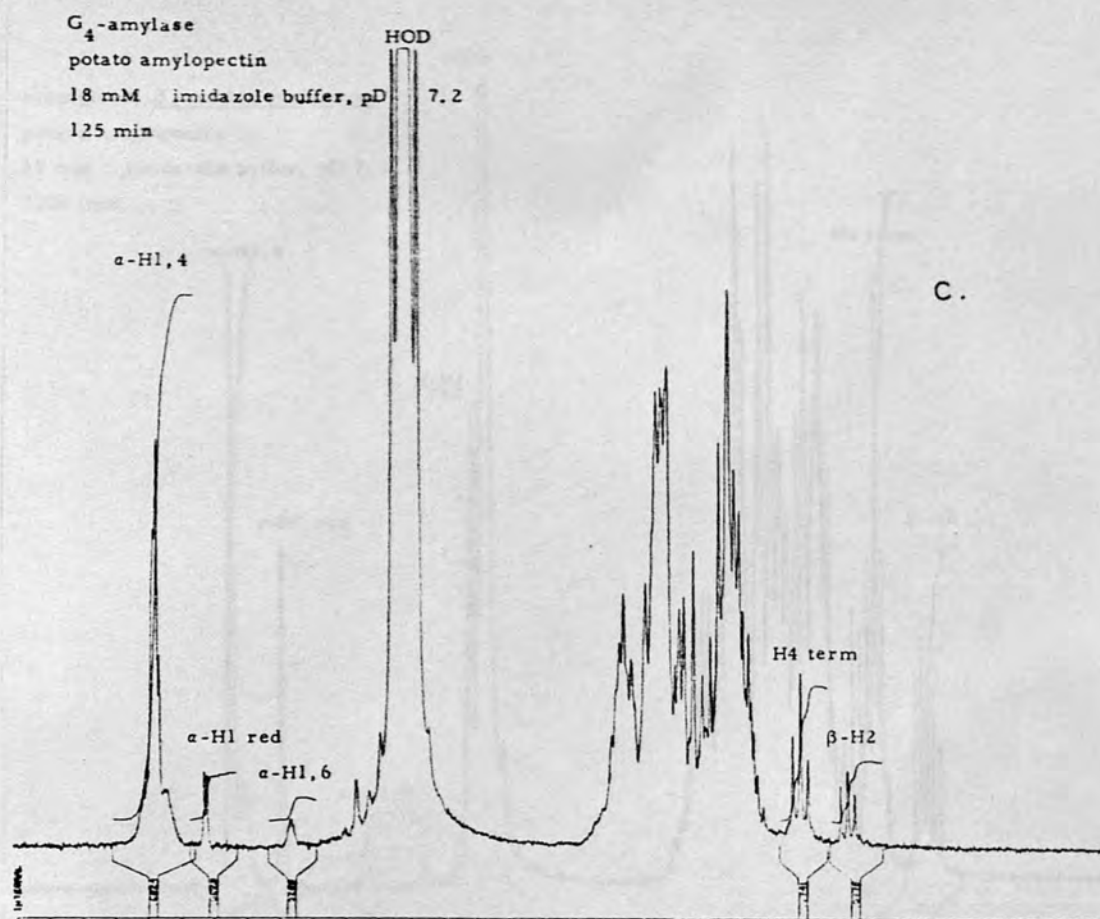
Fig. 4.2. Hydrolysis V. 400 MHz ^{1}H-nmr spectra of hydrolysis of potato amylopectin by G_4-amylase, 37°C. Internal standard: CH_3CN. (a) 2.5% potato amylopectin in D_2O and (b) 11 min after the addition of G_4-amylase and (c) the same after 125 min. The initial mixture contained 2% potato amylopectin, 10.5 Boeh U/ml G_4-amylase in 18 mM imidazole-DCl buffer containing chlorhexidene diacetate, p´H 6.8.

G_4-amylolysis of β-limit dextrin (Hydrolysis XI) similarly showed early spectra containing α-H1 red signals but no β-H2 signal. In many of the other hydrolyses early spectra showed both anomers but an excess of the α-anomer, though in several of high buffer concentration (Hydrolyses I, III, IV, and IX), the anomers were present at equilibrium concentrations.

α-amylase, B. amyloliquefaciens
potato amylopectin
15 mM imidazole buffer, pD 7.3
1200 min
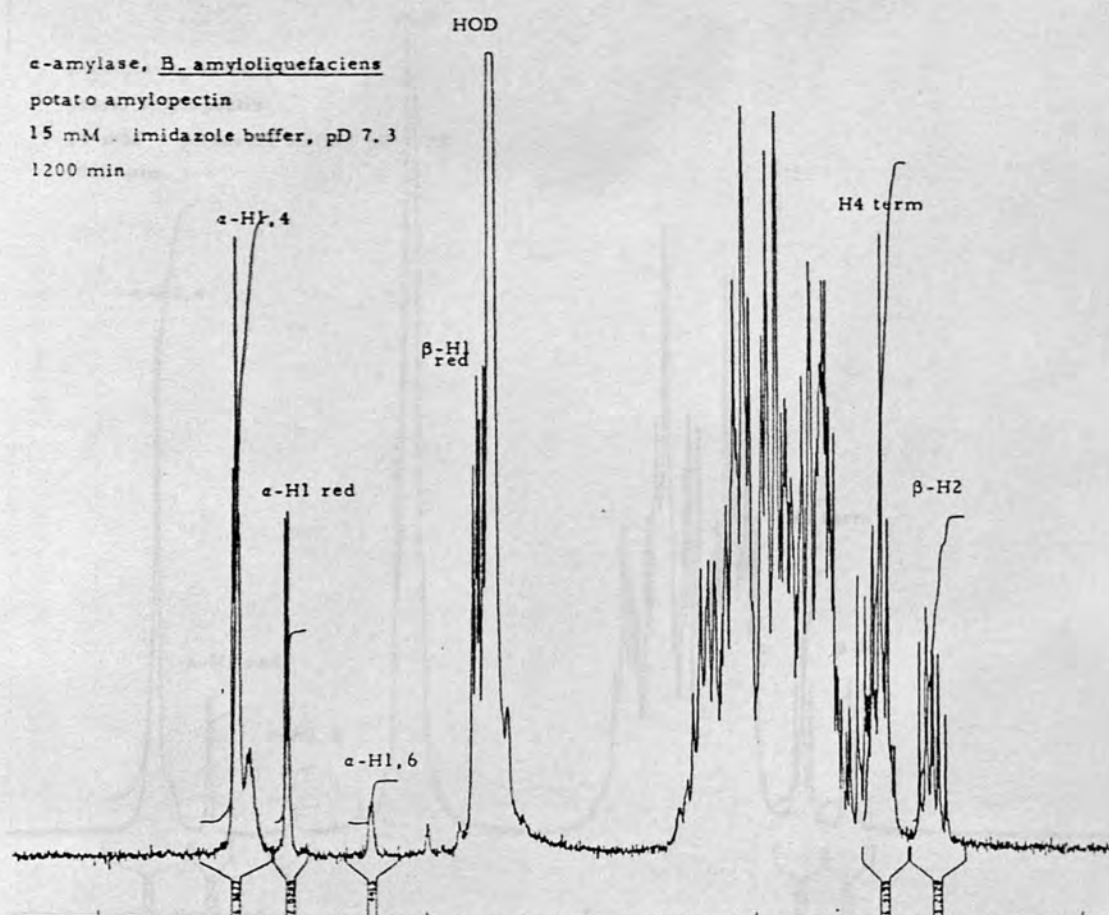
Fig. 4.3. Hydrolysis VIII. 400 MHz ^{1}H-nmr spectrum 1200 min after hydrolysis of potato amylopectin by α-amylase from *Bacillus amyloliquefaciens*, 37°C. Internal standard: CH_3CN. Hydrolysis of the potato amylopectin shown in Fig. 4.2. The mixture shown contained 1.7% potato amylopectin (initial concentration) and 52 Boeh U/ml α-amylase in 15 mM imidazole-DCl buffer, p´H 6.9.

The first spectra of amylolysis by α-amylase shows both anomers to be present but with the α-anomer initially in excess, rapidly decreasing from 59% at 7 min to an average equilibrium concentration of 40% from 34 min onwards. As shown in Fig. 4.3, the spectra were noticeably different from the spectra from G_4- and β-amylolysis. The
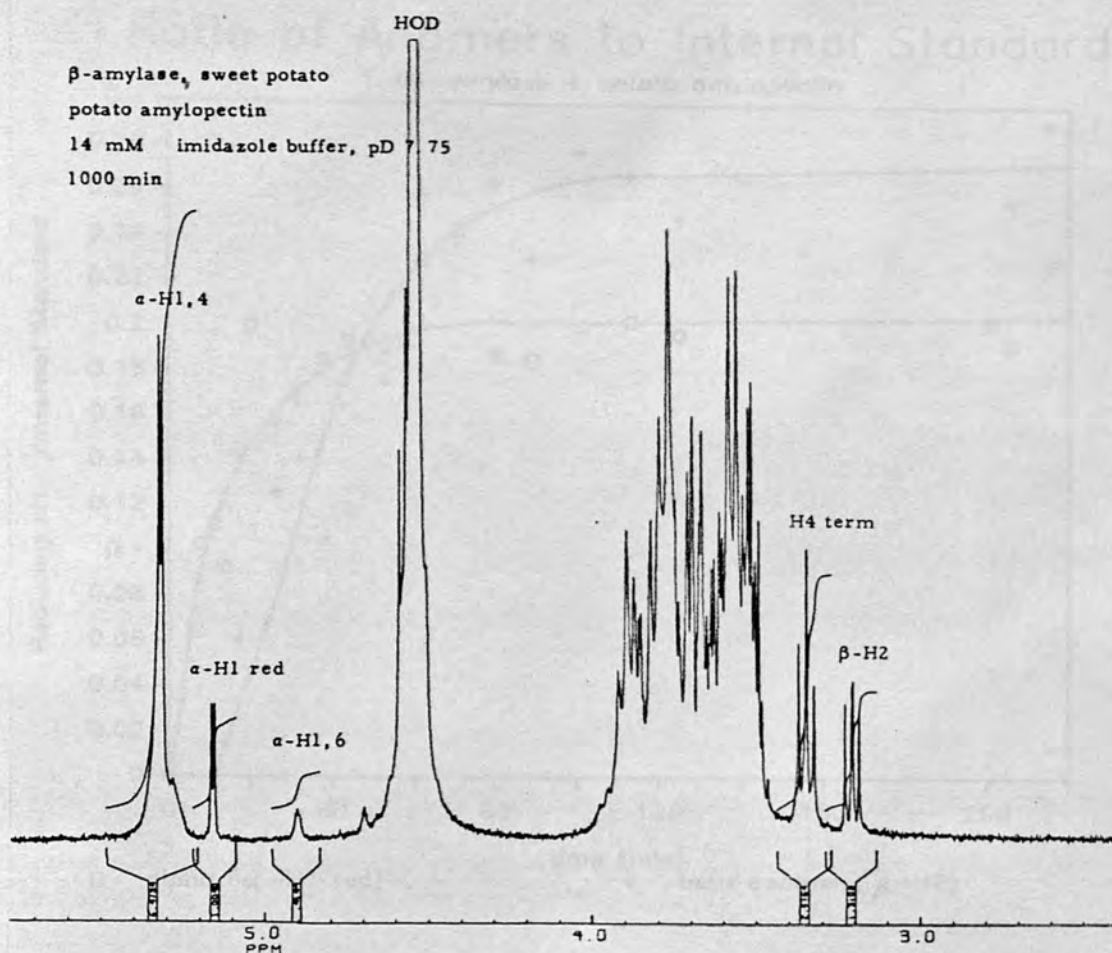
Fig. 4.4. Hydrolysis VI. 400 MHz ^{1}H-nmr spectrum 1000 min after hydrolysis of potato amylopectin by β-amylase from sweet potato, 37°C. Internal standard: CH_3CN. Hydrolysis of the potato amylopectin shown in Fig. 4.2. The initial mixture contained 2.3% potato amylopectin, 16.5 Boeh U/ml β-amylase in 14 mM imidazole-DCl buffer, p´H 7.4.

H4 term resonance was not resolved and could not be satisfactorily integrated. Both the β-H2 signal and the partially distinguishable β-H1 red signal show what appears to be two sets of overlapping signals, possibly also shown at the α-H1 red signal. Similar spectra were observed when glucose was added to the G_4-amylolysis mixtures in Hydrolyses IIc and IIIc (4.3.1.4.2 and 4.3.1.5.1). A significant
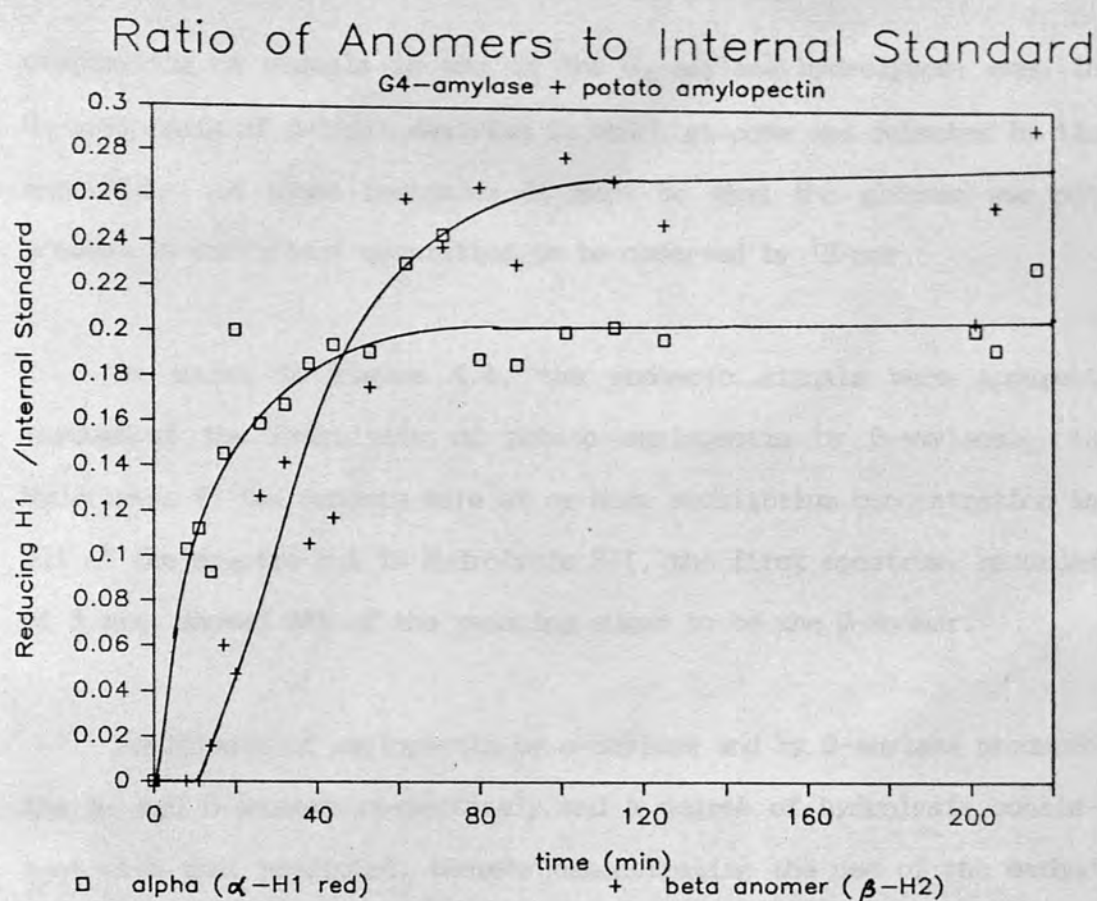
Fig. 4.5. Production of the α-anomer by G_4-amylolysis of potato amy-lopectin. Hydrolysis V, as in Fig. 4.2. The amount of the α-anomer (α-H1 red integral) and the subsequent production of the β-anomer (β-H2 integral) by mutarotation (the mutarotation rate constant for G_3 is given in Table 4.1), relative to the internal standard CH_3CN.

proportion of the α-amylolysis solution contained glucose (detected by tlc) and the overlapping signals were apparent in the first spectrum, recorded at 7 min when 22% of the glycosidic bonds had already been hydrolyzed (see 4.2.1.3.3 for further discussion of percent hydrolysis).

It should be noted that there was never any evidence of similar

overlapping of signals in any of the G_4-amylase hydrolyses, even in G_4-amylolysis of β-limit dextrins in which glucose was detected by tlc and HPLC. In those instances it must be that the glucose was not present in sufficient quantities to be observed by ^{1}H-nmr.

As shown in Figure 4.4, the anomeric signals were apparent throughout the hydrolysis of potato amylopectin by β-amylase. In Hydrolysis VI the anomers were at or near equilibrium concentration in all of the spectra but in Hydrolysis VII, the first spectrum, recorded at 5 min, showed 98% of the reducing sugar to be the β-anomer.

Amylolysis of amylopectin by α-amylase and by β-amylase produced the α- and β-anomers respectively and a degree of hydrolysis consistent with that predicted, thereby demonstrating the use of the method for studies of enzyme degradation of starch-type polysaccharides.

4.2.1.3.2.   Quantification of Product.
By standardizing the spectra to an internal standard, the integrals from different signals could be compared. The total product could be assessed from the sum of the α-H1 red signal and the β-H2 signal, P_a = α-anomer + β-anomer. This was routinely graphed as the amount of product relative to the internal standard. Of greater interest though was the amount of product produced per branch chain of the substrate. Because the small α-H1,6 signal gave a less accurate integral the error in the P_a/α-H1,6 would be greater, nevertheless, as shown in Fig. 4.6, a limit could be observed for the number of reducing sugar units produced per branch.

The total product could also be measured from the H4 term

integral. A new H4 term signal is produced each time a glycosidic bond is hydrolyzed and adds to the integral already present from the substrate. The number of non-reducing termini (H4 term) in the original starch substrate does not change and is equal to number of branches (α-H1,6), i.e. (H4 term)$_0$ at time zero = (α-H1,6)$_t$ at any time. (The α-H1,6 was shown to be constant in all of the hydrolyses by comparison to the internal standard and/or the total H1 signals. This could also be used to compare the quality of the spectra.) By subtracting the α-H1,6 integral (= H4 term$_0$) from the H4 term integral at time t, the product from hydrolysis is obtained, P_t = H4 term – α-H1,6. As shown in Fig. 4.6, P_t compares well with P_a, the total reducing sugar present.

From the plots of product vs time (as in Fig. 4.6) for each hydrolysis, it was possible to estimate the product limits for most of the hydrolyses. In most of the G_4-amylolysis studies there appeared to be a limit reached during the initial stages of hydrolysis. For example, in Hydrolysis V shown in Fig. 4.6, an average limit of 4.9 bonds were hydrolyzed per branch (P_a/α-H1,6) determined from the initial period measured from 80–216 min. A final spectrum of Hydrolysis V, at 1560 min, not shown in the figure, indicated additional hydrolysis and 6.3 reducing sugars per branch. This is discussed further in 4.2.1.3.4, in terms of average chain length. The spectra of most of the hydrolyses were recorded after 17 hours when it is probable that hydrolysis was essentially complete. Whereas maltotetraose is believed to be the exclusive product during the early stages of hydrolysis, other oligosaccharides can be detected after extended G_4-amylolysis.

Ratio of Product to Branch

G4—amylase + potato amylopectin
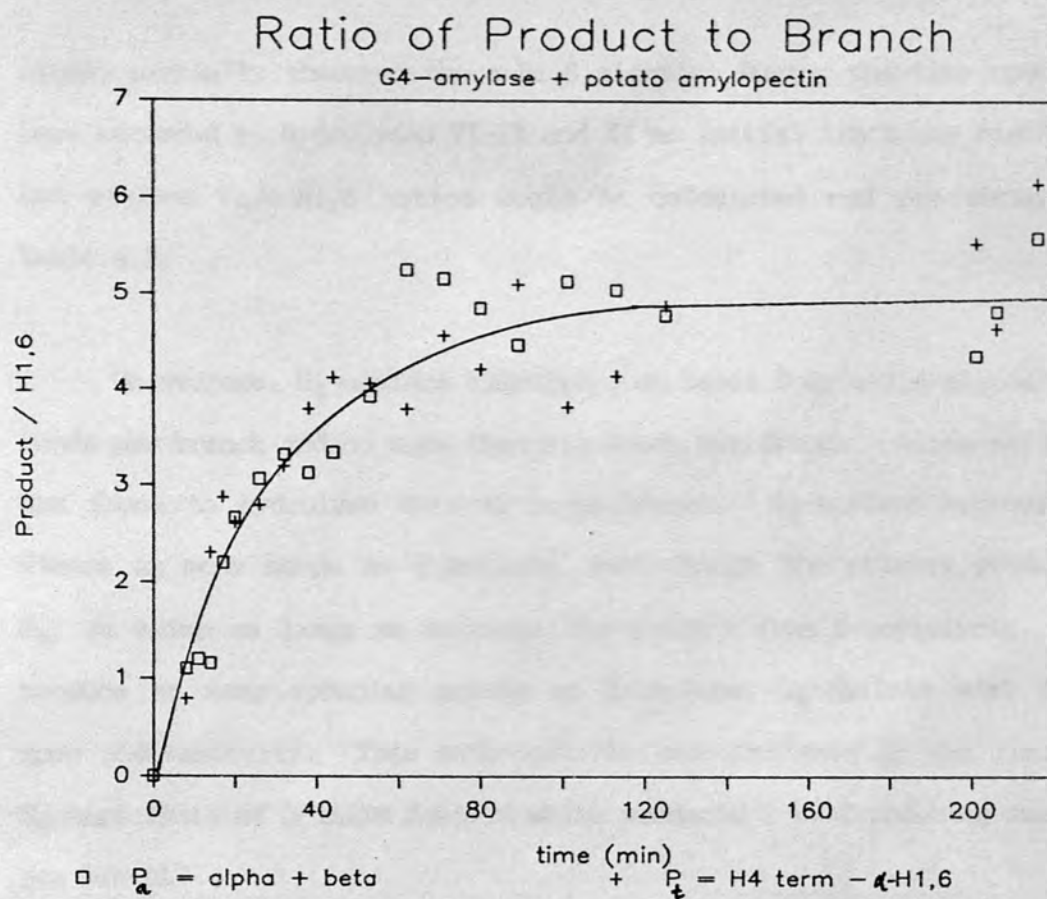
Fig. 4.6. Average number of molecules produced per branch during G_4-amylolysis of potato amylopectin, Hydrolysis V, as in Fig. 4.2. Product was calculated as either P_a = α-anomer (α-H1 red integral) + β-anomer (β-H2 integral) or as P_t = non-reducing termini (H4 term) – branches (α-H1,6 integral) relative to the branch α-1,6-linkage (α-H1,6 integral).

Table 4.3 summarizes the results from the twelve hydrolyses and the amount of reducing sugar (P_a) produced per branch. In Hydrolyses IV and XI the spectra were collected periodically for over 12 hours and the period over which the reducing sugar (P_a) remained constant is shown in parentheses. The P_a/α-H1,6 however, could not be determined for Hydrolysis IV (or for Hydrolyses IX and X) because the large HOD

signal partially obscured the α-H1,6 signal. During the time spectra were recorded in Hydrolyses VI-IX and XI no initial limit was reached, but minimum P_a/α-H1,6 ratios could be calculated and are shown in Table 4.3.

On average, G_4-amylase hydrolyzed at least four α-1,4-glycosidic bonds per branch and no more than six bonds per branch. Alpha-amylase was found to hydrolyze over 12 bonds/branch. G_4-amylase hydrolyzed almost as many bonds as β-amylase, even though the primary product, G_4, is twice as large as maltose, the product from β-amylolysis. To produce as many reducing groups as β-amylase, G_4-amylase must have some endo-activity. This endo-activity was confirmed by the limited G_4-amylolysis of β-limit dextrin which produced 1 to 2 reducing sugars per branch.

4.2.1.3.3. <u>Percent Hydrolysis</u>. The fraction or percent of all H-1 protons (Total H-1 protons = α-H1,4 + α-H1 red + α-H1,6 + β-H2) which is reducing sugar (P_a) can be determined for each spectrum (also see 5.9.4.5). Since reducing sugar was not detectable in the substrates (see Figs. 4.1.a and Table 4.2), the fraction present in any spectrum represents the degree of hydrolysis at a given time. Fig. 4.7 shows the percent hydrolysis from Hydrolysis V, plotted against time. An initial hydrolysis limit of 17% was reached at about 80 min. If G_4 were the exclusive product from G_4-amylolysis, then four glucosyl residues are removed each time a bond is hydrolyzed, or 68% (17% * 4) of the amylopectin was removed. The limit dextrin would be 100% - 68%, or 32%. However 17% was not the final hydrolysis limit and in Hydrolysis V the spectrum recorded at 1560 min showed that 24% of the glycosidic bonds had been hydrolyzed.

Maltotetraose was not the only product after extended hydrolysis for some G_3 and G_2 could also be detected by tlc, thus the amylolysis limit cannot be calculated using a factor of 4. These results are summarized in Table 4.4 along with the limits from the other hydrolyses.

From Table 4.4 it can be seen that 17-19% of the glycosidic bonds were initially (stage i) hydrolyzed by G_4-amylase, but after extended hydrolysis 24% of the bonds were found to be hydrolyzed in potato amylopectin. This was nearly the extent of hydrolysis observed for β-amylase. The β-hydrolysis limit of 26% would represent 52% (2*26%) of the potato amylopectin hydrolyzed as maltose (G_2). This limit is within the range reported in several reviews (79, 80) which list β-amylolysis limits, [β], for potato amylopectin ranging from 49-59%, depending on the type and size of the potato. As discussed in 4.2.2.1.1 the [β] for this potato amylopectin was found to be 52%.

If the initial stage of G_4-amylolysis is used, when G_4 may be the exclusive product, then a G_4-amylolysis limit of 72% (=4*18%) is found for both potato amylopectins. The G_4-amylolysis limit in Hydrolysis IX, for wheat amylopectin, was <72% since some G_3 and G_2 were detected.

The β-limit dextrins were hydrolyzed by G_4-amylase. The principal product from potato amylopectin β-limit dextrin, however, was not maltotetraose but maltose, and from wheat amylopectin β-limit dextrin a mixture of G_1-G_4. It is possible that G_4-amylase only hydrolyzed the maltosyl and maltotriosyl terminal chain stubs (47) which remain on the β-limit dextrins, however Sakano et al (15) have shown that G_4-amylase does not remove maltosyl stubs in branched glucooligomers.

Percent Hydrolysis

G4—amylase + potato amylopectin
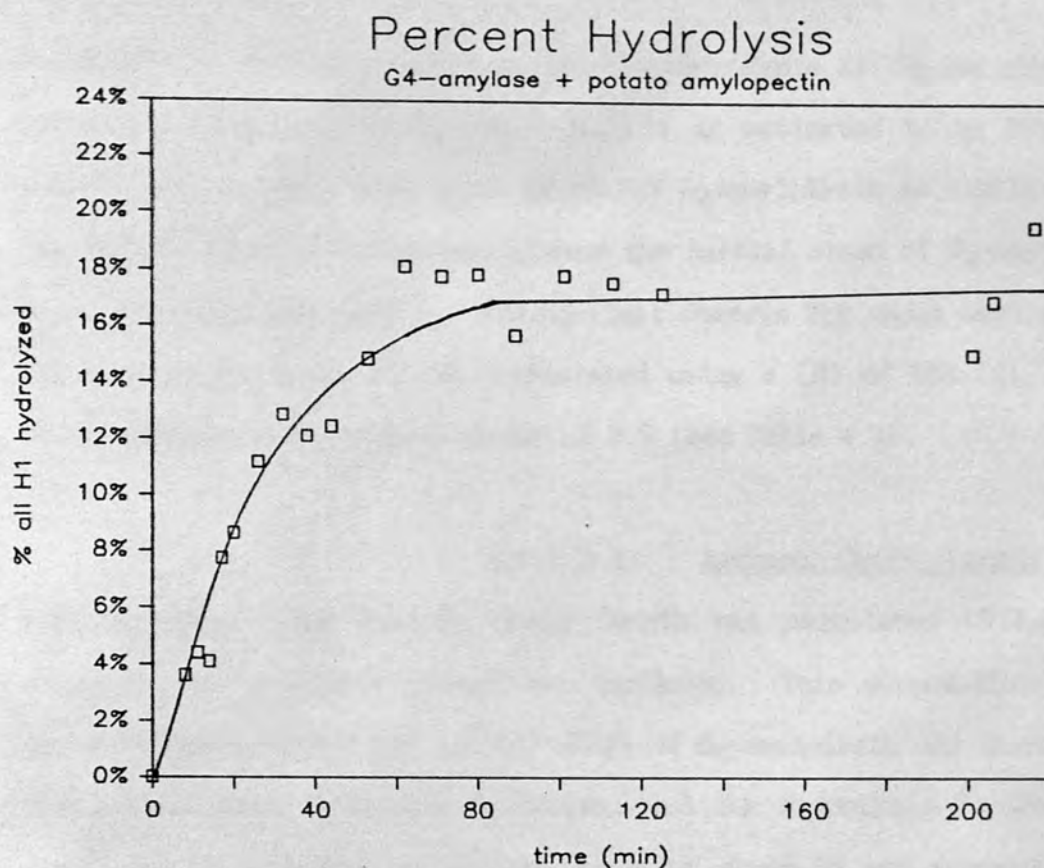
Fig. 4.7. Percent hydrolysis of glycosidic bonds during G_4-amylolysis of potato amylopectin, Hydrolysis V, as in Fig. 4.2. The percent of anomeric protons of all H-1 protons was calculated from the integrals: % Hydrolysis = 100 (α-H1 red + β-H2) / (α-H1 red + β-H2 + α-H1,4 + α-H1,6).

In conjunction with the other hydrolyses, it is more likely that interbranch chains were cleaved and further hydrolyzed. From the data, an estimation of the size of G_4-limit dextrin can be made. If β-limit dextrin = 48% of potato amylopectin, and 20% (Hydrolysis X) of the β-limit dextrin is hydrolyzed by G_4-amylase to produce primarily G_2, then 100% - (20%*2) = 60% of β-limit dextrin is G_4-limit dextrin. Therefore, 60% * 48% = 29% is G_4-limit dextrin of the original potato

amylopectin.   Recalculating from 14% for Hydrolysis XI, G_4-amylolysis of β-limit dextrin, the G_4-limit dextrin is estimated to be 35% of potato amylopectin.   This range of 65-71% G_4-amylolysis is similar to the percent hydrolysis determined from the initial stage of G_4-amylolysis of potato amylopectin.   The G_4-limit dextrin for wheat amylopectin was estimated to be 33%, calculated using a [β] of 55% (41, 46) and an average unit product length of 2.5 (see Table 4.4).

4.2.1.3.4.   <u>Average Chain Length of Dextrin (CL)</u>.   The dextrin chain length was calculated (5.9.4.6) assuming that a single product was produced.   This supposition was probably valid during the initial stage of G_4-amylolysis and throughout β-amylolysis.   As shown in Figure 4.8 for Hydrolysis V, the CL decreased rapidly from an initial value of about 28 and appeared to reach a limit of about 9 glycosidic bonds/ branch.   These CL's were calculated on the basis that the α-H1,4 integral contained contributions from three α-1,4-linkages for each G_4 produced; subtraction of $3P_a$ from the total α-H1,4 integral would leave the remaining α-H1,4 integral as that of the dextrin.   The average chain length of the G_4-limit dextrin was therefore calculated from CL = {(α-H1,4 - $3P_a$) + α-H1,6} / α-H1,6.   During the early stages of G_4-amylolysis the product appears to be G_4 exclusively; however after extensive hydrolysis (>1000 min) small amounts of G_3 and G_2 can also be detected which would give a low value for the average chain length of the residual dextrin.   In addition, in purified G_4-limit dextrin (see 4.2.2.1.1, Table 4.9, Fig. 4.12) signals from the anomeric protons could be detected in the hydrolysis mixture which would be calculated as P_a and subtracted from the α-H1,4 in the dextrin, giving an artificially low CL.   These effects could be observed in Hydrolysis V when, after

Average Chain Length of Dextrin
G4—amylase + potato amylopectin
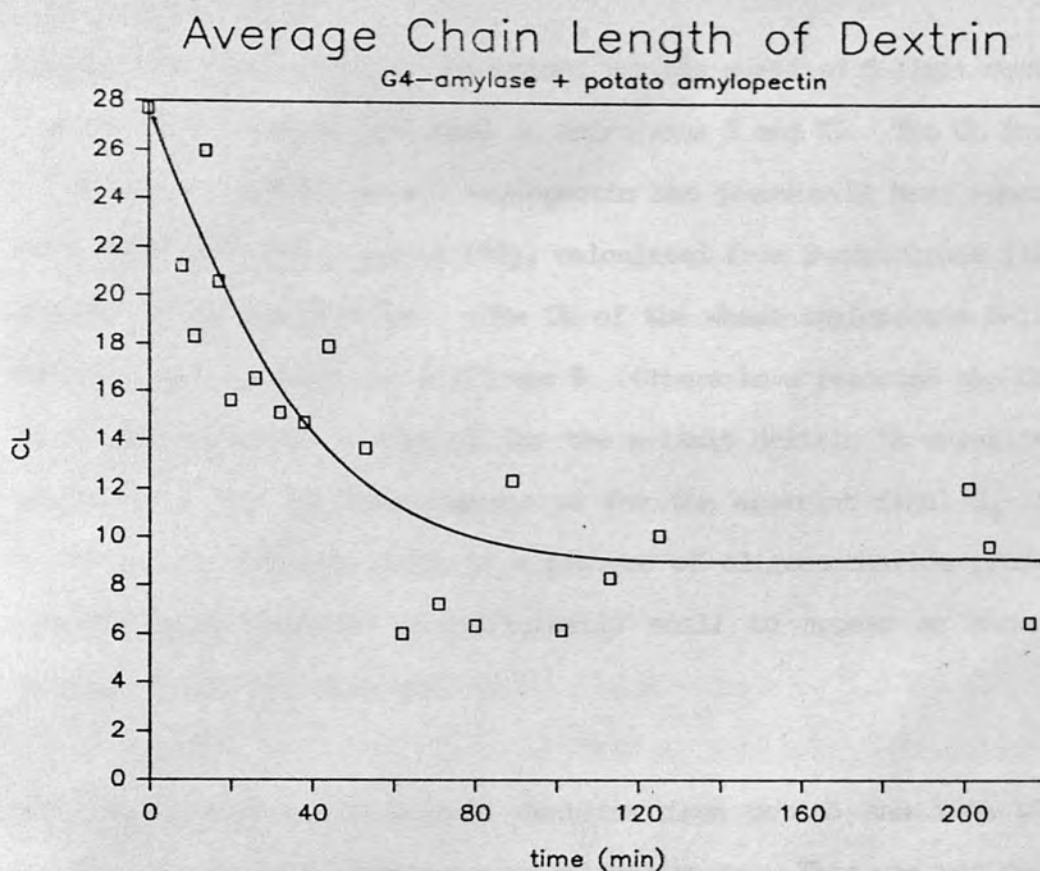
Fig. 4.8. The average chain length of the G_4-limit dextrin from potato amylopectin, Hydrolysis V, as in Fig. 4.2. Chain length (CL) was calculated assuming G_4 as the only product and reducing sugar. CL = (α-H1,4 - 3 P_a + α-H1,6) / α-H1,6 where P_a = α-H1 red + β-H2

extended hydrolysis (at 1560 min), the CL appeared to be 2.1, improbable since polysaccharide could be precipitated from G_4-amylolysis solutions and high or intermediate molecular weight polysaccharide could be detected by tlc.

As shown in Table 4.4, the chain length of the β-limit dextrin (from Hydrolysis VI) was 11 α-1,4-linkages/branch, an estimate which

agreed with the CL of 11.3 determined for the purified β-limit dextrin (see 4.2.2.1.1, Table 4.9) used in Hydrolyses X and XI. The CL for β-limit dextrin from potato amylopectin has previously been reported at 9 (46), 9-10 (81), and 11 (79), calculated from β-amylolysis limits and CL of the amylopectin. (The CL of the wheat amylopectin β-limit dextrin used for Hydrolysis XII was 9. Others have reported the CL to be 8 (82) or 9 (83).) The CL for the α-limit dextrin is unrealistically small for the same reasons as for the apparent final G_4-limit dextrin; i.e. because there is a mixture of oligosaccharide products and the limit dextrin is sufficiently small to appear as anomeric product in the calculations.

G_4-amylolysis of β-limit dextrins from potato and from wheat amylopectin produced little or no maltotetraose. This was not due to a contaminating enzyme since G_4-amylase from different purifications (and culture media) produced similar results when hydrolyzing potato amylopectin β-limit dextrin; yet gave different results when one of the G_4-amylase preparations hydrolyzed wheat amylopectin β-limit dextrin. G_4-amylolysis of potato amylopectin β-limit dextrin produced primarily maltose (G_3:G_2:G_1 = 2:9:2) and left a β,G_4-limit dextrin with a CL (calculated by subtracting one glycosidic linkage/product, n= 1) of 7.7. A very different oligosaccharide distribution was observed from hydrolysis of wheat amylopectin β-limit dextrin even though the same G_4-amylase preparation was used in one of the potato amylopectin β-limit dextrin hydrolyses. Almost equal proportions of G_4, G_3, G_2, and G_1 were produced from G_4-amylolysis of wheat amylopectin β-limit dextrin and the CL of the β,G_4-limit dextrin was 6.4 (calculated using n = 1.5). These results indicate a significant structural difference between potato amylopectin and wheat

amylopectin.

This difference may lie in the distribution of the branches, with potato amylopectin having more evenly spaced branching and wheat amylopectin having regions of closer branching interspersed with longer strands between these regions. (It is possible that G_4-amylase only hydrolyzed the remaining outer maltosyl and maltotriosyl stubs of the β-limit dextrins; however, this is unlikely since the oligosaccharide distribution was different between the two sources of β-limit dextrins.) If the wheat amylopectin had longer strands in some regions then it would be reasonable to have some G_4 and G_3 produced, and the remaining dextrin CL to be shorter. The lower percent hydrolysis would suggest that there are fewer regions in which G_4-amylolysis of wheat amylopectin can occur, results similar to that for the whole wheat amylopectin molecule (Hydrolysis IX).

Others (58, 84) have also suggested differences in the distribution of branching and the lengths of the B chains between potato and other amylopectins. From studies with β-amylase and cyclodextrin glucosyltransferase, Bender et al (84) concluded that potato amylopectin contained a looser structure than waxy maize amylopectin and clusters which were not as tightly packed.

Hizukuri (58) has compared the chain length distribution of 20 species of amylopectin, including potato and wheat amylopectin. The amylopectins were debranched by isoamylase; chromatography of the chains showed bimodal distributions of long and short chains in all but 3 of the specimens, including debranched wheat amylopectin, which showed trimodal distributions from 1 long and 2 short chain

populations. Wheat amylopectin was also exceptional in that it was one of 5 specimens which contained chains longer than 100, with some chains as long as 200. The CL of whole wheat amylopectin was 25, the CL of the longer chains (F1 fraction) was 56 (but the peak apex at CL 38), and the CL of the shorter chains (F2) was 17. Potato amylopectin had longer chains--an CL of 34, an F1 fraction with an CL of 62 (peak apex at CL 50), and an F2 fraction with an CL of 20. Wheat amylopectin had a much broader distribution of long chains than potato amylopectin, but most of the long chains in wheat amylopectin were shorter than those in potato amylopectin (CL 38 vs. 50). The molar ratio of F2/F1 chains in wheat amylopectin was 12, whereas in potato amylopectin it was 6, i.e. wheat amylopectin had twice as many short chains/long chain. One can imagine that the wheat amylopectin had a densely branched cluster of short interchain length such that the cluster would look something like a pitch-fork with A and B chains having similar chain lengths.

Several of the amylopectins, including potato amylopectin, were later reexamined by Hizukuri (45) and, through improved resolution of HPLC chromatography, shown to have a tetramodal distribution profile. Hizukuri attributed the F1 fractions to be composed of 3 fractions of B chains (of CL's 48, 75 and 104 for potato amylopectin) and the F2 fractions to be composed of A chains and short B chains (CL 16 and 24, respectively, for potato amylopectin). The A:B (all 4 B chain fractions) ratio was 0.89, a slightly lower ratio than previously reported (1.1 and 1.3 (57)) for potato amylopectin. If the clusters in potato amylopectin were less densely branched so that the interbranch distance in the cluster were longer, then a higher proportion of long B chains (F2 fraction) would be required in order to extend into the

crystallite regions proposed in cluster models (42, 48, 54). This longer interbranch chain length was observed here in the CL of the β,G_4-limit dextrin (CL 7.7 vs. 6.4 for wheat β,G_4-limit dextrin) produced in Hydrolysis XI.

The wheat amylopectin (and potato) examined by Hizukuri (45, 58) had an unusually long CL; however, 7 other cultivars of wheat with an average CL of 18 (85) similarly showed trimodal distributions and it was concluded that the chain length distributions were characteristic of a given species. (The chain length of wheat amylopectin is most frequently reported to be 19-21 (46, 80).)

Since G_4-amylase does not hydrolyze 6-α-maltosylglucose, 6^2-α-maltosylmaltose, or 6^3-α-glucosylmaltotriose (15) it is likely that the oligosaccharides smaller than G_4 arise from interbranch hydrolysis and that maltosyl and perhaps maltotriosyl stubs remain on the outer branches similar to the stubs found on β-limit dextrins. G_4-amylase does hydrolyze pullulan containing 5% maltotetraosyl units (10). Glucans with α-1,6-linkages, such as pullulan and dextran, are competitive inhibitors (9, 10) of G_4-amylase. Binding at the branch residue in amylopectin may affect the endo-hydrolysis since G_4-amylase will not hydrolyze the Schardinger dextrins (7, 10) implying that the branch may be required for endo-activity. G_4-amylolysis may occur at the third residue in from the branch, towards the reducing end (6^3-α-glucosylmaltotriose is not hydrolyzed, pullulan is), and the stub remaining on the core dextrin is then hydrolyzed in an exo-fashion, leaving at least a maltotriosyl stub. If the internal chain length were 8, for example, then maltose could have been removed from the interbranch chain. The average internal chain length of amylopectins,

determined by sequential enzymatic degradation (48), is reported to be between 5-9 (42, 46, 48). (The CL determined from the nmr studies are not internal chain lengths but would include outer chains as well.) If potato amylopectin has shorter intercluster chain lengths, then only maltose might be produced from endo-amylolysis. If wheat amylopectin had longer interbranch regions between clusters of more highly branched regions then G_4-amylase would produce G_4 and G_3 from, perhaps, an interbranch section of >9 glucosyl units, and any interbranch region <6 units would produce no linear oligosaccharide. Spatial restrictions may also be important in order for the catalytic site to accept the branches.

4.2.1.3.5. <u>Changes in the α-H1,4 Signal</u>. During amylolysis the appearance of the α-H1,4 signal changed as shown in Figuress 4.2-4.4 from a single asymmetric peak to what appeared to be at least two overlapping peaks. The two signals could be distinguished only after extensive hydrolysis by β-amylase, but were distinct in the earliest spectra recorded during α-amylolysis. G_4-amylolysis appeared to be intermediate in its effect on the α-H1,4 signals and never reached the resolution observed in α-amylolysis.

The enhanced resolution of the α-H1,4 signals is apparent in the spectra of the purified G_4- and β-limit dextrins shown in Figures 4.9-4.12. The chemical shifts of these protons are given in Table 4.5. Although the separate peaks could not be integrated it appears that only one set of glycosidic linkages are hydrolyzed, those at 5.33 ppm, and that relatively few of these glycosidic linkages remain in the G_4-limit dextrin.
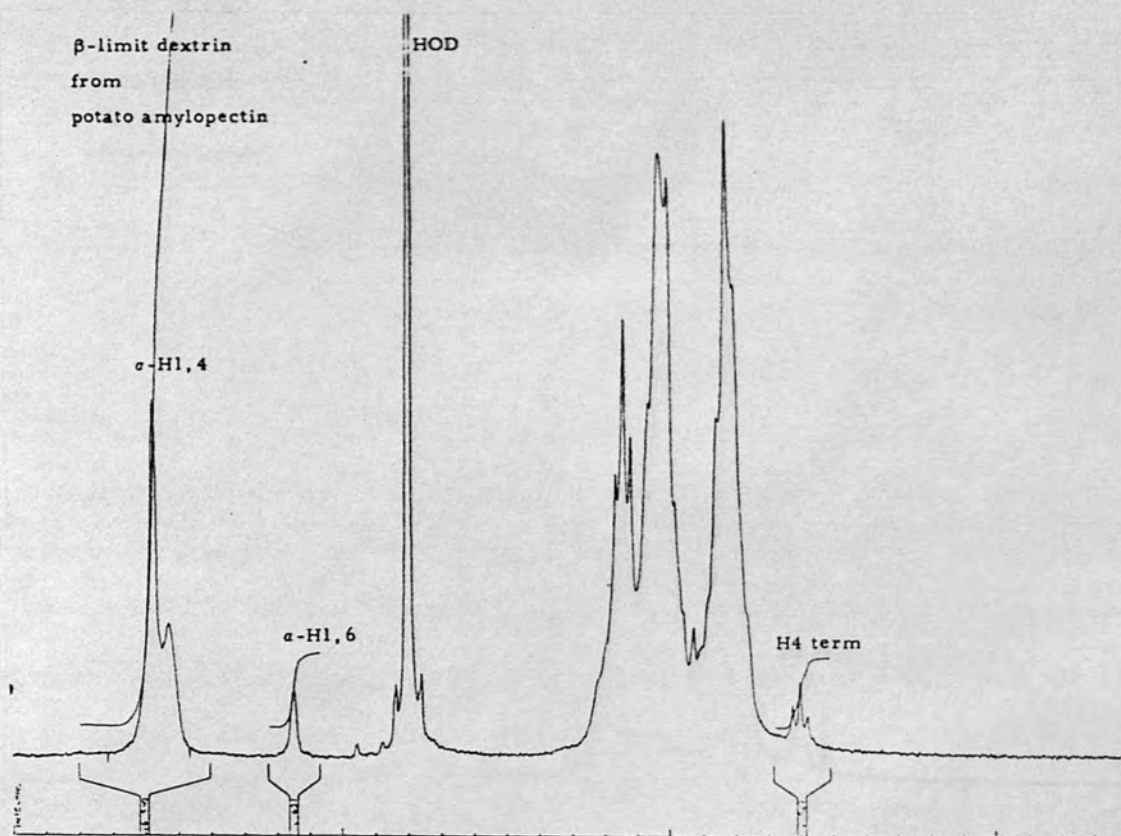
Fig. 4.9. 400 MHz ^{1}H-nmr spectrum of β–limit dextrin from potato amylopectin, 37°C, internal standard CH_3CN.
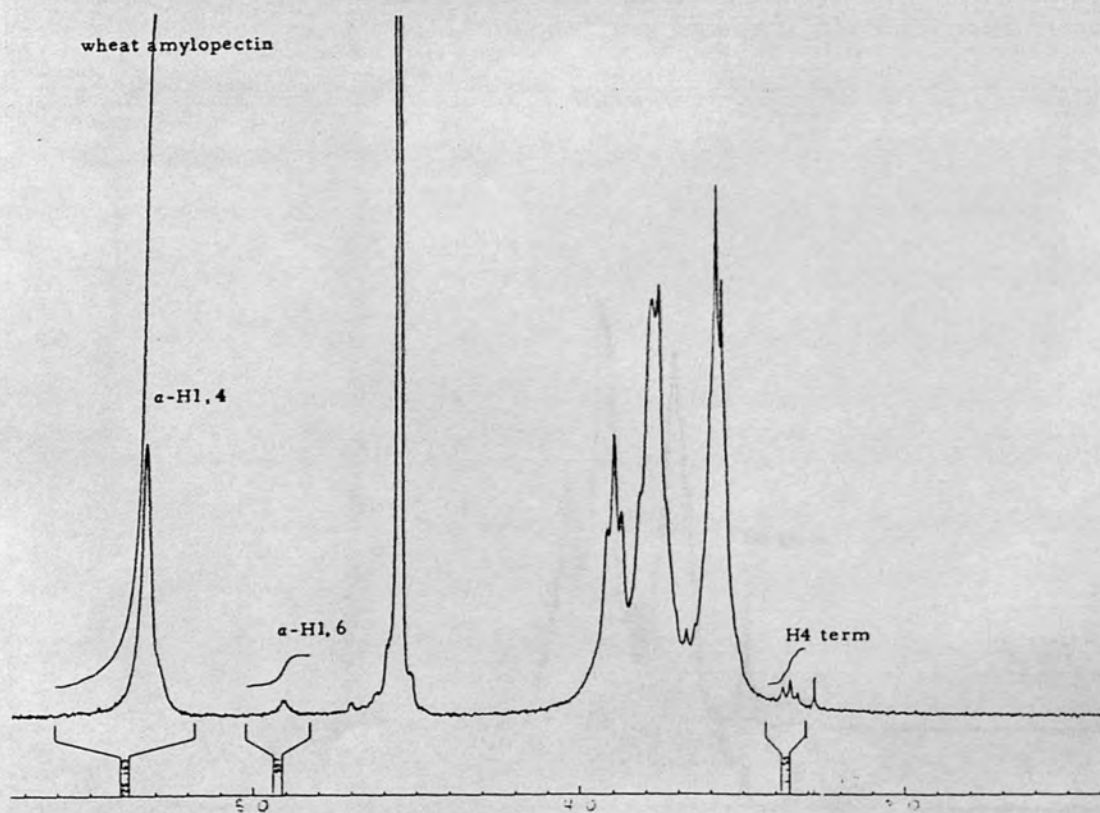
Fig. 4.10. 400 MHz ^{1}H-nmr spectrum of wheat amylopectin, 37°C, internal standard CH_3CN.
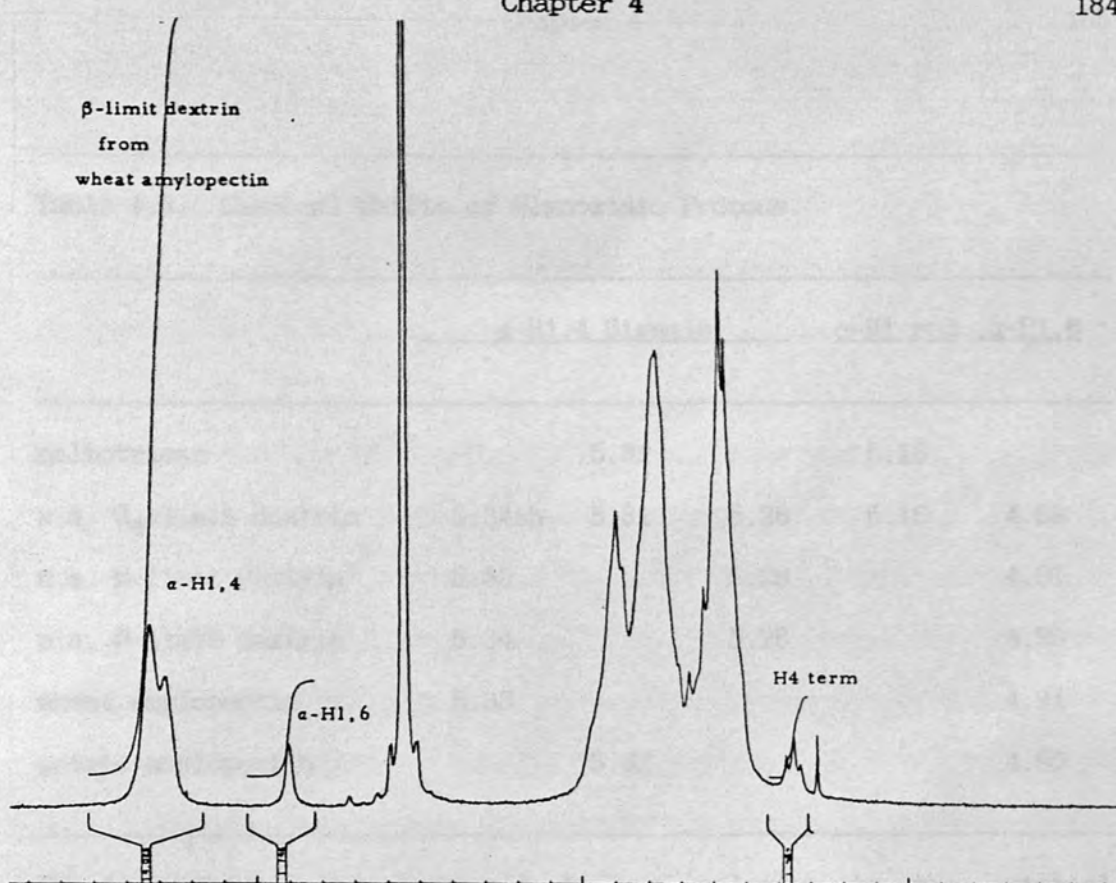
Fig. 4.11. 400 MHz ^{1}H-nmr spectrum of β-limit dextrin from wheat amylopectin, 37°C, internal standard CH$_3$CN.
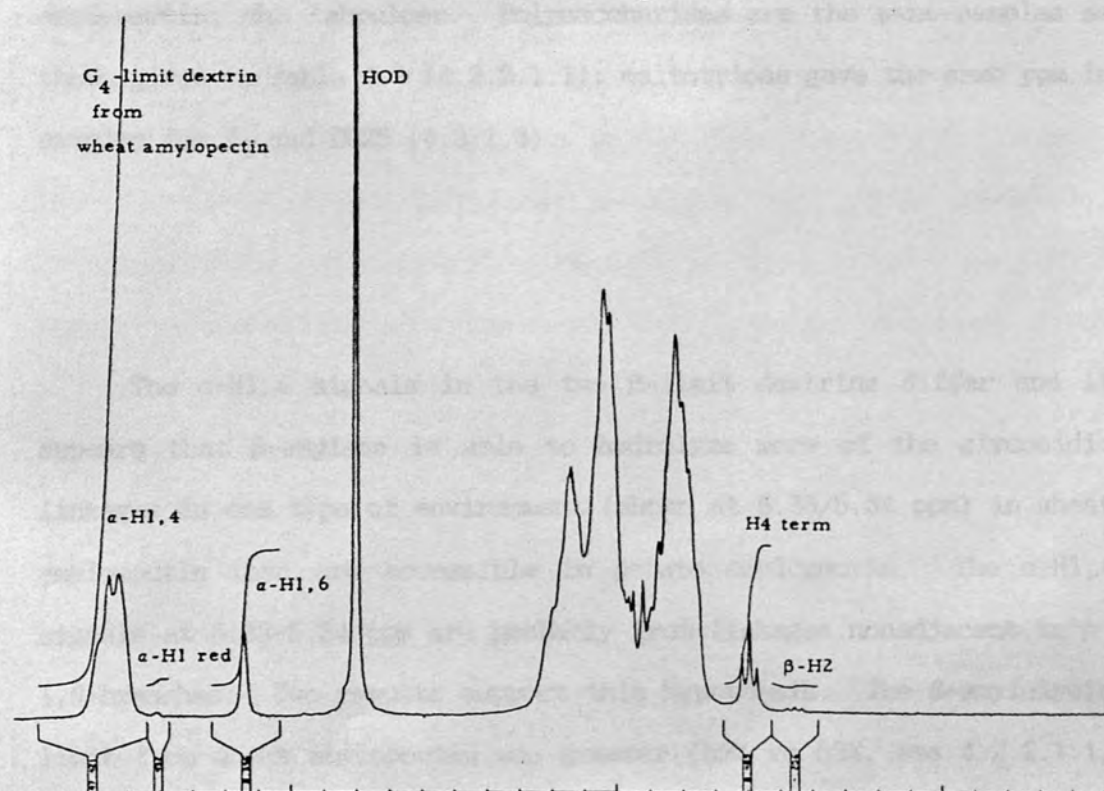
Fig. 4.12. 400 MHz ^{1}H-nmr spectrum of G$_4$-limit dextrin from wheat amylopectin, 37°C, internal standard CH$_3$CN.

Table 4.5. Chemical Shifts of Glycosidic Protons.

	α-H1,4 Signals			α-H1 red	α-H1,6
maltotriose		5.31		5.16	
w.a. G_4-limit dextrin	5.34sh	5.31	5.28	5.16	4.89
w.a. β-limit dextrin		5.33	5.29		4.91
p.a. β-limit dextrin		5.34	5.28		4.90
wheat amylopectin		5.33			4.91
potato amylopectin		5.32			4.90

Chemical Shifts are expressed in ppm relative to the internal standard, CH_3CN, 2.00 ppm; w.a., wheat amylopectin; p.a., potato amylopectin; sh, shoulder. Polysaccharides are the same samples as those given in Table 4.8 (4.2.2.1.1); maltotriose gave the same ppm in samples for T_1 and DS25 (4.3.1.3).

The α-H1,4 signals in the two β-limit dextrins differ and it appears that β-amylase is able to hydrolyze more of the glycosidic linkages in one type of environment (shown at 5.33/5.34 ppm) in wheat amylopectin than are accessible in potato amylopectin. The α-H1,4 signals at 5.33-5.34 ppm are probably from linkages nonadjacent to α-1,6-branches. Two results support this hypothesis. The β-amylolysis limit from wheat amylopectin was greater (59% vs 52%, see 4.2.2.1.1, Preparation of Limit Dextrins) than from potato amylopectin, suggesting more linear, outer glucosyl residues were removed from wheat

amylopectin. In addition, the CL of wheat amylopectin β-limit dextrin was shorter (Hydrolysis XII, preparation in 4.2.2.1.1) at 9 units when compared to a CL of 11 for potato amylopectin β-limit dextrin. Potato amylopectin β-limit dextrin has more residues between branches.

Therefore the other glycosidic signals, upfield at 5.30 ppm and 5.28, must represent linkages near branches which β-amylase is unable to remove. In wheat amylopectin G_4-limit dextrin, very few protons are found at 5.33 ppm, again supporting the contention that extended unbranched chains are not found in the G_4-limit dextrin. Potato amylopectin G_4-limit dextrin remains to be purified in order to confirm the contention that potato amylopectin has slightly longer internal chain lengths within its cluster.

There have been other reports of the upfield shift in signals from α-H1,4 protons near α-1,6 linkages. Bock and Pedersen (86) were able to assign all of the protons in the model branch point trisaccharide, methyl β-D-(6^1-α-glucosyl)maltoside, and found the α-H1,4 signal in the model compound to be 0.06 ppm upfield from the α-H1,4 signal in methyl β-D-maltoside. The α-H1,4 signal in panose, 6^2-α-glucosylmaltose, similarly showed a slight shift upfield from the α-H1,4 signal in maltoheptaose (76).

4.2.1.3.6. <u>Hydrolysis Limits</u>. The hydrolysis limits observed in the β-and G_4-amylase nmr studies were not due to loss of enzyme activity since both of the amylases were found to remain active in the mixtures for at least 6 days even after being kept at 37°C and room temperature. Even in slightly acidic buffer, in which G_4-amylase is less stable (11), there was little loss in

activity (3% loss in Hydrolysis X, no loss in XI, 38% in XII; compositions given in Table 4.2). Considerable β-amylase activity remained in the Hydrolysis VII mixture after storage for 1 week (4.8 Boeh U/ml).

One of the G_4-amylolysis mixtures for which the amylase activity had been measured after storage, Hydrolysis XI, was also examined for product specificity by incubating the G_4-amylase-β-limit dextrin hydrolyzate mixture with soluble starch. The product specificity of each of the G_4-amylase preparations used for hydrolysis of the β-limit dextrins was also examined. In each instance the G_4-amylase was shown to produce primarily G_4 from soluble starch although little G_4 had been produced from the β-limit dextrins. The same G_4-amylase preparations were also used for HPLC studies of G_4-amylolysis which are described in 4.2.2.1.2.b and c.

The limit dextrins from Hydrolyses X, XI, and XII were also examined by HPLC. The results are discussed with other HPLC studies in 4.2.2.1.2.a.

4.2.1.4. <u>Summary and Conclusions</u>. Using high field ^{1}H-nmr spectroscopy it is possible to follow the hydrolysis of glucans and to determine the specificity of the amylase, the anomeric configuration of the product, and the percent hydrolysis of the glucan. Several properties of G_4-amylase were ascertained or confirmed, as well as structural differences between wheat and potato amylopectin.

a. Properties of G_4-amylase.

1.   Only α-1,4-linkages and not α-1,6-linkages are hydrolyzed in starch.

2.   The anomeric configuration of the principal product is the α-anomer.

3.   Hydrolysis of potato amylopectin occurs in two stages. In an initial stage, an apparent hydrolysis limit is reached which represents about 18% hydrolysis of the glycosidic linkages and the primary product is G_4. It can be concluded that some endo-amylolytic activity occurs during this stage since less than 8 molecules of G_2 are produced per branch from β-amylolysis of potato amylopectin, yet an average of 4.5-4.9 molecules are produced from G_4-amylolysis. After the final stage of G_4-amylolysis, 24% of the bonds were hydrolyzed, nearly as many as β-amylase and about half as many as α-amylase.

4. G_4-amylase hydrolyzes the α-1,4-glycosidic bonds which are farthest downfield, at 5.34 ppm, in the [1]H-nmr spectrum. Few of this type of α-1,4 bond remains in the limit dextrin.

b. Differences between Potato and Wheat amylopectin.

1. G_4-amylase hydrolyzes more α-1,4-bonds in potato amylopectin than in wheat amylopectin.

2. G_4-amylase hydrolyzes more α-1,4-bonds in potato amylopectin β-limit dextrin than in wheat amylopectin β-limit dextrin.

3.   Potato amylopectin β-limit dextrin has a longer average chain length (CL 11) and contains more α-H1,4 protons at δ5.34 than does wheat amylopectin β-limit dextrin (CL 9).

4. G_4-amylolysis of the wheat amylopectin β-limit dextrin produces oligosaccharide of slightly longer DP (degree of polymerization) than from potato amylopectin β-limit dextrin. Calculations of

the G_4-limit dextrin in the hydrolysis mixtures indicate a shorter CL (wheat β,G_4-limit dextrin, CL 6.4) for the G_4-limit dextrin from wheat amylopectin than from potato amylopectin (potato β,G_4-limit dextrin, CL 7.7).

These differences between potato and wheat amylopectin indicate that potato amylopectin probably contains regions of low branch density which remain resistant to G_4-amylolysis. There is a slightly more even distribution of branching throughout potato amylopectin. Although G_4-amylase is able to hydrolyze more linkages it is primarily maltose which is produced from endo-amylolysis suggesting a shorter interbranch chain between clusters.

Wheat amylopectin probably contains more defined regions of high density branching than potato amylopectin. G_4-amylase is able to hydrolyze between clusters of highly branched regions and to produce G_4 and G_3 from endo-hydrolysis, suggesting a longer intercluster chain length than potato amylopectin.

4.2.2. Hydrolysis of α-1,4-Glucans.

4.2.2.1. Preparation of Limit Dextrins.

4.2.2.1.1. Experiment I: Preparation of β-Amylase and Maltotetraohydrolase Limit Dextrins from Amylopectin.

Wheat amylopectin, potato amylopectin, and a commercial potato amylopectin from Sigma, referred to as Sigma potato amylopectin, were hydrolyzed by β-amylase, the β-amylolysis limits determined, and the β-limit dextrins purified. Wheat amylopectin and Sigma potato amylo-

pectin were also hydrolyzed by maltotetraohydrolase, the G_4-amylolysis limits determined, and the G_4-limit dextrins purified.

Part A.   Amylolysis of Sigma Potato Amylopectin.   A solution of Sigma potato amylopectin (3.65 mg carbohydrate/ml) was hydrolyzed by β-amylase from sweet potato (24 Boeh U/ml) and by G_4-amylase (0.88 Boeh U/ml).   Both enzymes remained active during the first incubation period of 48 h, retaining more than 13% and 68% of their respective activities and producing high molecular weight dextrins and maltose or maltotetraose.   Tlc analysis of the G_4-amylase digest showed both intermediate (streaking near the origin) molecular weight dextrin and some maltotriose in addition to maltotetraose.   Fractionation of the β-amylolysis mixture by ethanol precipitation gave a supernatant (Supernatant 1 in Table 4.6.) which contained 43% of the original carbohydrate, 18% of which was reducing sugar, shown by tlc to be primarily maltose.   Supernatant 1 from G_4-amylolysis contained much more of the original carbohydrate (72%), <14% of which was reducing sugar, shown by tlc to be primarily G_4; this from an original amylase activity which was a thirtieth of the β-amylase activity.

In a second incubation of the β-dextrin with β-amylase, an additional 4.7% (calculated from original carbohydrate, Table 4.6. Supernatant 2) of maltose was produced and tlc indicated the presence of some intermediate (unexpected from β-amylolysis) as well as high molecular weight limit dextrin.   Analysis by HPLC of the limit dextrin, described in Part D, also shows intermediate weight polysaccharide.   The total low molecular weight carbohydrate contained in Supernatants 1 and 2, the β-amylolysis limit, represented 48% of the original carbohydrate and had an average DP of 2.5.

Table 4.6. Carbohydrates Found in the Supernatants from Ethanol Precipitation after β- and G_4-Amylolysis of Amylopectin.

Amylopectin Digest (mg carbohydrate)	Supernatant 1			Supernatant 2			Total Hydrolysis	
	RS	TC	DP	RS	TC	DP	H1	[L]
	(μmole)			(μmole)				
wheat (201 mg)								
+ β-amylase	318	690	2.2	19.3	48.0	2.5	29%	59%
potato (243 mg)								
+ β-amylase	292	722	2.5	26.3	58.6	2.2	21%	52%
Sigma potato (912 mg)								
+ β-amylase	989	2431	2.5	85.5	262.	3.1	19%	48%
Sigma potato (511 mg)								
+ G_4-amylase	448[1]	2255	5.0[1]	11.5[1]	47.2	4.1[1]	15%[1]	73%
wheat (198 mg)								
+ G_4-amylase	145[1]	573	4.0[1]	>32[1,2]	>192[2]	10.4	>15%[1]	>62%[2]

RS: μmole reducing sugar found in the supernatant, determined using alkaline ferricyanide reagent, standardized with G_2; [1] G_3 and probably G_4 were found to give higher RS values than G_2, see Chap. 5.8.3.2.

TC: total carbohydrate, μmole glucose equivalents in supernatant;

DP: average degree of polymerization = TC/RS

Total Hydrolysis: calculated from the sum of Supernatants 1 and 2;

  H1   % H1,4 bonds hydrolyzed = 100 (μmole RS) / (mg substrate/0.162)

  [L] β-amylolysis limit or G_4-amylolysis limit = % TC, as in H1 except μmole TC instead of RS.

[2] calculated from remaining the supernatant, which was refractionated into fractions of DP 14.4 and 4.4 with [L] >57%.

G$_4$-amylolysis of Sigma potato amylopectin was essentially complete in the first incubation and only a further 1.5% carbohydrate (calculated from Table 4.6. Supernatant 2) was hydrolyzed in a second incubation, shown by tlc to be mainly G$_4$, with some G$_3$ and G$_2$. However, the total carbohydrate in the two Supernatants, the amylolysis limit, represented 73% of the original carbohydrate, having a DP of 5.0, yet fewer reducing groups (15%) than were produced by β-amylase (19%). Since the reducing sugar reagent was later found to be over-reduced by G$_3$ and probably by G$_4$, the actual number of reducing groups was probably <15%. If the maltotetraohydrolase were acting solely by an exo-mechanism the total carbohydrate fraction found in the supernatant could not exceed that of β-amylase by such a factor, even allowing for removal of the G$_2$ or G$_3$ β-limit dextrin branch stubs (87), which could contribute only an additional 5%.

Sigma potato amylopectin was readily soluble in water, forming a clear 5% solution. Tlc showed intermediate as well as high molecular weight polysaccharide, suggesting that Sigma potato amylopectin was already partially degraded. An intermediate weight fraction was also detected by tlc in each of the supernatants from both β- and G$_4$-amylolysis; consequently the amylolysis limits may be slightly high, reflecting intermediate weight polysaccharide as well as oligosaccharide. Refractionation of the supernatant could have confirmed this.

Part B. Amylolysis of Wheat Amylopectin and Potato Amylopectin. Solutions of wheat amylopectin (1.25% w/v carbohydrate) and potato amylopectin (gift from E. Percival, 1.5% w/v carbohydrate) were hydrolyzed by β-amylase (37 Boeh U/ml), and, in addition, the solution of wheat amylopectin was hydrolyzed by G$_4$-amylase (0.6 Boeh U/ml). After

42 hours, the solutions containing β-amylase remained distinctly turbid, similar to the controls without amylase, whereas the solution containing G_4-amylase was only slightly turbid.

Tlc of the samples from β-amylolysis showed maltose and high molecular weight material at the origin, from controls only high molecular weight material, and from G_4-amylolysis of wheat amylopectin much G_4, faint G_3, and high and intermediate molecular weight material. The amylopectin-amylase digests were precipitated with ethanol after 48 hours amylolysis, the high molecular weight precipitate removed for incubation with additional amylase, and the supernatant (Table 4.6., Supernatant 1) analyzed for carbohydrate content.

The wheat and the potato β-dextrins were each incubated with additional β-amylase, the dextrins removed by ethanol precipitation and analyzed as discussed in Part D. The supernatants (Supernatants 2, Table 4.6.) were also analyzed. Each of the supernatants were shown by tlc to be primarily maltose although the DP of two of the supernatants was somewhat high, having a DP of 2.5. This may have been due to incomplete precipitation (none of the supernatants were reprecipitated for purification). Most of the hydrolysis occurred during the first incubation with only 3.9% additional carbohydrate produced from either the wheat or the potato β-dextrin, giving a final β-amylolysis limit of 59% from wheat amylopectin and 52% from potato amylopectin.

The β-amylolysis limits, [β], from the two potato amylopectins were quite similar, 48% and 52%, and somewhat higher from wheat amylopectin, 59%. These compare reasonably well with the β-amylolysis

limits, 51% to 57% (46, 79) conversion into maltose, reported for various sizes and sources of potato amylopectin, and the 54% and 56% [β] reported for wheat amylopectin (46).

Supernatant 1 from G_4-amylolysis of wheat amylopectin contained 47% of the original carbohydrate with a DP of 4.0, shown by tlc to be primarily maltotetraose but also some maltotriose. Tlc of the second G_4-amylolysis digest showed high and intermediate polysaccharide and, again, primarily G_4 and a little G_3. Unfortunately part of Supernatant 2 was lost after ethanol precipitation, but the remaining supernatant contained a surprising 15.7% of the original carbohydrate in the wheat amylopectin (Table 4.6.), suggesting that, in all, much more than 15% (and not 12%) of the glycosidic bonds had been hydrolyzed, and an amylolysis limit of more than 62%. Carbohydrate analysis of Supernatant 2 showed a higher than expected DP, 6.0 and intermediate molecular weight fraction on tlc. Supernatant 2 was refractionated as discussed in Part C.

Part C. Analysis of Supernatant 2 from G_4-amylolysis of Wheat Amylopectin. Supernatant 2 from G4-amylolysis of wheat amylopectin dextrin was further fractionated by ethanol precipitation and the supernatant, Supernatant 2S, found to have 26.9 μmole reducing sugar, 118.8 μmole ge total carbohydrate, giving a DP of 4.4; tlc showed primarily G_4 but some G_3, G_2, and oligosaccharides longer than G_6. Oligosaccharides G_4-G_1 were separated by HPLC in a ratio of 25:9:4:1, only by this method was glucose detected. Estimations from this refractionation indicate that at least 57% of the wheat amylopectin was converted into fractions with a DP of 4.4 or less, but perhaps not as much as the 62% indicated in Table 4.6. Nmr analysis showed that

some G_4-limit dextrin was present with Supernatant 2S which was probably the intermediate polysaccharide >G_6 shown by tlc. (Nmr showed 7.6% branching, one α-1,6-linkage /2.1 reducing groups or /3.2 nonreducing termini or /10.2 α-1,4-linkages, and an average of 6.4 glucose residues/reducing group.) Even one highly branched molecule would greatly affect the averages shown by the nmr analysis. For further, future analysis it would be better to analyze a sample which had first been purified by chromatography.

The precipitate from refractionation of Supernatant 2, an intermediate molecular weight G_4-limit dextrin, contained 5.1 μmole reducing sugar, 73.5 μmole ge total carbohydrate, giving a DP of 14.4; tlc showed predominately intermediate molecular weight carbohydrate >G_8 and a little G_4. HPLC (Figure 4.13b) showed a peak at about 5000 daltons. Nmr (Table 4.7, wheat G_4-limit dextrin, Supernatant 2P) showed a highly branched average dextrin with an average of 13% branching, 3.4 branches per molecule, a CL of 7.4, and a DP of 26 giving an average molecular weight of 4230. Carbohydrate analysis had given a lower DP of 14.4 but that may reflect nonlinearity of the reducing sugar test for polysaccharides (see 5.8.3). The ´little G_4´ present would markedly affect the DP and the nmr averages; yet these averages still indicate minima for a densely branched molecule resistant to G_4-amylolysis.

Part D. Analysis of Limit Dextrins. The results of nmr analysis are summarized in Table 4.7. The 3.5% and 3.8% branching found in potato and wheat amylopectin agree well with the 4-5% determined by methylation studies (46). The average chain lengths for potato and wheat amylopectin are somewhat high but within the range of 18-28

Table 4.7. Nmr Analysis of Amylopectins and Limit Dextrins.

Polysaccharide	% Branching	CL	DP	Branches/ Molecule
potato amylopectin	3.5%	28.7	I	I
p.a. β-limit dextrin	8.9%	11.3	I	I
wheat amylopectin	3.8%	26.2	I	I
w.a. β-limit dextrin	11.1%	9.0	I	I
w.a. G_4-limit dextrin[1]	14.8%	6.8	88	13
w.a. G_4-LD, Supernatant 2P[1]	13.0%	7.7	26	3.4
Sigma potato amylopectin	5.7%	17.6	248 *	14 *
Sigma p.a. β-LD	11.0%	9.1	161 *	18 *
Sigma p.a. G_4-LD	11.7%	8.6	52	6

% Branching: % H1,6 = 100 * H1,6 / Sum of All H1 protons.

CL: average Chain Length, (All H1 Protons) / H1,6 = Total H1 / Branch.

DP: Glucose Units/Molecule; Sum of All H1/ Sum of Reducing H1 α- and β- anomers.

Branches/Molecule: H1,6/ Sum of Reducing H1 α- and β- anomers.

I: Indeterminate, reducing sugar not detected; * reducing sugar integrals very small, figures estimated.

[1] tlc indicated that these solutions contained some G_4.

reported for different sources of potatoes (80), and fractions of wheat amylopectin (46).

The G_4-limit dextrins have a higher degree of branching and a shorter average chain length than β-limit dextrins indicating denser, more highly branched molecules. The major component of wheat G_4-limit dextrin shows a very compact molecule of 88 glucose residues with 13 branches in each molecule, an average molecular weight of 14300; found only at the origin on tlc. HPLC of this component (Figure 4.13a) indicated a molecular weight of about 10000 (Dextran standards). A second fraction of wheat G_4-limit dextrin, Supernatant 2P described in Part C, was similar in % branching and average chain length but with an average molecular weight of 4230 (determined by nmr) or ≈5000 (by HPLC).
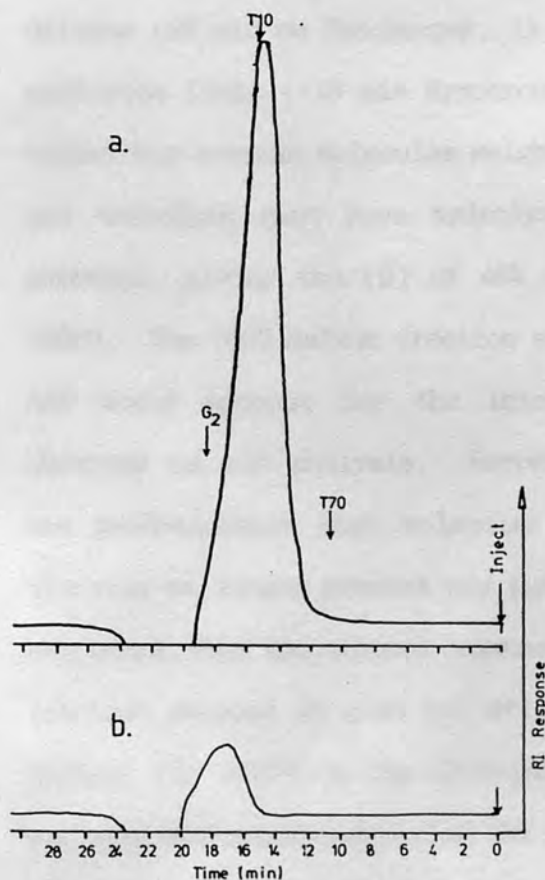
Figure 4.13. HPLC chromatograms of two fractions of G_4-limit dextrin from wheat amylopectin in D_2O. Synchropak column, eluent H_2O 0.2 ml/min, RI detection. The negative peak at 21 min is D_2O. Standards: Maltose, 18.4 min; Dextran T10, 15.4 min.

a. Wheat G_4-limit dextrin in D_2O, major fraction.

b. Supernatant 2P wheat G_4-limit dextrin, from reprecipitation of ethanol Supernatant 2.

The Sigma potato amylopectin is clearly unique, showing some, albiet a low concentration of, reducing groups even before amylolysis. Even so, β-amylolysis gives a β-limit dextrin similar in branching and CL to wheat β-limit dextrin. The Sigma G_4-limit dextrin appears to be a half to a third the size of the β-limit dextrin, but similar in branching and CL. The DP of Sigma G_4-limit dextrin is 21% that of the amylopectin which correlates well with the amylolysis limit of 73%.

HPLC analysis of the Sigma potato samples, shown in Figure 4.14, contrasts with the nmr analyses of the same samples, and also indicates marked differences between the G_4-limit dextrin and Sigma amylopectin or Sigma β-limit dextrin. Chromatography on two HPLC gel permeation columns showed both Sigma amylopectin and its β-limit dextrin to have two approximately equal populations, one at about 7000 daltons (16 min on Synchropak, 11 min on Chrompak) and another at the exclusion limit (<10 min Synchropak). Apparently, β-amylase did not effect the average molecular weight of the amylopectin at 7000 daltons and therefore must have hydrolyzed only the high molecular weight material, giving the [β] of 48% and nmr molecular weight average of 26000. The 7000 dalton fraction would greatly affect the nmr averages and would account for the intermediate molecular weight fraction observed on tlc analysis. Surprisingly, the Sigma G_4-limit dextrin was predominately high molecular weight material. The 7000 dalton fraction no longer present may have been hydrolyzed by G_4-amylase and extracted into the ethanol supernatant, and the high molecular weight fraction reduced in size but still near the exclusion limit of 70000 daltons (or 40000 on the Chrompak). The nmr average weight of 8400 was very different suggesting the possibility of crosslinked molecules although not in sufficient concentration to be observed by nmr. In
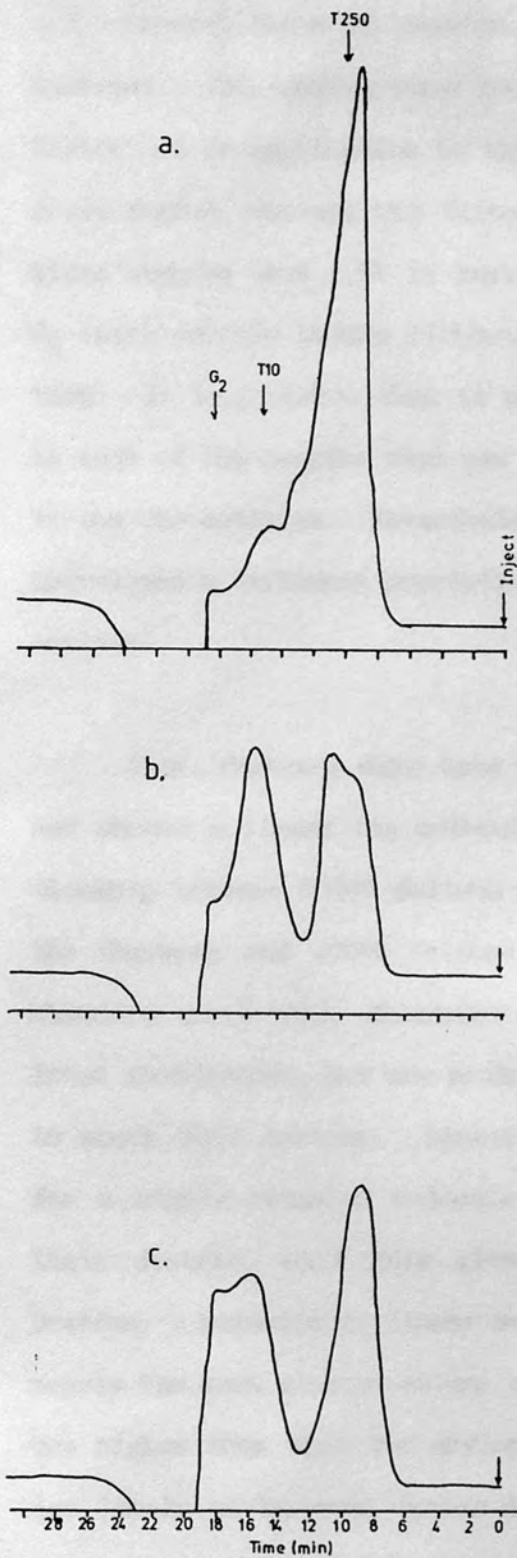
Figure 4.14. HPLC chromatograms of Sigma potato amylopectin and its β- and G_4-limit dextrins in D_2O. Synchropak column, as in Figure 4.13.

a. Sigma G_4-limit dextrin.

b. Sigma β-limit dextrin.

c. Sigma potato amylopectin.

any case, G_4-amylase hydrolyzes specific populations leaving a unique limit dextrin.

Several notes of caution must be noted in discussing the HPLC analyses. The samples were routinely filtered through a 0.45 micron filter before application to the columns, and not all of the carbohydrate passes through the filter. The carbohydrate contents of the Sigma samples were 1.9% in amylopectin, 2.2% in β-limit dextrin, 1.7% G_4-limit dextrin before filtration and 1.2-1.3% in each after filtration. It is probable that it was very high molecular weight material in each of the samples that was removed by the filter and not observed in the chromatogram. Nevertheless, it is apparent that the G_4-amylase hydrolyzed a different population in the Sigma amylopectin than did β-amylase.

Also, dextrans were used as standards for the two HPLC columns and showed a linear log molecular weight versus elution volume relationship between 70000 daltons on the Synchropak or 40000 daltons on the Chrompak and 10000 daltons, the lowest average molecular weight standard available. Molecular weights less than 10000 were extrapolated from graphs, but are probably valid for the Chrompak column down to about 5000 daltons. Linear dextrans, however, used as standards for a highly branched molecule like amylopectins, or especially G_4-limit dextrin, will only give relative molecular weight averages. Dextran, a principally linear α-1,6-glucan with some 1,3 branches, has nearly the same elution volume as amylose of similar molecular weight, but higher than that for amylopectin (88). Since the limit dextrins are likely to be even denser if the outer chains have been removed, then the average molecular weights are probably higher than indicated by gel permeation chromatography. In accordance with the higher than indicated molecular weight is the estimation that ethanol precipitation (2:1 v/v) of a 2% aqueous solution fractionates polysaccharides

at about 10000 daltons (88) which suggests that none of the ethanol precipitate should contain significant amounts less than 10000 daltons and that all of the HPLC weight estimates are minimum molecular weight averages for limit dextrins.

Neither wheat amylopectin nor its β-limit dextrin would filter through a 0.45 micron filter, even when solubilized at 1% and diluted to 0.5%. The clear solution of wheat G_4-limit dextrin did filter and 98% of the material eluted at 10000 daltons (Figure 4.13a). Tlc of the wheat G_4-limit dextrin showed only material at the origin.

Part E. Summary. The β-amylolysis limits for wheat and potato amylopectin, 59% and 52%, and are similar to those [β] reported in the literature (41, 46, 79, 89). The [β] of 48% for Sigma potato amylopectin was low, reflecting a population shown by HPLC to be resistant to β-amylolysis. Maltose was the major oligosaccharide produced.

The β-limit dextrin from potato amylopectin had 8.9% branching and an average chain length, CL, of 11.3. Wheat and Sigma potato β-limit dextrins were more highly branched, 11%, and had a shorter average chain length of 9. The Sigma β-limit dextrin contained intermediate molecular weight polysaccharide, as did the parent Sigma potato amylopectin. Sigma potato amylopectin was probably a partially degraded amylopectin.

The G4-amylolysis limit for Sigma potato amylopectin, 73%, was considerably higher than the [β], approaching the 75% conversion reported for G4-amylolysis of soluble starch (10). The oligosaccharide fraction was predominately maltotetraose, with some G_3, G_2, and inter-

mediate molecular weight polysaccharide giving an average DP of 4.9. Nmr analysis showed that the Sigma G_4-limit dextrin was only slightly more compact with 11.7% branching, CL 8.6, than the Sigma β-limit dextrin, but appeared to have only 52 glucose units per molecule giving an average molecular weight of 8433. However HPLC excluded the dextrin indicating >70000 daltons. Possibly the HPLC sample was aggregated or a portion of the Sigma potato amylopectin was cross-linked. (It was not a different polysaccharide which would have been detected in the nmr spectrum, and it is unlikely that it was non-carbohydrate material since the % carbohydrate in Sigma potato amylopectin was similar to that measured for other carbohydrates, see Section 5.8.2.)

Sigma potato amylopectin, a commercial preparation of potato amylopectin, believed to be partially degraded amylopectin illustrated the unique specificity of G_4-amylase. The cold water-soluble Sigma potato amylopectin had a shorter (17.6) average chain length than found in standard potato amylopectin (28.7), and chromatographed on HPLC as two approximately equal populations in weight. Beta-amylolysis did not markedly affect the population distribution but did decrease the average chain length to 9.1. G_4-amylase hydrolyzed most of the population at 7000 daltons, according to HPLC, leaving only the high molecular weight material at >70000 daltons. Incongruously, this G_4-limit dextrin appeared to have a DP of 52 by nmr, and an average chain length of 8.6. Nevertheless, Sigma potato amylopectin and its β-limit dextrin contained two populations of molecules, only one of which was observed after G_4-amylolysis. And, both β- and G_4-limit dextrins were shown by nmr to have shorter average chain lengths than the parent Sigma potato amylopectin.

Because the commercial Sigma potato amylopectin appeared to modified and was not well characterized, it was not included in further studies reported here. It demonstrated that the specificity of G_4-amylase was quite different from β-amylase, but because it is not a typical amylopectin it was not included in the discussion on the fine structure of amylopectin.

The G_4-amylolysis limit of wheat amylopectin was at least 48% and perhaps greater than 57%, calculated from carbohydrate having a DP of 4.4. Maltotetraose was the major oligosaccharide produced with some G_3-G_1. An intermediate molecular weight dextrin was fractionated which was compact (13% branching, CL 7.7) with a molecular weight of about 5000. The major portion of the wheat G_4-limit dextrin showed a narrow distribution on HPLC at 10000 daltons, and an average molecular weight of 14300 from nmr analysis. Nmr also showed a densely branched molecule with low internal CL. The implications from these results with regard to the structure of amylopectin are discussed in 4.2.4.3.

4.2.2.1.2. <u>Experiment II</u>: <u>HPLC Studies of Amylolysis by Maltotetraohydrolase</u>. The G_4-limit dextrins from G_4-amylolysis of β-limit dextrins produced in the nmr studies described in Section 4.2.1 were examined by HPLC. The same G_4-amylase preparations were then used for G_4-amylolysis of wheat amylopectin and of soluble starch and the products from the G_4-amylolyses were monitored by high pressure liquid chromatography (HPLC).

a. *Analysis of Dextrins from NMR Studies*. Three hydrolysis mixtures from nmr studies, Hydrolyses X-XII, G_4-amylolyses of β-limit dextrins (see Tables 4.2-4.4), were further analyzed 6 days after the

nmr studies.   The hydrolysis mixtures retained between 62% and 97% of their initial G_4-amylase activity.   The G_4-amylase in the hydrolysis mixtures hydrolyzed soluble starch to produce maltotetraose (although the principal products from β-limit dextrins, in the nmr studies, discussed in 4.2.1.3.4, were G_3-G_1).

The solutions from Hydrolyses X-XII were precipitated with ethanol, dried, and solubilized in HPLC solvent for chromatography (see Figure 4.15).   Most of the G_4-limit dextrin from wheat amylopectin β-limit dextrin (wheat β,G_4-dextrin) chromatographed at about 8000 daltons with some additional dextrin at 30,000 daltons. Both potato β,G_4-dextrins showed the majority of the dextrin at about 20,000 daltons, with an additional population at about 8000 daltons. All three precipitates also contained oligosaccharide.

The wheat β,G_4-dextrins at 30,000 and larger were unexpected since only a single population at about 10000 daltons was observed in Experiment I, and the wheat β,G_4-dextrin solution had been subjected to extended hydrolysis.   The higher molecular weight dextrin may not be a limit dextrin or may be aggregated dextrin; this has direct implications on the results for potato β,G_4-dextrin.   However, since both potato β,G_4-dextrins (from Hydrolyses X and XI) showed identical chromatograms, although one hydrolysis mixture contained 2.5 times more G_4-amylase, the potato β,G_4-dextrins at 20000 probably are limit dextrins.   (The hydrolysis solution containing more G_4-amylase would be expected to contain more limit dextrin and the chromatograms would not be identical.)

amount of the total carbohydrate in the filtrate indicated that only 25% (10.7 μmole ge/ml) of the carbohydrate was contained in the filtrate. HPLC of the filtrate showed two peaks; one peak corresponding to maltotetraose, and the second peak being skewed with a peak apex at 10000 and a long tail extending to the exclusion limit of 50000.

Samples removed after 45 min and after 90 min contained 76% and 38%, respectively, (117 μmole ge/ml and 58.7 μmole ge/ml) of the original carbohydrate. HPLC of each filtrate showed two peaks, the high molecular weight peak which remained skewed but decreased in size and the average molecular weight from ca. 10000 to the peak apex corresponding to G4 which showed at least a four-fold increase in size.

Fig. 4.15. HPLC gel permeation chromatogram of G_4-limit dextrins from (a) potato amylopectin and from (b) wheat amylopectin, Synchropak column, eluent H_2O containing 0.02% NaN_3, 0.2 ml/min, RI detection. The G_4-limit dextrin solutions were prepared from the dried ethanol precipitates of Hydrolyses X and XII respectively.

the average molecular weight of the peak, at 10000 was smaller than the molecular weight of 15000 determined by that method in Chapter 3 in Experiment 3.

b. Amylolysis of Wheat Amylopectin. G_4-amylolysis of an aqueous solution of wheat amylopectin (30.8 μmole ge/ml, 1% w/v) was followed by HPLC. The wheat amylopectin solution was slightly turbid and clogged a 45 micron filter. The small amount of filtrate contained <0.1 μmole ge/ml carbohydrate and was not detected on HPLC.

Within 5 min after the addition of G_4-amylase, the solution was clear and appeared to readily pass through the filter although mea-

surement of the total carbohydrate in the filtrate indicated that only 35% (10.7 µmole ge/ml) of the carbohydrate was contained in the filtrate. HPLC of the filtrate showed two peaks; one peak corresponding to maltotetraose, and the second peak being skewed with a peak apex at 10000 and a long tail extending to the exclusion limit of 70000.

Samples filtered after 28 min and after 55 min contained 56% and 59%, respectively, (17 µmole ge/ml and 18.2 µmole ge/ml) of the original carbohydrate. HPLC of each filtrate showed two peaks, the high molecular weight peak which remained skewed but decreased in amount and the average molecular weight from ca. 10000 to 7000 and a peak corresponding to G_4 which showed at least a four-fold increase in area.

When the 55 min filtrate was left for 4 days, then chromatographed, the skewed peak remained at 7000 but the tail was shorter, extending only to 40000 daltons. This peak contained approximately 46% of the material chromatographed, an amount corresponding to the 43%-52% estimated in Experiment I for wheat G_4-limit dextrin. The average molecular weight of the peak, at 7000, was slightly lower than the molecular weight of 10000 determined for purified G_4-limit dextrin in Experiment I.

HPLC of the filtrates showed only two peaks at any time, one a higher molecular weight fraction which decreased in amount and average molecular weight from ca. 10000 to 7000, and a peak corresponding to maltotetraose which increased in size during the experiment. The G_4-amylase used in this experiment was the same used to produce β,G_4-limit dextrin in the nmr study, Hydrolysis XI, which showed an addi-

tional peak at 30000 daltons, as discussed in Section *a* above.

    c. Amylolysis of Soluble Starch. In another experiment, amylolysis products from soluble starch were examined at 1.5 h and 1 week after hydrolysis. The 1% soluble starch solution clogged the filter-- the small amount of filtrate gave a small broad peak between the exclusion limit and 50000 daltons.

    At 1.5 h after hydrolysis there was a skewed peak at 30,000- 35000 containing ≈15% of the material with a tail extending to the exclusion limit of 70,000 containing 41%. A second, narrow peak corresponding to G_4 contained 42% of the material in the filtrate.

    After one week approximately 63% of the material chromatographed at G_4, but was shown by tlc to contain some G_3 and G_2; another 12% chromatographed at <5000 and was observed as a shoulder on the G_4 peak. The remaining material (19%) was contained in a skewed peak at 18000 with a tail extending to the exclusion limit at 70000.

    The G_4-limit dextrin from soluble starch does not appear to be as well defined as G_4-limit dextrin from amylopectin; however the presence of a peak which decreases in molecular weight supports both the cluster model for amylopectin and the evidence for endo-amylolysis by G_4-amylase. Approximately 75% of the material chromatographed at <5000 daltons. This would probably appear as an amylolysis limit of 75%. Schmidt and John (10) previously reported 75% conversion of soluble starch by G_4-amylase. G_4-amylase probably first removed clusters from the main starch molecule and subsequently further hydrolyzed the cluster by removing G_4 and/or by further endo-amylolysis.

d. *Summary and Comments*. Molecules of amylopectin were expec-
ted to pass through a 45 micron filter which is large enough to permit
some microorganisms to filter; even the smaller molecules of the β-
limit dextrins consistently aggregate or interact with the filter and
are retained. However, once G4-amylase was added to the polysaccha-
ride, changes rapidly occurred, not only in the physical appearance
changing from a turbid to a clear solution, but in the amount of sac-
charide that passed through the filter. In both G_4-amylolysis experi-
ments, the high molecular weight fraction which passed through the
filters after G4-amylolysis, was further hydrolyzed so that distinct,
high and intermediate molecular weight fractions could be detected by
HPLC.

The G_4-amylases used in the two G_4-amylolysis studies examined
by HPLC were from the same preparations of G_4-amylase used in nmr
studies of G_4-amylolysis of β-limit dextrins. Analysis of the β,G_4-
limit dextrins from potato and wheat amylopectin showed limit dextrin
at 8000 daltons as well as higher molecular weight peaks. The wheat
β,G_4-limit dextrin at 30000 was a secondary peak and may have been
aggregated dextrin. The potato β,G_4-limit dextrin at a molecular
weight of 20000 was the major peak in two preparations using different
concentrations of G_4-amylase and is probably not aggregated dextrin.

4.2.2.1.3. Experiment III: Hydrolysis
of Wheat Amylopectin by G_4-Amylase. G_4-amylolysis of wheat amylopectin
was followed for 31 hours as shown in Figure 4.16 and the G_4-limit
dextrin purified for analysis.

a. *G_4-amylolysis*. As shown in Figure 4.16, wheat amylopectin

was rapidly hydrolyzed during the first 6 hours, reaching a limit thereafter. At 24 h, the G_4-amylase retained 53% (0.13 Boeh U/ml) of its activity but no further hydrolysis occurred. During the first six hours the appearance of the wheat amylopectin changed from a turbid, homogeneous dispersion to a clear solution containing a fine white suspension in the lower quarter of the test tube. The rapid change from a turbid, milky solution to a clear solution had previously been observed in all G_4-amylase-starch solutions. The precipitate observed was not present in controls but was present in the wheat amylopectin hydrolysis solutions, including those from β-amylolysis. The white precipitate was not bacterial cells. It was insoluble in lower alcohols, CH_2Cl_2, and boiling water. It contained <3% by weight carbohydrate, and was not detectable by tlc. Elemental analysis gave 41.9% C, 6.5% H, 0.7% N.

An average hydrolysis limit of 26% is shown in Fig. 4.16; the 26% being calculated from the amount of reducing sugar produced in the hydrolysis mixture. This figure is, however, probably too high since maltotetraose appears to be overoxidized by the alkaline ferricyanide reagent, giving an artificially high reducing value (see 5.8.3.2).

b. *Analysis of the Hydrolyzate*. A sample was removed at 28 h and the insoluble white precipitate removed by centrifugation leaving a clear solution containing 25% μmole reducing sugar/μmole total carbohydrate, shown by tlc to be high or immediate molecular weight polysaccharide (streaking from the origin) and G_4. All of the carbohydrate passed through the 0.45 micron filter and through the HPLC Chrompak column. Chromatography and analysis of the hydrolyzate is shown in Fig. 4.17. The broad peak eluting between 50,000 daltons and

Hydrolysis of Wheat Amylopectin

Figure 4.16
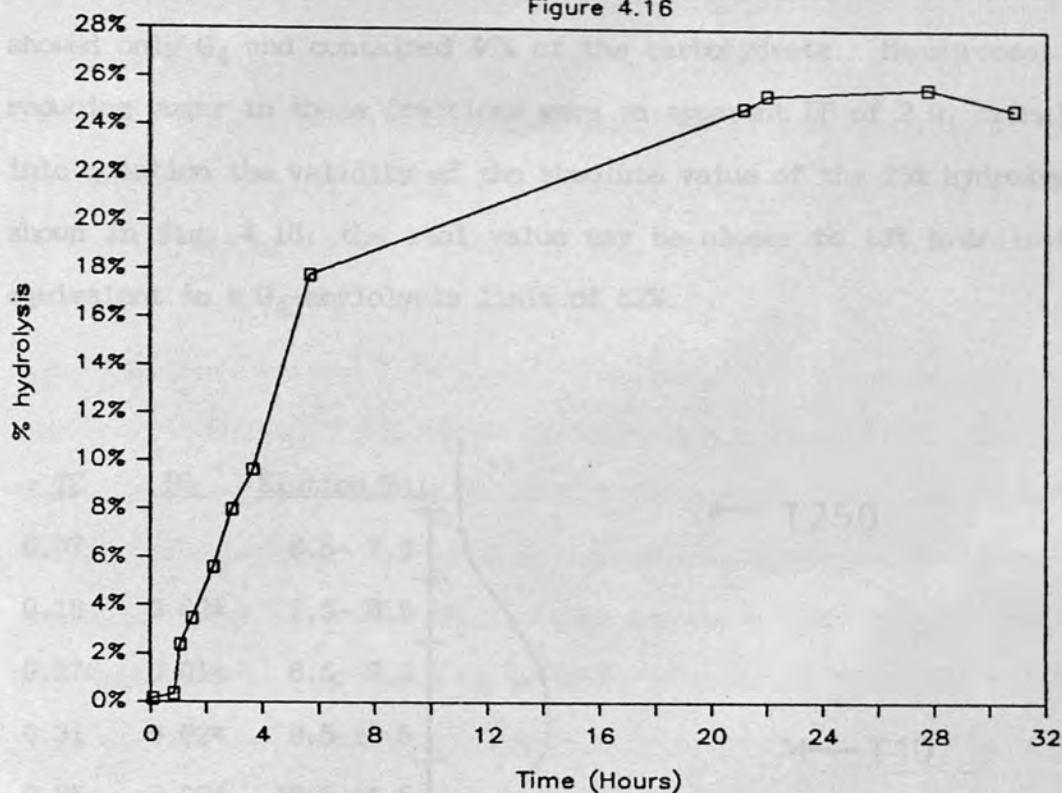
Fig. 4.16. G_4-amylolysis of wheat amylopectin (0.24 Boeh U/ml, 2.5% TC/ml) in 50 mM imidazole buffer containing 0.002% chlorhexidene, pH 7 at 37°C, except 0–1 h at 20°C. Percent hydrolysis calculated from the reducing sugar (RS) produced (µmole/ml, alkaline ferricyanide method, maltose standard) in the wheat amylopectin solution containing 157.7 µmole ge/ml total carbohydrate (TC).

% Hydrolysis = 100 RS/TC

≈7000 daltons, median 15,000 daltons (9.5 ml), contained 54% of the total carbohydrate, and appeared as high molecular weight material on tlc (fractions at 9.5 ml and 11.5 ml, no streaking was evident). Fractions from 10.5–12.2 ml (11.2 pa) and 12.2–15 ml (12.6 pa) contained uv (254 nm) material; that at 12.6 ml having a spectrum similar

to imidazole. Tlc of fractions collected at 12.5 ml and 13.5 ml showed only G_4 and contained 46% of the carbohydrate. Measurement of reducing sugar in these fractions gave an apparent DP of 2.0, bringing into question the validity of the absolute value of the 25% hydrolysis shown in Fig. 4.16; the real value may be closer to 13% hydrolysis, equivalent to a G_4-amylolysis limit of 52%.

TC	RS	Elution Vol
0.07		6.5- 7.5
0.18	0.02*	7.5- 8.5
0.27	0.01*	8.5- 9.5
0.31	0.02*	9.5-10.5
0.25	0.02*	10.5-11.5
0.32	0.07*	11.5-12.5
0.75	0.44	12.5-13.5
0.44	0.20	13.5-14.5
0.04	ND	14.5-15.5

$\longleftarrow$ T250

$\longleftarrow$ T10

G_2
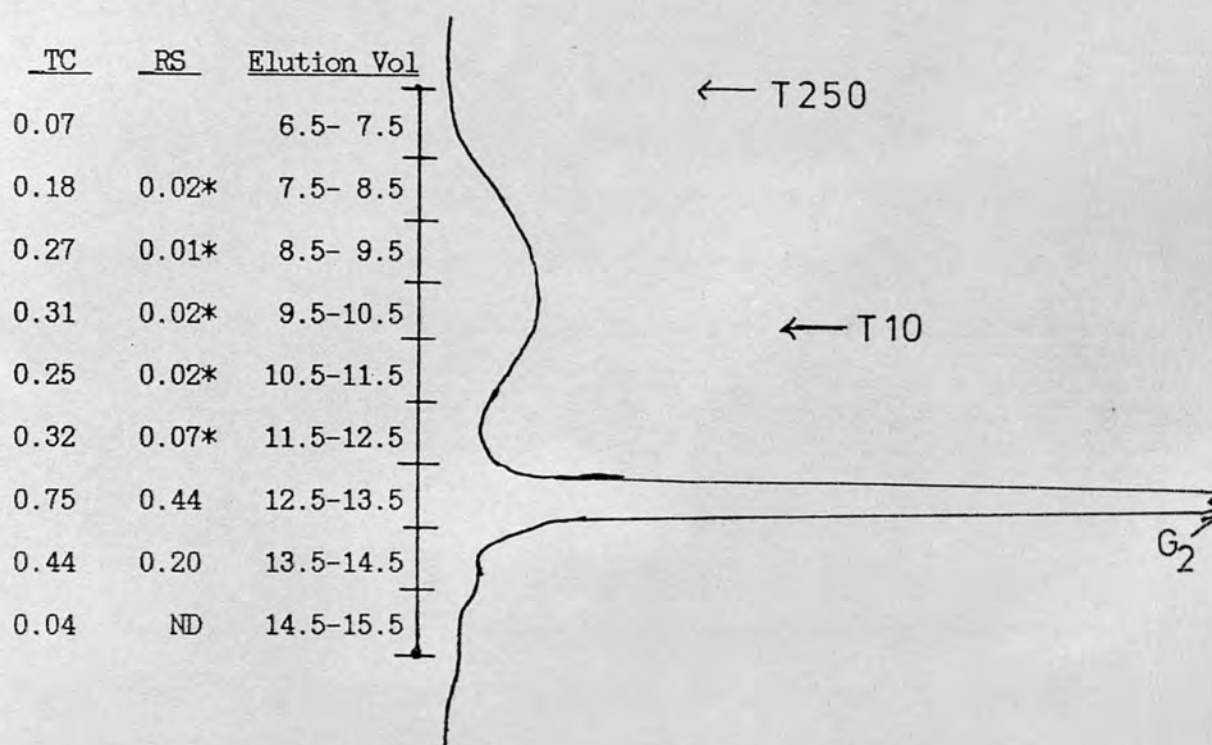
Fig. 4.17. HPLC chromatogram of G_4-amylase-wheat amylopectin hydrolyzate. Chromatography on Chrompak TSK G2000SW, 300 mm x 7.5 mm; mobile phase: H_2O containing 0.02% NaN_3, 0.5 ml/min, RI detection. Sample from the hydrolyzate shown in Fig. 4.16, removed at 28 h, heated, centrifuged, filtered. Fractions of 1.0 ml were collected (|——|) and examined by tlc, for uv absorbance, total carbohydrate and reducing sugar. Symbols: TC, μmole/ml total carbohydrate;

RS, μmole/ml reducing sugar, * estimated; Elution Volume in ml of collected fraction.

Ethanol (2 vol.) precipitated 35% of the carbohydrate in the hydrolysis mixture from 28 h, which chromatographed as the broad peak shown in Fig. 4.17 with peak apex at 14000 daltons. The remaining carbohydrate, that from the ethanol supernatant chromatographed only at elution volume >12 ml.

The hydrolyzate from 31 h (2038 μmole ge, 492 μmole RS) was similarly purified (1987 μmole ge, 514 μmole RS after heating and centrifugation, see scheme in 4.3.2.1.3) except that the G_4-limit dextrin was precipitated with 3 volumes of ethanol. The ethanol supernatant was primarily G_4 (nothing at the origin on tlc, and on heavy spotting of the mixture a very small amount of G_2, again indicating that G_4 was over-oxidized by the alkaline ferricyanide reducing sugar reagent) and contained 55.3% (1126 μmole ge, 475 μmole RS) of the carbohydrate in the original hydrolyzate.

Some of the ethanol precipitate, the G_4-limit dextrin, was lost on freezedrying, however the remaining 150.6 mg contained 4.8 μmole ge/mg (i.e. 77% carbohydrate) or 35% (718 μmole ge) of the carbohydrate in the original hydrolyzate, and 0.0 μmole RS/mg (although tlc showed a small amount of G_4, and material at the origin, but no streaking). Chromatography (HPLC, Chrompak) showed a single symmetrical carbohydrate peak extending throughout the fractionation range ($\approx$5000-40000 daltons) with a peak apex at 14000 daltons.

The G_4-limit dextrin was reprecipitated with ethanol and the nmr spectrum recorded as shown in Fig. 4.18. It was not possible to accurately integrate the signals but it is apparent that the proportion of reducing termini was very small and the degree of branching

SAMPLE DS39 - WITH INTEGRALS
Wheat amylopectin G_4-amylase limit dextrin
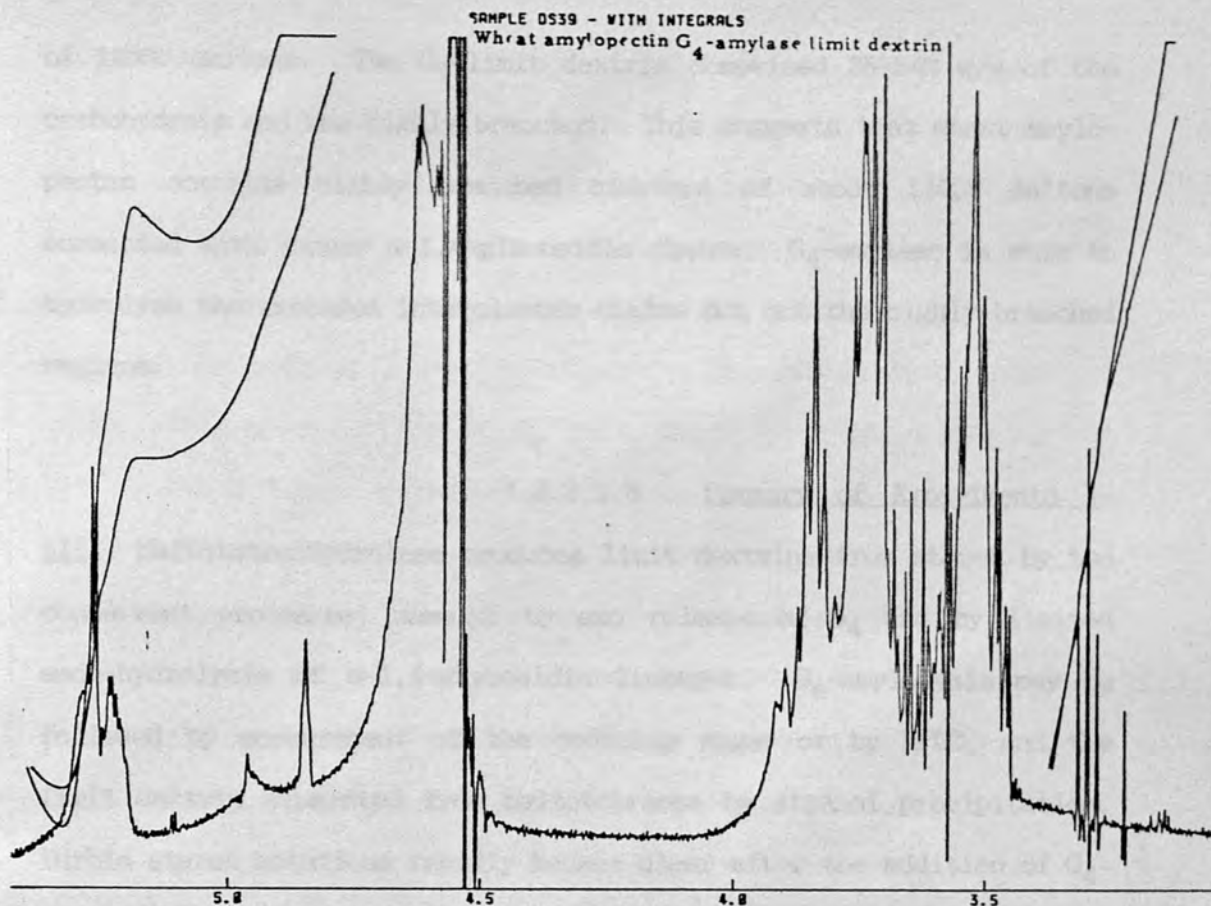
Fig. 4.18. 500 MHz ^{1}H-nmr spectrum of wheat amylopectin G_4-limit dextrin in D_2O.

high. At least five α-1,4-glycosidic protons in slightly different environments are detectable. They are probably protons contiguous to branch points since the α-1,4-glucosidic protons in the branched oligomers, panose and methyl-β-(6-α-glucosyl)maltoside, were shifted upfield relative to G_3 and G_7 (76, 86). The two glycosidic protons in G_3, one on the nonreducing terminal and one adjacent to the reducing terminal unit are coincident as shown in Fig. 4.1.

In summary, maltotetraohydrolase hydrolyzed between 12-25% of the glycosidic linkages in wheat amylopectin, producing maltotetraose almost exclusively and a limit dextrin with a median molecular weight

of 14000 daltons. The G_4-limit dextrin comprised 35-54% w/w of the carbohydrate and was highly branched. This suggests that wheat amylopectin contains highly branched clusters of about 14000 daltons connected with longer α-1,4-glucosidic chains. G_4-amylase is able to hydrolyze the extended intercluster chains but not the highly branched regions.

4.2.2.1.4. <u>Summary of Experiments I-III</u>. Maltotetraohydrolase produces limit dextrins from starch by two concurrent processes; namely, by exo release of G_4 and by limited endo-hydrolysis of α-1,4-glycosidic linkages. G_4-amylolysis may be followed by measurement of the reducing sugar or by HPLC, and the limit dextrin separated from maltotetraose by ethanol precipitation. Turbid starch solutions rapidly became clear after the addition of G_4-amylase. G_4-amylase-starch solutions at 37°C retained >50% of the amylase activity for >48 hours.

Hydrolysis of starch into G_4 and a limit dextrin could be monitored by HPLC of aliquots taken at various time intervals during G_4-amylolysis. During the early stages of G_4-amylolysis there is a major population of dextrin at <35000 daltons which gradually decreases in average molecular weight to a G_4-limit dextrin whose molecular weight may depend on the source of starch. In each instance, however, the principal G_4-limit dextrin seems to be restricted to one or two major populations of narrow mean molecular weight.

Wheat amylopectin was hydrolyzed by G_4-amylase in three separate experiments. A G_4-amylolysis limit of at least 46% oligosaccharide, possibly as high as 62%, is produced from wheat amylopectin. The

higher amylolysis limit was determined from ethanol precipitation which, under the conditions used here, did not precipitate all of the G_4-limit dextrin. This intermediate G_4-limit dextrin (Supernatant 2P, Table 4.7), probably <5% of the total carbohydrate, had an average molecular weight estimated to be 5000 daltons. This G_4-limit dextrin had a DP >26 with at least 13% branching, an average chain length of <7.7. The major portion of G_4-limit dextrin, at least 35% of the total carbohydrate, chromatographed in a single peak, which varied between different experiments, between 7000 and 14000 daltons. The G_4-limit dextrin had a narrow weight distribution and no G_4-limit dextrin >40000 daltons was detected. Nmr showed that the G_4-limit dextrin had a DP >88 and >15% branching, an average chain length of <6.8.

Maltotetraose was the major or only oligosaccharide detected, with G_3 as a secondary oligosaccharide, G_2, and G_1 found in some hydrolysis mixtures.

The results of these experiments can be explained using the cluster model for the structure of amylopectin and by the unique specificity of maltotetraohydrolase. G_4-amylase hydrolyzes amylopectin by an endo-mechanism only at regions of extended chains between clusters of highly branched regions. The endo-amylolysis gives clusters which are further hydrolyzed by G_4-amylase, probably by an exo-mechanism in which G_4 is removed from the nonreducing termini, decreasing the average molecular weight of the cluster to the G_4-limit dextrin of between 7000-14000 daltons in wheat amylopectin. In wheat amylopectin the G_4-limit dextrin, and hence the clusters, had a very narrow weight distribution, suggesting clusters of very uniform

branching and internal chain length.

        4.2.2.2. <u>Hydrolysis of Blue Amylose</u>. Blue amylose can be used to descriminate between exo- and endo-amylase activity, as well as to detect contaminating endo-activity in preparations of classical exo-amylases. Blue amylose, a water insoluble commercial preparation of potato amylose covalently bonded with Remazolbrilliant Blue R dye, produces water soluble blue coloured saccharide when hydrolyzed by endo-amylolysis. The blue dye marker prevents exo-amylases from releasing dye-saccharide product. Dellweg *et al* (9) first used a blue starch as substrate for G_4-amylase, selecting *Pseudomonas stutzeri* for culture and subsequent purification of G_4-amylase on the basis of blue starch hydrolysis. Nakakuki *et al* (14) studied several exo-amylases and found G_4-amylase to be the only exo-amylase which hydrolyzed blue starch. Cross-linked blue starch has been used for clinical determination of α-amylase activity and has been calibrated to International Units of activity (90, 91) and the optimal conditions described (91). Using those optimal conditions (10 mg/ml, i.e. saturating substrate, for up to 4.5 IU/ml in phosphate buffer, pH 7, 37°C) for blue amylose and α- and G_4-amylase, it has been possible to show that different preparations of G_4-amylase all partially hydrolyze blue amylose to produce G_4-limit dextrins. The G_4-limit dextrins can be further hydrolyzed by α-amylase.

As shown in Figure 4.19, G_4-amylase partially hydrolyzes blue amylose producing <57% of the absorbance of α-amylase. At a concentration of 0.1 Boeh U/ml, an hydrolysis limit was rapidly approached by α-amylase and in <1 h was within 6% of the maximum absorbance. The rate of G_4-amylolysis was lower and a limit approached only after 2 h.
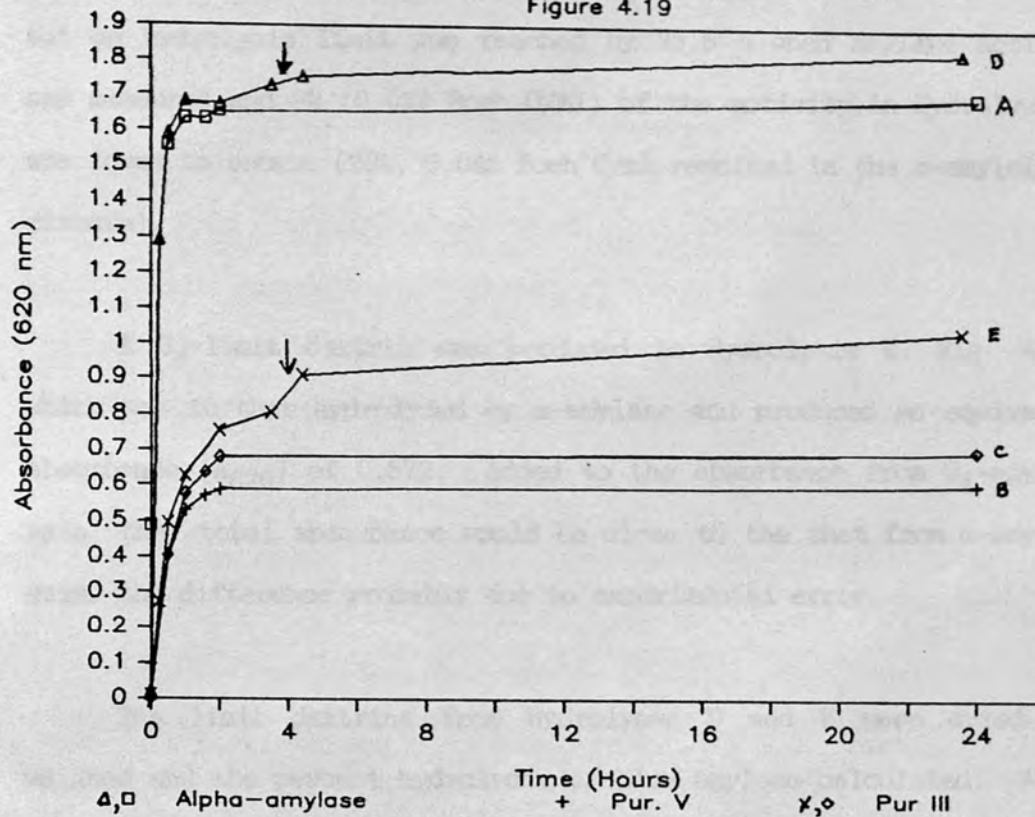
Fig. 4.19. Hydrolysis of blue amylose by α-amylase and G₄-amylase.
Blue amylose (10 mg/ml 100 mM phosphate buffer, pH 7.0) was hydrolyzed
at 37°C by 0.1 Boeh U/ml of α-amylase from *Bacillus amyloliquefaciens*
and G₄-amylase from different cultures and purification steps.

α-amylase: Hydrolyses A and D;

G₄-amylase: Hydrolysis B, Na Culture B Purification V Step 4b;

Hydrolysis C, Trypticase I Culture Purification III Step 4;

Hydrolysis E, Trypticase I Culture Purification III Step 8 from
affinity chromatography.

At 3.6 h, 0.125 Boeh U/ml of the respective amylase was added to
Hydrolyses D and E, indicated by ↓ .

Further G_4-amylolysis occurred after the addition of 0.125 Boeh U/ml, but an hydrolysis limit was reached by 23.5 h when amylase activity was measured and 9% (0.019 Boeh U/ml) of the activity in Hydrolysis E was found to remain (20%, 0.045 Boeh U/ml remained in the α-amylolysis mixture).

A G_4-limit dextrin was produced in Hydrolysis E, Fig. 4.19, which was further hydrolyzed by α-amylase and produced an equivalent absorbance (A_{620}) of 0.572. Added to the absorbance from G_4-amylolysis, the total absorbance would be close to the that from α-amylolysis, the difference probably due to experimental error.

The limit dextrins from Hydrolyses D and E were dried and weighed and the percent hydrolysis of blue amylose calculated. About 44% of the blue amylose remained from α-amylolysis (9.6 mg from 22 mg blue amylose) and 51% remained from G_4-amylolysis (10.3 mg from 20.3 mg). Since these figures are based on the blue amylose remaining after removal of 8 samples, and since some precipitation occurred even as samples were being removed, the final sample which was dried probably contained blue amylose precipitate from previous samples, and the final percent hydrolysis was probably higher than 56% hydrolysis for α-amylase and 49% hydrolysis for G_4-amylase. It is apparent that α-amylase hydrolyzes blue amylose to a greater extent than does G_4-amylase and also that blue amylose contains regions which α-amylase from *B. amyloliquefaciens* will not hydrolyze.

The amylolysis products were examined by tlc and a marked difference noted between the two amylases. After chromatography, blue colour was observed only at the tlc origin from G_4-amylolysis but from

α-amylolysis contained heavy blue streaking through ≈G_6 (R_G 0.44) and two spots between G_2 and G_4 (R_G 0.79 and 0.70). The same substances were detected after charring. The hydrolyzate from G_4-amylolysis showed only G_4 (R_G 0.66) and no additional substances from amylolysis. A mixture of oligosaccharides containing dye marker was apparently produced by α-amylolysis, suggesting that G_4-amylase hydrolyzed only regions of amylose chains containing no dye markers leaving significantly larger dye-saccharide molecules (yet small enough for some to be water soluble) and maltotetraose without dye markers. Nakakuki *et al* (14) reported similar products from degradation of cross-linked blue starch by α-amylase from *B. licheniformis* and by G_4-amylase.

It appears that G_4-amylase hydrolyzes blue amylose by an endo-mechanism but only in regions containing no dye marker. More than 49% of the dye-saccharide fragments from G_4-amylolysis were sufficiently small to be water soluble, but large when compared to fragments from α-amylolysis. G_4-amylase was able to hydrolyze the dye-saccharide fragments, possibly by an exo-mechanism, to produce maltotetraose containing no dye marker. This unique action of G_4-amylase to hydrolyze only extended regions of linear glucosyl chains could similarly limit G_4-amylase to hydrolysis of chains between highly branched clusters of glucosyl residues in amylopectin. This directly supports the cluster model and the data from the nmr and HPLC studies.

It is unlikely that the endo-activity found in G_4-amylase is due to contaminating α-amylase, since three different preparations of G_4-amylase gave similar hydrolysis limits. The final absorbances for Hydrolyses B and C shown in Figure 4.19 is lower than that in Hydrolysis E, probably due to loss in G_4-amylase activity. G_4-amylase showed

rapid loss in activity in the presence of blue amylose when compared to the stability of G_4-amylase in the presence of amylopectin (4.2.1.3.6, 4.2.2.1, and 4.2.3). In Hydrolysis E, additional G_4-amylase was added at 3.6 h and only 8% of the activity remained at 23.5 h; and in the control study of G_4-amylase with blue amylose G_4-limit dextrin only 22% of the activity remained after 1.5 h. Almost no difference was detected between Hydrolysis B and Hydrolysis C although one G_4-amylase preparation came from a culture in Trypticase I medium and the other from a culture in Na medium; and both preparations were only partially purified (both principally concentrated over a 10000 dalton membrane and frozen, thawed, centrifuged). The G_4-amylase used in Hydrolysis E had been purified by affinity chromatography, had a very high specific activity ($\approx$2000 Boeh U/mg); yet showed similar hydrolysis of blue amylose.

The rate of amylolysis of blue amylose was measured using the amylase preparations as in Hydrolyses A, B, and C except at 0.01 Boeh U/ml. The two G_4-amylases gave exactly the same absorbances (A_{620}) and a decrease in hydrolysis rate after 60 min. During the first 60 min absorbance increased at a rate of 0.0034 A/min (intercept 0.0002). With α-amylase, the rate was linear from 30-120 min at 0.0053 A/min however the initial rate of hydrolysis was so rapid at 0.01 Boeh U/ml that at 0 min there an absorbance of 0.56 A. It is clear that under these conditions the endo-activity of G_4-amylase is considerably less than that of α-amylase.

4.2.3. **Preparation of Maltotetraose.** G_4-amylase from Trypticase I Cultures, Purifications I and II was used for preparation of maltotetraose. As shown in the summary in Table 4.8, the yield of

Table 4.8. Summary of Maltotetraose Preparations.

Experiment[1]	Filtrate Weight[2] (g)	Total Carbohydrate[2] (μmole ge/mg)	Reducing Sugar[3] (μmole/mg)	HPLC Areas[4] $G_4:G_3:G_2$	% w/w[4] G_4
I	1.22	4.9	1.42	165:11:6	91%
II	3.54	4.1	1.53	264:1:3	99%
III	1.51	4.4	1.42	17:3:4	71%

[1]G_4-amylase from Purifications I (Exp. I) and II (Exp. II and III) was incubated with substrate in an ultrafiltration cell and filtrate collected through an Amicon PM-10 membrane; G_4-amylase activity, DNS assay, 37°C.

Experiment I: 2.0 Units G_4-amylase, incubated at room temperature with a total of 1.6 g cornstarch solution in 10 mM Buffer A and 5 g potato amylose (as shown in Table 4.9);

Experiment II: 125 Units G_4-amylase, incubated with 21 g potato amylose slurry in H_2O at 35°C, collected continuously for 16 h;

Experiment III: 69 Units G_4-amylase incubated with 5 g potato amylose slurry in H_2O at 35°C, collected continuously for 19 h.

[2]Weight and total carbohydrate of lyophilizate from ultrafiltrate.

[3]Reducing sugar in lyophilizate, alkaline ferricyanide (AF) reagent.

[4]HPLC Area Ratios, APS-Hypersil column, oligosaccharides detected; % w/w G_4 calculated from oligosaccharide HPLC areas.

G_4 was high in all three experiments, particularly in Experiments I and II. At no time were oligosaccharides longer than G_4 observed by HPLC; on tlc, some material was found near the origin (light streaking), indicating higher molecular weight saccharide which could pass through the ultrafiltration membrane (10000 dalton retention, globular protein standard).

Experiments II and III were expected to give similar results in G_4 production. The G_4-amylase in both experiments was from the same culture and were from parallel purification fractions (see Table 3.2, Purification II, Step 4), except solubilized in different buffers but with similar specific activities and not expected to differ in their properties or purity. In both experiments, substrate and conditions were similar. The higher in rate of filtration ($\approx$60 ml/h in II, $\approx$150 ml/h in III) in Experiment III does not seem likely to have caused the lower proportion of G_4 shown in Table 4.8.

The filtrates from Experiments II and III were lyophilized and 2% w/v solutions were found to be partially insoluble in H_2O, giving slightly cloudy solutions. Lyophilizate from Experiment I was partially insoluble at 0.5%. In Experiment I the precipitate may be calcium salt from the hydrolysis buffer, however, in Experiments II and III buffer was not present and could not account for the precipitate.

HPLC of the first four filtrate fractions from Experiment III, collected during 19 h of hydrolysis, showed similar proportions of G_4-G_2 to be produced throughout the hydrolysis of potato amylose. From the areas of G_4 there appeared to be little loss in G_4-amylase

activity and the hydrolysis reaction could probably have been continued. However, some material, probably short chains of amylose, was found at the origin on tlc, presumably the proportion of this material to that of G_4 would have increased with time as the longer amylose chains were hydrolyzed. During the 19 h of hydrolysis approximately 24% w/w of the potato amylose was hydrolyzed to oligosaccharide.

In Experiments II and III, in which potato amylose was the only substrate, G_4 was produced in high yields but G_3 and G_2 were also detected in up to 29% w/w (of the oligosaccharide weight). It is unlikely that the G_3 and G_2 were from the end of amylose chains since G_1 was not detected in any of the filtrates and should be found with equal probability. (Glucose is a product from G_4-amylolysis of G_5 (12).) If maltotetraose were produced exclusively, then >169 G_4 would be produced for each possible G_3-G_1 from potato amylose, reported to have an DP of 4850-6340 and an CL of 510-670 (44, 92).

In Experiment I, summarized in Table 4.9, G_4-amylase again produced G_3 and G_2. The principal substrate was cornstarch, and G_3 was the secondary oligosaccharide rather than G_2 as in Experiments II and III. In Experiments II and III, with the substrate potato amylose, slightly more G_2 was produced than G_3. The proportions of G_3:G_2 may be coincidental, but G_2 was also found to be the predominant secondary oligosaccharide from G_4-amylolysis of potato amylopectin.

In Experiment I, reducing sugar was produced during the first 24 h at an average rate of 151 µmole/h. The rate decreased to 45 µmole/h during the next 93 h of incubation. After the addition of potato amylose, the rate increased to >154 µmole/h (Filtrate I,3). Filtrate

Table 4.9. Scheme for Maltotetraose Preparation, Experiment I, and Analysis of the Ultrafiltrates.

Filtrate[3]
I,1; 24 h

      20 ml (0.4 g) cornstarch solution[1]
       + 1.75 ml G_4-amylase[2]
       + 30 ml (0.6 g) cornstarch solution at 24 h
       ⟶ 36 ml (2.52 mmole RS)

      15 ml (1.05 mmole RS remaining)

I,2; 117 h
       + 30 ml (0.6 g) cornstarch solution at 94.5 h
       ⟶ 29 ml (3.39 mmole RS)

      16 ml (1.87 mmole RS remaining)

I,3; 143.5 h
       + 35 ml H_2O containing 5.0 g potato amylose at 119 h
       ⟶ 30 ml (3.51 mmole RS)

      21 ml (2.46 mmole RS remaining)

I,4; 167 h
       ⟶ 3 ml (0.05 mmole RS)

      18 ml (0.32 mmole RS remaining)

Filtrate	Volume (ml)	RS (μmole/ml)	HPLC G_4 : G_3 : G_2
I,1	36	70	73 : 5 : 0
I,2	29	117	101 : 7 : 1
I,3	30	117	87 : 8 : 4
I,4	3	18	35 : 12 : 2

[1]Substrate: 2% cornstarch solution in 10 mM glycerophosphate buffer containing 5 mM $CaCl_2$, pH 7; potato amylose slurry in H_2O.

[2]G_4-amylase (2 Units, DNS assay, 25°C) was incubated at room temperature with substrate in an ultrafiltration cell.

[3]Filtrate was collected (at 24h, 117h, 143.5h, and 167h) through an Amicon PM-10 membrane and analyzed for reducing sugar (RS) with dinitrosalicylate reagent and by HPLC using the APS-Hypersil column.

I,4 showed a sharp decrease in reducing sugar even though 117 μmole/ml
of reducing sugar should have remained from the previous hydrolysis.
After the extended period of incubation without sterile conditions (>6
days), it is probable that the substrate solution was contaminated.
No further filtrate was collected.

The filtrates from Experiment I were combined and lyophilized.
Some of the results are shown in Table 4.8. A portion of the lyophi-
lizate was insoluble, even at concentrations as low as 0.5% w/v. With
heavy spotting, tlc showed material at the origin, G_4, G_3, and G_2.

In order to determine whether the material at the tlc origin was
high molecular weight carbohydrate, a portion of the lyophilizate was
filtered through a Millipore PS ultrafiltration membrane (≈1000 dalton
retention), the concentrate was washed with H_2O, and the three frac-
tions (concentrate, filtrate and wash) examined. Tlc of the turbid
concentrate, clear wash, and clear filtrate all showed G_4 and material
at the origin; therefore, the material at the tlc origin was not
necessarily high molecular weight carbohydrate. The material may have
been glycerophosphate from the hydrolysis buffer. The three solutions
were lyophilized and weighed (concentrate, 321 mg; wash, 22 mg;
filtrate, 37 mg). Solutions (1%) of all three lyophilizates were
slightly cloudy. A 4% solution from the concentrate lyophilizate
formed a gel which settled within a few minutes of mixing. The lyoph-
ilizate from the concentrate contained 4.8 μmole ge/mg, DP 3.2; from
the wash 3.6 μmole ge/mg, DP 3.1; and from the filtrate 2.9 μmole
ge/mg, DP 2.9. The percent carbohydrate in the concentrate (78% w/w)
was well within the range found for other carbohydrates (see 5.8.2)
and implies the insoluble suspension was not carbohydrate or was a

minor component with regard to weight.

Another portion (500 mg) of the lyophilizate from Experiment I was examined by gel filtration chromatography (Figure 4.20). Again, part of the lyophilizate was insoluble and remained in the applicator cup as a gel when applied to the column. The gel was lyophilized (4.0 mg) and insoluble at 0.4 mg/ml, was not detected by tlc, contained 14% w/w protein, 0.79 µmole ge/mg (14% carbohydrate) and 0.2 µmole reducing sugar/mg. The gel may be, at least partly, a calcium glycerophosphate matrix.

Maltotetraose was eluted from the Bio-Gel P-4 column in fractions 24-27 (see Table 4.10) which contained 262 mg, >52% of the weight and carbohydrate applied to the column. As shown in Table 4.10, G_3 and G_2 were also detected, and in fractions 28 and 29 only G_2 was observed. From chromatography it is estimated that the total G_2 and G_3 was <20 mg (<4%) of the sample weight, or 7% of the oligosaccharide weight (comparable to the 9% determined by HPLC, see Table 4.8). At no time was glucose detected. Neither was any maltopentaose or maltohexaose identified. Tlc of the fractions showed only the oligosaccharides indicated in Table 4.10.

Carbohydrate was detected at the void volume (fractions 3-5) which, along with fractions 6-14, showed material at the origin on tlc. On standing, a cloudy precipitate formed in fractions 2, 3, 15-20 (very slightly turbid), and 21-29 (slightly turbid), although fractions 4-14 and 30-40 remained clear. The cause of the turbidity was not determined. It was not from the buffer, except possibly in fractions 24-29. Conductivity measurements (see Fig. 4.20) indicated

Maltotetraose Prep. from Experiment I

Figure 4.20
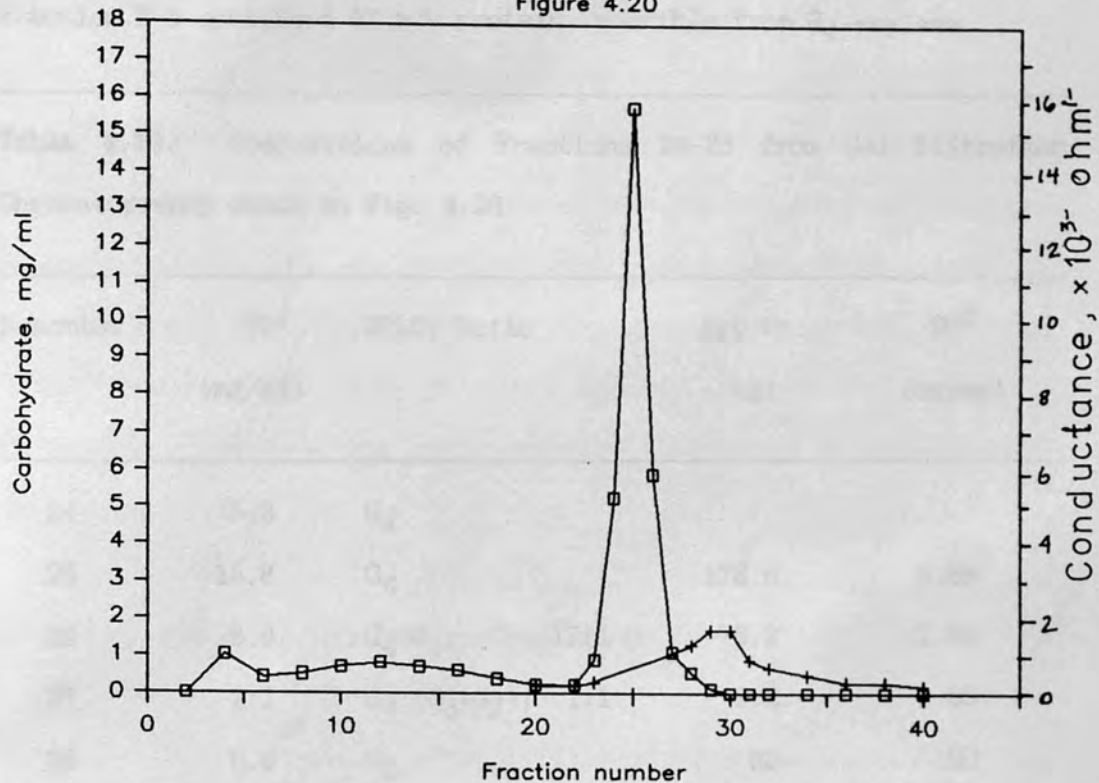
Fig. 4.20. Chromatography of Ultrafiltrate from Maltotetraose Preparation, Experiment I. Lyophilizate (0.5 g) from Experiment I described in Tables 4.10 and 4.11, in 2 ml H_2O was chromatographed on a jacketed, 65°C, Bio-Gel P-4, 100-200 mesh column, 26 mm x 96 cm, flow rate 48 ml/h. Fractions (9.6 ml) were collected after 150 ml; carbohydrate, mg/ml (—□—); conductance, x 10^{-3} ohm^{1-} (— +—).

that the majority of the hydrolysis buffer, sodium glycerophosphate and calcium chloride, eluted in fractions 26-32. It is unlikely that the precipitate was retrograded short amylose chains since fractions 4-14 remained clear. After lyophilization fraction 24-25 was soluble at 20 mg/ml, fractions 26 and 27 were partially soluble at 10 mg/ml

(soluble at 2 mg/ml), and fraction 2-3 partially soluble at 2 mg/ml. Fraction 2-3 contained 5% w/w protein, possibly from G_4-amylase.

Table 4.10. Compositions of Fractions 24-28 from Gel Filtration Chromatography shown in Fig. 4.20.

Fraction	TC^1	HPLC; Ratio		Dry Wt.	TC^2
	(mg/ml)			(mg)	(mg/mg)
24	5.3	G_4			
25	15.8	G_4		176.6	5.65
26	6.0	G_4:G_3;	17:1	76.2	2.85
27	1.1	G_4:(G_3+G_2);	1:1	9.2	2.85
28	0.6	G_2		ND	ND

TC, total carbohydrate; TC^1 glucose equivalents/ml fraction; TC^2, glucose equivalents/mg lyophilizate dry weight; HPLC, APS-Hypersil column; ND, not determined.

In summary, it can be concluded that G_4-amylase does not produce maltotetraose exclusively but does produce a high proportion of G_4, up to 99% w/w of the oligosaccharide weight. A high proportion of G_4 can be produced from a slurry of amylose or from a solution of cornstarch. Secondary oligosaccharides, G_3 and G_2, are also produced. At no time were G_5 or G_1 detected. In buffer G_4-amylase continued to produce G_4 for at least 5 days and in distilled H_2O remained active for at least 19 hours.

These were preliminary studies but it is likely that considerable G_4 can readily be produced by G_4-amylase. In one experiment using 2 Units of G_4-amylase and in which hydrolyzate was chromatographed, at least 0.58 g of G_4 was produced. In a second experiment with 125 Units of G_4-amylase and potato amylose as the sole substrate, an estimated 1.8 g of G_4 was produced in 16 hours, and it is likely that the hydrolysis reaction could have been continued. G_4-amylase hydrolyzed the insoluble slurry of potato amylose, thereby obviating solubilization of amylose and circumventing interference from retrogradation of an amylose solution.

4.2.4. Summary and Conclusions.

4.2.4.1. <u>Properties of Maltotetraohydrolase</u>. G_4-amylase is able to hydrolyze starch by an endo-mechanism. The principal product has an α-anomeric configuration, produced from hydrolysis of α-1,4-glucosidic bonds. This *Pseudomonas* enzyme, therefore, fits the general pattern of endo-glycanases which act with retention of anomeric configuration. Branch α-1,6-glucosidic linkages were not hydrolyzed. The extent of endo-amylolysis of branched α-1,4-glucans is, however, restricted and high molecular weight limit dextrins are produced. Even though endo-amylolysis has been shown to be an integral part of the activity of G_4-amylase, it still remains likely that the principal mode of hydrolysis is that of an exo-mechanism.

It had been implied that at low concentrations of G_4-amylase, hydrolysis occurred only by an exo-mechanism (7, 10). However, even at low (0.01 Boeh U) concentrations of G_4-amylase, endo-amylolysis was demonstrated by the hydrolysis of blue amylose by G_4-amylase.

Although blue amylose was a substrate for G_4-amylase, blue amylose was a much better substrate for α-amylase from *Bacillus amyloliquefaciens* which hydrolyzes blue amylose at more than twice the rate of G_4-amylase. In addition α-amylase hydrolyzes more of the bonds in blue amylose than does G_4-amylase, and hydrolyzes a blue amylose limit dextrin produced by G_4-amylase.

Endo-amylolysis was confirmed by G_4-amylolysis limits of >11% from β-limit dextrin. If G_4-amylase acted only by an exo-mechanism, and removed the outer maltosyl and maltotriosyl stubs on β-limit dextrins, the G_4-amylolysis limit from β-limit dextrin could not be greater than the percent of outer stubs (= % branching) of the β-limit dextrin. Potato amylopectin β-limit dextrin contained 9% branching but G_4-amylase hydrolyzed 20% of the glycosidic bonds in the β-limit dextrin. The higher molecular weights observed for wheat G_4-limit dextrins (10000-14000) than for the majority of the wheat β,G_4-limit dextrin (8000) are consistent with longer outer stubs on G_4-limit dextrin than on β,G_4-limit dextrin. In addition, Sakano *et al* (15) have shown that maltosyl stubs cannot be removed by G_4-amylase from branched oligosaccharides. The length of the outer stubs was not determined but is probably greater than G_3.

Endo-amylolysis is an integral part of G_4-amylolysis and occurs at all stages of hydrolysis. Immediately after the addition of G_4-amylase to starch solutions there is a decrease in turbidity, a property associated with an endo-mechanism. More importantly, this can be observed by HPLC as several distinct populations (macroclusters) are produced immediately after the addition of G_4-amylase. The molecular weight of these populations decreases with time, probably due to

removal of G_4 from the chain ends, but the bi- or trimodal distribution, of limit dextrin and oligosaccharide, remains the same.

It was originally reported that at low concentrations of G_4-amylase (7, 10) or during the initial stages of amylolysis (10), only maltotetraose was produced from hydrolysis of amylopectin. Results presented here also indicate that the principal mode of action of G_4-amylase is that of an exo-mechanism with a high specificity for G_4. Hydrolysis of linear α-1,4-glucans such as amylose produced up to 99% G_4. After endo-amylolysis of blue amylose, G_4 was the only oligosaccharide produced from segments containing no dye marker. Hydrolyses of amylopectin consistently showed only G_4 during the early stages of hydrolysis. Although endo-amylolysis occurs during the early stages of G_4-amylolysis, it is speculated that only G_4 is readily removed from the non-reducing terminus.

G_4-amylase binds α-1,6-linkages at its catalytic site (9, 10) with pullulan being a better inhibitor (K_i 0.6 mg/ml) than soluble dextran (K_i 5 mg/ml) (10). G_4-amylase does not hydrolyze the α-1,6-linkage although it does hydrolyze some of the α-1,4-linkages in pullulan. G_4-amylase probably also binds to α-1,6-linkages in amylopectin and hydrolyzes interior chains before releasing the branch point and then hydrolyzing the new non-reducing terminus by an exo-mechanism. Endo-amylolysis probably occur at least three units from the branch linkage since 6^3-α-glucosylmaltotriose is not hydrolyzed by G_4-amylase (15).

4.2.4.2. Description of G_4-Limit Dextrin. Properties of the G_4-limit dextrin were determined by nmr, from amylolysis

limits, and by HPLC. The results from these preliminary studies are summarized in Table 4.11. Most of these results are from single experiments and figures are approximate but show a definite pattern. In each instance G_4-amylase forms a limit dextrin intermediate in molecular weight between those of β- and α-limit dextrins. There are significant differences between the G_4-limit dextrins from different sources of starch.

The G_4-limit dextrin from wheat amylopectin was between 38%–54% (G_4-amylolysis limit, 46%–62%) of the weight of the amylopectin. It is probably 45% and the G_4-amylolysis limit 55%. This is surprisingly less than the β-amylolysis limit (59%, Exp I), particularly since a further 11% of the glycosidic linkages in β-limit dextrin could be hydrolyzed by G_4-amylase. If G_4-amylase hydrolyzes amylopectin and leaves branch stubs longer than maltotriosyl, then, even though oligosaccharides are produced from endo-amylolysis, the G_4-amylolysis limit could be less due to the longer non-reducing stubs. This would explain the smaller % β,G_4-limit dextrin (33% of amylopectin) obtained from G_4-amylolysis of β-limit dextrin. If β-amylase had shortened the branch stubs to G_3 and G_2 before G_4-amylolysis, the resulting G_4-limit dextrin would be smaller than the G_4-limit dextrin from amylopectin. This would also explain the slightly lower molecular weight (8000) observed for G_4-limit dextrin from β-limit dextrin than observed from G_4-limit dextrin from amylopectin (10000–15000).

Two of the wheat amylopectin G_4-limit dextrin preparations were purified and showed a narrow weight distribution corresponding to an apparent molecular weight of 10000–15000, as shown in Figs. 4.13 and 4.17. Nmr spectra (Figs. 4.11 and 4.18) show α-1,4-protons in at

Table 4.11. Yield and Molecular Sizes of G_4-Limit Dextrins.

Study	Chapter	% w/w Substrate	CL	Average Molecular Weight	HPLC
Wheat Amylopectin					
Nmr	4.2.1.	>28%[a]			
Exp I	4.2.2.1.	38%-43%	6.8	10000[b], 14300[c]	Figs. 4.11, 4.13
		≈5%	7.7	5000[b], 4230[c]	Fig. 4.13
Exp II	4.2.2.2.	≈46%[b]		7000[b]	
Exp III	4.2.2.3.	45%[a], 54%[d]		14000[b], 15000[b]	Figs. 4.17, 4.18
*β-LD	4.2.1.	33%[a]	6.4	8000 (1°)[b] + 30000[b]	Fig. 4.15b
Potato Amylopectin					
Nmr	4.2.1.	28%[a]			
*β-LD	4.2.1.	29%-35%[a]	7.7	8000[b] + 20000 (1°)[b]	Fig 4.15a
Soluble Starch					
Exp II	4.2.2.2.	19%[d]		18000[b]	
		12%[d]		<5000[b]	

[a] calculated from hydrolysis limit;

[b] HPLC, peak apex;

[c] calculated from DP, nmr spectrum;

[d] HPLC area.

*from hydrolysis of β-limit dextrin.

least five different environments, shifted upfield from the majority of α-1,4-protons in amylopectin and β-limit dextrin. Signals from the anomeric protons are small but the DP and molecular weight calculated from the integrals (DP = 88, MW = 14300) were similar to the results from HPLC. The G_4-limit dextrin contained >14.8% branching, >13 branches/molecule, and an average chain length of <6.8.

The average chain length of a limit dextrin (CL) is the average chain length of A and B chains and 1 chain containing a reducing unit (R), which in G_4-limit dextrin was originally a B chain. The A chains are branch stubs, in β-limit dextrin these are maltosyl or maltotriosyl units, linked to the dextrin by α-1,6-bonds. The B chains contain other branches and are in turn linked by α-1,6-bonds to the dextrin. The average chain length in G_4-limit dextrin can be represented by

$$CL = (cl_s A + cl_B B + cl_B) / (A + B + 1)$$

where cl_s is the average chain length of the A stubs, cl_B is the average chain length of the B chains and the R chain, and A and B are the number of A and B chains in the dextrin. If A = B in amylopectin, in the G_4-limit dextrin where R was formerly a B chain in amylopectin A = B + R. In G_4-limit dextrin from wheat amylopectin, the nmr spectrum showed A + B = 13 (13 branches/molecule, Table 4.7), therefore B = 6 and A = 7. Using the equation for CL and CL from Table 4.7/4.11, cl_B could be calculated if cl_s were known. In G_4-limit dextrin from β-limit dextrin the outer stub length, cl_s, is probably the same as β-limit dextrin which is 2.5. If so, then the average chain length of the B chain, cl_B, is 10.3 units long. If it is assumed that the cl_s in G_4-limit dextrin from amylopectin is 3.5, then cl_B is again 10.1 units long. In a smaller fraction of G_4-limit dextrin (Supernatant 2P Table 4.7, MW 4000-5000) containing 3.4 branches per molecule, cl_B

was calculated to be 8.4 units long. Many more studies need to be completed on these G_4-limit dextrins, in particular the cl_s needs to be determined, but from the G_4-limit dextrin the chain length of the B chain in G_4-limit dextrin could be calculated. This is not expected to be the chain length of the B chain in amylopectin or even the inner chain length since the branch points on the B chain in G_4-limit dextrin are probably several units in from the nonreducing termini.

G_4-limit dextrin was not purified from potato amylopectin or from soluble starch but estimations were made from hydrolysis mixtures. G_4-limit dextrin was at least 28% of the potato amylopectin weight but is probably not as high as the 45% from wheat amylopectin. More α-1,4-glycosidic bonds were hydrolyzed in potato amylopectin (24%) and in potato amylopectin β-limit dextrin (20%) than in the respective wheat amylopectin (18% and 11%, see Table 4.4), consequently the G_4-limit dextrin is expected to be a smaller proportion of potato amylopectin. HPLC indicated that the major population of G_4-limit dextrin from potato amylopectin β-limit dextrin had a molecular weight of 20000 and a minor population at 8000. It is not known if the 20000 daltons population is a true limit dextrin since in the sample was not precipitated for a second hydrolysis with G_4-amylase. (In the equivalent sample from wheat amylopectin β-limit dextrin, a 30000 dalton population was not present in rehydrolyzed dextrin preparations.)

The G_4-limit dextrin from soluble starch contained 19% of the starch weight at about 18000 daltons, similar to the percent dextrin weight from potato amylopectin. Approximately 75% of the hydrolyzate from soluble starch had molecular weights of <5000 and would generally be included in the G_4-amylolysis limit. A G_4-amylolysis limit of 75%

has been reported for soluble starch (10).

   4.2.4.3.   <u>Implications on the Fine Structure</u>
<u>Amylopectin</u>. G_4-amylolysis of wheat and potato amylopectin showed
major structural differences in the two amylopectins.   There were also
similarities and in both cases G_4-limit dextrins of narrow weight
distributions were produced.

   a.   Differences Between Wheat and Potato Amylopectin.   Table
4.12 summarizes the results of analysis of wheat and potato amylo-
pectin and the products from amylolysis.   The high β-amylolysis limit
for wheat amylopectin indicated that wheat amylopectin contains longer
outer chains than potato amylopectin.   A maximum average of 7.7 units
of β-maltose per chain were produced from potato amylopectin.   If an
average of 2.5 units remain on the β-limit dextrin, then the average
outer chain length is 18 units long.   This is a little longer than the
exterior chain length of 13-17 (79, 81) generally reported for potato
amylopectin.

   The β-limit dextrin from wheat amylopectin had a shorter average
chain length and a higher degree of branching than the β-limit dextrin
from potato amylopectin.   The nmr spectra also indicated that wheat
amylopectin had proportionally fewer α-H1,4-protons at 5.33 ppm than
potato amylopectin.   This chemical shift is believed to represent the
α-1,4-linkages non-adjacent to branch points.   The CL, percent branch-
ing, and α-1,4-proton signals all suggest that wheat amylopectin β-
limit dextrin has a higher branching density than potato amylopectin
β-limit dextrin.

Table 4.12. Comparison of Results from Wheat and Potato Amylopectin.

	Wheat		Potato	
Amylopectin[a]	CL=26.2 3.8% Br.		CL=28.6 3.5% Br.	
After Hydrolysis by:	β-amylase	G_4-amylase	β-amylase	G_4-amylase
Amylolysis Limit	59%[b]	46%-62%[a,b]	52%[b]	65%-72%[a,b]
Product/Branch			6.3-7.7	4.5-6.3
Limit Dextrin,				
CL[b,a]	9.0	6.8	11.3	
% Br.	11.1%	14.8%	8.9%	
α-H1,4[d], δ	5.33>5.29		5.34>>5.28	
		5.34<5.31≈5.28		
From β-limit dextrin				
G_4-amylolysis Limit		27.5%		28%-40%
CL of β,G_4-limit dextrin		6.4		7.7
Maltodextrin Product DP		2.5		2.0
Product/Branch		1.0		1.5
MW of Major β,G_4-Limit Dextrin		8000		20000

[a], calculated from nmr spectra;

[b], from 4.2.2.1, Experiment I;

[c], from HPLC;

[d] comparison of intensities of H-1 signals of α-1,4-linkages, chemical shifts from Table 4.5, Figs. 4.9, 4.11, 4.12.

% Br., % branching;

Product/Branch, from Table 4.3, reducing sugar product/α-1,6-linkage;

G_4-amylolysis Limits = % Hydrolysis * Product DP, calculated from Table 4.4; Maltodextrin Product DP, from Table 4.4.

The G_4-amylolysis limit was lower for wheat amylopectin than for potato amylopectin, even though the β-amylolysis limit was higher. G_4-amylase hydrolyzed fewer linkages in wheat amylopectin than in potato amylopectin. Since potato amylopectin must have longer outer chains than wheat amylopectin, the difference must arise from increased hydrolysis of the intrabranch chains in potato amylopectin. This is confirmed by the higher G_4-amylolysis limit for potato amylopectin β-limit dextrin. More extensive endo-amylolysis occurs in potato amylopectin, an average of 1.5 glycosidic linkages/branch are hydrolyzed in potato amylopectin β-limit dextrin. In wheat amylopectin β-limit dextrin only an average of 1.0 linkage/branch is hydrolyzed. This is an average and many interbranch chains will not be hydrolyzed.

Since the major maltodextrin product from G_4-amylolysis of potato amylopectin β-limit dextrin is maltose, it is likely that those interbranch chains which are hydrolyzed are only long enough to then be hydrolyzed by an exo-mechanism to yield G_2. Therefore, more interbranch chains must have been hydrolyzed and more product molecules produced than in wheat amylopectin β-limit dextrin. In wheat amylopectin G_4 as well as G_3 were produced in amounts equal to G_2. The interbranch chain must have been longer, perhaps even long enough to produce G_4 as well as a smaller maltodextrin from a single interbranch chain. Since the average chain length in wheat amylopectin β-limit dextrin is shorter than in potato amylopectin β-limit dextrin, the wheat amylopectin must contain clusters of more highly branched regions separated by longer chains between clusters.

The CL of wheat amylopectin G_4-limit dextrin was shorter and the nmr spectra showed fewer signals at 5.34 ppm, however signals at

5.31 ppm as well as 5.28 ppm, believed to be signals adjacent to the branch residue, were resolved. The CL of potato amylopectin G_4-limit dextrin was estimated from hydrolysis mixtures to be longer (ca. 9), and the molecular weight to be larger (about 25000, calculated from β,G_4-limit dextrin, HPLC studies).

In summary, these results indicate that wheat amylopectin has shorter outer chains, and more highly branched clusters joined by longer intercluster chains than found in potato amylopectin. The molecular weight of the wheat amylopectin clusters is estimated to be between 14000-25000. In potato amylopectin the branches in clusters are slightly farther apart, most of the clusters are larger (possibly as large as 29000) and the intercluster chains shorter.

b.   General Properties of Amylopectin. G_4-amylase hydrolyzes amylopectin to produce limit dextrin. This in itself is strong evidence for the cluster model for amylopectin.

During the initial stages of G_4-amylolysis, macroclusters are produced. These have a broad molecular weight distribution, nevertheless they show a median molecular weight which for soluble starch, and probably for wheat amylopectin, was 30000 daltons. With continued hydrolysis the molecular weight distribution becomes narrower and shifts to a G_4-limit dextrin which may depend upon the source of amylopectin.

Different amylopectins may contain clusters of similar molecular weights. G_4-amylolysis of both wheat and potato amylopectin produced limit dextrin with a median molecular weight of 10000-14000, although in potato amylopectin this G_4-limit dextrin was not the major

component. It also needs to be verified that the potato amylopectin 20000 dalton G_4-limit dextrin is a limit dextrin, that it cannot be degraded after purification and further G_4-amylolysis, and/or that it is not aggregated dextrin.

Most of the amylopectin molecule consists of highly branched clusters and few, if any, singly or doubly branched regions. Wheat amylopectin does yield a minor G_4-limit dextrin, <5% w/w of the amylopectin, which is multiply branched, with a molecular weight of about 5000. Amylopectin appears to have either multiply branched clusters or long chains connecting clusters, with few lightly branched regions.

4.3. Experimental.

4.3.1. High Field Proton NMR. ^{1}H-nmr spectra (400 MHz) were recorded on a Bruker spectrometer as described in Methods 5.9. Sample preparation, hydrolysis procedures, and calculations are also described in Chapter 5.9.

4.3.1.1. <u>Determination of T_1 Values for Maltotriose</u>. The spin-lattice relaxation time (T_1) of maltotriose (Sigma, twice exchanged with D_2O, 13% w/v D_2O solution) at 35°C was determined using the inversion-recovery pulse sequence (180°-τ-90°-acquire). The integrals of the α-1,4-glycosidic (5.4 ppm), the α-anomeric (5.2 ppm), the non-reducing H4 terminal (3.4 ppm), and the β-anomeric H2 (3.3 ppm) peaks were measured from spectra at twenty different taus (20s, 12s, 8s, 6s, 5s, 4s, 3.6s, 3.2s, 2.8s, 2.4s, 2.0s 1.7s, 1.4s, 1.1s, 0.9s, 0.7s, 0.5s, 0.3s, 0.1s, 0.000001s) and the T_1s calculated by Kevin Kittle using a program provided by Dr. Duncan Gilles.

4.3.1.2.   <u>Mutarotation Measurements for NMR</u>
<u>Studies</u>. The rates of mutarotation of glucose, maltose and maltotri-
ose in H_2O, D_2O and several buffers were determined at 37°C from
changes in the optical rotation of the sugar solutions. Procedures
for measurement of the optical rotation and calculations for determin-
ation of the mutarotation rate and specific rotation are described in
Methods 5.8.4.   Preparation of deuterium exchanged buffers is
described in 5.9.2.

4.3.1.2.1. <u>Optical Rotation of Maltose</u>.
D-(+)-maltose monohydrate (Aldrich: $[\alpha]_D$ +131 (c=4, H_2O, 48h)).

    a. *Maltose in 56 mM phosphate buffer, pH 7.0*. i.) Maltose
(80.3 mg) was dissolved in 2 ml of buffer (prepared by diluting
standard 100 mM phosphate buffer, pH 7.0, see Methods 5.2; the pH of
200 ml was adjusted to pH 6.6 by the addition of potassium dihydrogen
phosphate) and the optical rotation followed at 436 nm for 6.5 min.
ii.) Maltose (800.6 mg) was dissolved in 20 ml buffer and the optical
rotation followed at 589 nm for 102 min; the pH of the sugar solution
was confirmed to be 7.0.

    b. *Maltose in 56 mM phosphate buffer, pH 6.6*. Maltose (800.5
mg) was dissolved in 20 ml of buffer (described in a.) and the optical
rotation followed at 436 nm for 31 min. At 123 min there was no
change in optical rotation; the pH of the maltose solution was
measured and found to be 6.67.

    c. *Maltose in 56 mM deuterium-exchanged phosphate buffer, pH
7.0*. (pH 7 prior to deuterium exchange). Maltose (81.3 mg) was
dissolved in 2 ml exchanged buffer and the optical rotation followed
at 436 nm for 41.0 min.

    d. *Maltose in 56 mM deuterium-exchanged phosphate buffer, pH*

6.6. (pH 6.6 prior to deuterium exchange). Maltose (81.4 mg) was dissolved in 2 ml exchanged buffer and the optical rotation followed at 436 nm for 20 min. The pH of the buffer was later found to be p´H 7.2.

e. Maltose in H_2O. Maltose (803.1 mg) was dissolved in 20 ml purified water (Elga Water Purifier; the purified water was boiled for 15 min, stoppered, cooled to and incubated at 37°C) and the optical rotation followed at 589 nm for 25 min.

f. Maltose in deuterium oxide. Maltose (80.3 mg) was dissolved in 2 ml of D_2O and the optical rotation followed at 589 nm for 59 min.

4.3.1.2.2. <u>Optical Rotation of Glucose</u>. Glucose (Analar BDH; 1.009 g) was dissolved in 25 ml of 56 mM phosphate buffer, pH 7.0 and the optical rotation followed at 589 nm for 30 min.

4.3.1.2.3. <u>Optical Rotation of Malto-</u> <u>triose</u>. Crystalline maltotriose (Sigma; 95% O–α-D-glucopyranosyl-(1-4)-O–α-D-glucopyranosyl-(1-4)–α-D-glucose) was stored in a dessicator over Silica Gel. Prior to each measurement the polarimeter microcell was cleaned with water, followed by methanol, and air dried at 37°C.

a. Maltotriose in deuterium oxide. Maltotriose (80.5 mg) was dissolved in 2 ml of D_2O and the optical rotation followed at 589 nm for 129 min.

b. Maltotriose in H_2O. Maltotriose (84.3 mg) was dissolved in 2 ml of distilled, deionized water and the optical rotation followed at 589 nm for 130 min.

c. Maltotriose in 18 mM imidazole-DCl, pD 7.2 buffer. Imida-zole-DCl buffer containing 0.002% chlorhexidene (0.5 ml of 100 mM,

preparation described in 5.9) was diluted to 2.750 ml with D_2O. Maltotriose (82.9 mg) was dissolved in 2 ml of 18 mM imidazole-DCl buffer and the optical rotation followed at 589 nm for 180 min. The p´H of the solution was found to be 6.8, similar to the nmr study Hydrolysis V.

4.3.1.3. <u>Spectra of Maltotriose and Maltose</u>. Crystalline α-D-maltotriose and β-D-maltose monohydrate were dissolved in several buffers (prepared as described in 5.9) and the spectra recorded at various time intervals (see 5.9.4).

Maltotriose (5.6 mg) was dissolved in 0.5 ml of 200 mM imidazole-DCl buffer, p´H 7.35 and 28 spectra (DS21, see 5.9.4) recorded from 5 to 53 minutes after the addition of buffer.

Maltotriose (13.3 mg) was dissolved in 0.67 ml of 81 mM imidazole-DCl buffer containing CH_3CN and the spectrum recorded periodically (DS25) for 1.5 h and again after 24 h. The p´H of the sugar solution was 6.2.

Maltose (5.1 mg) was dissolved in 56 mM phosphate buffer (pH 7.0 prior to deuterium exchange) and several spectra (DS14) recorded after 10 min. The p´H of the sugar solution was 7.2.

4.3.1.4. <u>G_4-amylolysis of Soluble Starch</u>. Hydrolysis procedures and calculations are described in 5.9.3 and 5.9.4.

4.3.1.4.1. <u>Hydrolysis I</u>: 1.6% soluble starch, 8.2 Boeh U/ml, 77 mM phosphate buffer, p´H 7.2.

a. G_4-amylase. Partially purified G_4-amylase (1.0 ml of 1.5 ml sample) from Na Culture A Purification IV, Step 4 (Table 3.6 & 3.3.2.1) containing 8.9 Boeh U/ml in 30 mM phosphate buffer, pH 7 was lyophilized and exchanged with D_2O. To the lyophilized amylase was

added 0.2 ml of 250 mM exchanged phosphate buffer, pH 7.0, a final concentration of 415 mM phosphate. The solution contained some insoluble material. The G_4-amylase solution remaining (0.1 ml) after use for Hydrolysis I was lyophilized, stored at -20°C. The lyophilizate was later dissolved in 0.45 ml of D_2O containing CH_3CN and the spectrum was recorded (DS23, 100 scans).

A similar lyophilized G_4-amylase sample from the same purification step was used for nmr and product specificity studies described in 4.3.1.9.2, Hydrolysis XI.

   b.   Hydrolysis (DS12 and DS15).   The spectrum (DS12) of 2% w/v soluble starch (a clear solution, D_2O-exchanged once) was recorded at 310°K. The temperature was increased to 313°K, which shifted the HOD peak upfield. The spectrum (DS15.001) was recorded before the addition of 0.100 ml of G_4-amylase (4.45 Boeh U) to the 0.44 ml of starch solution. Spectra of the hydrolysis were recorded as described in 5.9.4, every 1.8 min from 6 min after the addition of enzyme through 43 min (DS15.003-DS15.023). The spectrum of the amylase-starch solution was also recorded 36 hours later. The p´H of the solution was 7.2.

                                   4.3.1.4.2.   <u>Hydrolysis II</u>:   2.0% soluble starch, 14.8 Boeh U/ml, 30 mM phosphate buffer, p´H 6.15.

   a. G_4-amylase.   Partially purified G_4-amylase (0.800 ml) from Na Culture B, Purification V, Step 8 (3.3.2.2 & Table 3.8) containing 10.2 Boeh U/ml in approximately 20 mM partially D_2O-exchanged phosphate buffer, pH 7 (see 3.3.3.2) was exchanged with D_2O. At the time of the nmr study, 0.1 ml D_2O was added but did not dissolve all of the lyophilizate, some of which adhered to the glass.

   b.   Hydrolysis (DS16).   The spectrum of 0.45 ml of 2.5% soluble

starch (49.0 mg exchanged in 1.96 ml D$_2$O) was recorded. All of the above G$_4$-amylase (0.1 ml, 8.15 Boeh Units) was added to the soluble starch and the spectra recorded from 5 min after the addition of amylase. The nmr tube was removed from the spectrometer after 128 min and TSP added. After equilibration at 37°C the spectrum was recorded at 138 min.

    c.   *Addition of Glucose (DS17).*   At 200 min 9.5 mg of α-D-glucose was added to the hydrolysis mixture (DS16) and several spectra recorded over 30 min. The pH of the sugar solution measured p´H 6.15.

    4.3.1.5.   G$_4$-amylolysis of Potato Amylopectin. Potato amylopectin refers to potato amylopectin B.V. 0.18 (see Materials 5.1).

    4.3.1.5.1.   Hydrolysis III: <2.5% potato amylopectin, 15.5 Boeh U/ml, 44 mM phosphate buffer.

    a.   *G$_4$-amylase.*   Partially purified G$_4$-amylase (1.20 ml) from Na Culture B, Purification V, Step 8 (3.3.2.2 & Table 3.8) containing 7.06 Boeh U/ml in approximately 20 mM partially D$_2$O-exchanged phosphate buffer, pH 7 (see 3.3.3.2) was exchanged with D$_2$O. At the time of the nmr study, 0.1 ml D$_2$O was added but did not dissolve all of the lyophilizate; a white precipitate was left.

    b.   *Hydrolysis (DS18).*   D$_2$O-exchanged potato amylopectin (53.3 mg in 0.535 ml) contained insoluble material which was partially removed by filtration. The spectrum of the cloudy filtrate was recorded. All of the G$_4$-amylase (0.1 ml, 8.48 Boeh Units) was added to the potato amylopectin solution and the spectra recorded from 7 min after the addition of amylase. At 48 min the nmr tube was removed; the solution appeared clear and gelatinous but on cooling to room temperature insoluble material sedimented in the tube. The solution

in the nmr tube was incubated at 30-40°C and the spectrum recorded at 1685 min.

c. *Addition of Glucose (DS18AG)*. At 1693 min 7.8 mg of α-D-glucose was added to the hydrolysis mixture and several spectra recorded over 50 min.

4.3.1.5.2. Hydrolysis IV: 1.4% potato amylopectin, 10.0 Boeh U/ml, 88 mM imidazole-DCl buffer, p'H 7.35.

a. *G_4-amylase*. The same preparation of G_4-amylase used for hydrolysis of wheat amylopectin (described in 4.3.1.5.8, DS19, Hydrolysis IX) was used for the hydrolysis of potato amylopectin.

b. *Hydrolysis (DS24)*. Potato amylopectin which had been prepared and the spectrum recorded (DS22) as described in 4.3.1.6.1, Hydrolysis VI, was used for G_4-amylolysis. Amylase (0.350 ml) was added to potato amylopectin (0.450 ml) and the spectra recorded (overnight) from 4 min after the addition of amylase through 770 min. The α-H1,6 signal was partially obscured by the broad HOD signal and though visible was not integrated. After 8 days the nmr sample which contained precipitate was examined by tlc for product specificity.

4.3.1.5.3. Hydrolysis V: 2.0% potato amylopectin, 10.5 Boeh U/ml, 18 mM imidazole-DCl buffer containing chlorhexidene diacetate, p'H 6.8.

a. *G_4-amylase*. Partially purified G_4-amylase (2.0 ml, 13.8 Boeh U/ml) from Na Culture C Purification VI, Step 8b (3.3.2.3. and Table 3.10) was concentrated by microfiltration (Centricon T30) to ≈0.2 ml and washed three times with 0.5 ml of 100 mM imidazole-DCl buffer containing 0.002% chlorhexidene diacetate (see 5.9.2.2) by reconcentrating. The final microconcentrate was diluted with the same

buffer to ≈0.35 ml and found to contain 57.7 Boeh U/ml.

b. Hydrolysis (DS27). The spectrum of 0.35 ml of G_4-amylase was recorded. Potato amylopectin (0.450 ml of a 2.5% solution described in 4.3.1.7, DS26 and also used for Hydrolysis VIII) was added to 0.10 ml of G_4-amylase and the spectra were recorded from 8 min after the addition of G_4-amylase through 125 min. At 125 min the sample was removed and found to have some sediment similar to that in Hydrolysis VIII, DS26. The pH was measured and found to be p´H 6.8. At 202 min the spectrum was recorded and an additional 0.100 ml G_4-amylase was added to the hydrolysis mixture (1.7% potato amylopectin, 17.8 Boeh U/ml, 31 mM buffer, p´H 6.7) and several spectra recorded. The pH was measured and found to be p´H 6.7. The spectrum was also recorded at 26 h. The sample was later examined by tlc for product specificity. The mutarotation rate constant for maltotriose in a parallel dilution of the same buffer was determined in 4.3.1.2.3.

4.3.1.6. <u>β-amylolysis of Potato Amylopectin</u>.

4.3.1.6.1. <u>Hydrolysis VI</u>: 2.3% potato amylopectin, 16.5 Boeh U/ml, 14 mM imidazole-DCl buffer, p´H 7.35.

a. β-amylase. Crystalline β-amylase from sweet potato, in ammonium sulfate, was added to 2 ml of 200 mM imidazole-DCl buffer, p´H 7.35 (Chapter 5.9), concentrated to 0.3 ml by microfiltration (Centricon T30) and diluted to 1.5 ml with buffer. Assay showed 229.8 Boeh U/ml.

b. Hydrolysis (DS22). The spectrum of 0.45 ml of 2.5% potato amylopectin (55.7 mg in 2.23 ml D_2O containing CH_3CN) was recorded. Beta-amylase (0.035 ml, 8 Boeh Units) was added to the potato amylopectin and the first spectrum recorded after 64 scans. Additional spectra were recorded in the standard way (32 scans) from 10 min

through 65 min, and one spectrum after 1000 min. After 8 days the slightly milky (similar to soluble starch) sample was examined by tlc for product specificity.

4.3.1.6.2. <u>Hydrolysis VII</u>: 2.3% potato amylopectin, >10 Boeh U/ml, imidazole-DCl-sodium acetate, p´H 7.5.

a. β-amylase. Beta-amylase (≈0.2 ml) from the Hydrolysis VI preparation was concentrated by microfiltration (T30) and washed twice with D_2O and once with D_2O-exchanged acetate buffer (acetate buffer, pH 4.9 exchanged by lyophilization two times---probably leaving only 20 mM NaAc and no acetic acid). The pH of the concentrate (0.300 ml, >>100 Boeh U/ml) was measured at p´H 6.45.

b. Hydrolysis (DS28 & DS29). The spectrum (DS28) of 0.45 ml of 2.5% potato amylopectin (36.3 mg in 1.45 ml D_2O containing CH_3CN) was recorded. Amylase (0.050 ml) was added to the potato amylopectin and the spectra recorded (DS29) from 4 min after the addition of amylase through 27 min when the pH was measured and found to be p´H 7.5. The spectra were also measured at 308 min and at 23 h (108 scans). The salts added with the amylase were estimated to be <5 mM imidazole-DCl and <2 mM sodium acetate in the hydrolysis solution.

After 6 days the hydrolysis mixture was stored at 4°C and further analyzed for amylase activity, product specificity, by tlc and HPLC. For HPLC analysis (5.7.3), 0.200 ml was removed and precipitated with 0.400 ml ethanol (5.4.2). The supernatant was chromatographed on the Spherisorb column.

4.3.1.7. <u>α-amylolysis of Potato Amylopectin</u>. <u>Hydrolysis VIII</u>: 2.0% potato amylopectin, 31 Boeh U/ml, 1.8 mM imidazole-DCl buffer, p´H 8.2.

a. α-amylase. Alpha-amylase (0.5 mg) was dissolved in 0.450 ml of 10 mM imidazole-DCl buffer (from a 1 M solution prepared from ly-ophilized 200 mM imidazole-DCl, p´H 6.2 and diluted with D_2O containing CH_3CN). Amylase (0.100 ml) was used for the initial DS26 hydrolysis; then 0.035 ml of 1 M imidazole-DCl buffer was added to the remaining amylase. Three days later the solution was found to contain 169 Boeh U/ml.

b. Hydrolysis (DS26). The spectrum of 0.45 ml of 2.5% potato amylopectin (40.3 mg in 1.61 ml D_2O containing CH_3CN, remained cloudy) was recorded. Alpha-amylase (0.100 ml) was added to the potato amylo-pectin (1.8 mM imidazole-DCl buffer) and the spectra recorded from 7 min through 46 min when the nmr tube was removed and it was noted that white precipitate sedimented. The pH was found to be p´H 8.2. At 234 min the spectrum was recorded and then an additional 0.100 ml of α-amylase containing 100 mM imidazole-DCl buffer was added to the nmr hydrolysis solution (1.7% potato amylopectin, 52 Boeh U/ml, 15 mM imidazole-DCl buffer). The spectra were recorded from 250 min after the initial addition of amylase through 268 min, when the pH was found to be p´H 6.9. The spectrum was also recorded at about 1200 min. The sample was later examined by tlc for product specificity.

4.3.1.8.   G4-amylolysis of Wheat Amylopectin.

Hydrolysis IX: 1.8% wheat amylopectin, 6.4 Boeh U/ml, 56 mM imidazole buffer, p´H 7.4.

a. G4-amylase. Partially purified G_4-amylase (1.83 ml, 16.5 Boeh U/ml) from Na Culture C Purification VI, Step 8b (3.3.2.3. and Table 3.10) was concentrated by microfiltration (Centricon T30) to ≈0.2 ml and washed two times with 1.0 ml of 200 mM imidazole-DCl buffer, p´H 7.35 by reconcentrating. The final microconcentrate was

diluted with buffer to ≈0.65 ml and found to contain 22.77 Boeh U/ml (48% recovery of G_4-amylase). The spectrum of the amylase in buffer was recorded (DS20).

b. Hydrolysis (DS19). The spectrum of 0.45 ml of 2.5% wheat amylopectin (49.5 mg in 1.98 ml D_2O containing CH_3CN; dissolved readily to a slightly milky solution). The G_4-amylase in imidazole-DCl buffer (0.175 ml, 4.0 Boeh Units) was added to the wheat amylopectin and the spectra recorded from 5 min to 127 min after the addition of amylase. The nmr tube and sample were incubated and the spectra recorded at 250 min and 1000 min. After 8 days the slightly milky sample containing a little precipitate was examined by tlc for product specificity.

4.3.1.9. <u>G_4-amylolysis of β-Limit Dextrin from Potato Amylopectin</u>. The β-limit dextrin prepared (4.3.2.1, Experiment I) from potato amylopectin B.V. 0.18 was examined by nmr (4.3.1.11) and hydrolyzed by G_4-amylase.

4.3.1.9.1. <u>Hydrolysis X</u>: 1.7% β-limit dextrin, 3.8 Boeh U/ml, 15 mM imidazole-DCl buffer containing chlorhexidene, p´H 6.25.

a. G_4-amylase. Partially purified G_4-amylase (0.600 ml, 20.1 Boeh U/ml) from Trypticase I Culture, Purification III, Step 10 (3.2.1.3, Table 3.5) was concentrated by microfiltration (Centricon T10) and washed two times with 50 mM imidazole-DCl buffer containing 0.002% chlorhexidene diacetate (5.9.2.2.). The concentrate was diluted to 0.450 ml and found to have 12.7 Boeh U/ml (48% recovery) of amylase activity.

The specificity of this G_4-amylase preparation was examined after storage at <9°C for 5 weeks. The product specificity from

hydrolysis of 0.5 ml of 1% soluble starch in 20 mM Buffer D (imidazole with chlorhexidene) by 0.05 ml (0.6 Boeh U) amylase was examined by tlc (at 1.5h, 21h, 120h, 1 week) and by HPLC (at 1 week) using the Synchropak column. The HPLC experimental and results are discussed in 4.3.2.1.2 and 4.2.2.1.2.

       b.   Hydrolysis (DS30B). To 0.46 ml of 2.5% potato amylopectin β-limit dextrin (4.3.1.11, Spectrum DS30; p´H 5.85) was added 0.200 ml of G_4-amylase. The spectra were recorded from 15 min after the addition of G_4-amylase through 227 min when the pH was measured and found to be p´H 6.25. The spectrum was also recorded at 1290 min (106 scans).

       After 6 days the hydrolysis mixture was stored at 4°C and further analyzed as described in 4.3.1.6.2, Hydrolysis VII.

       4.3.1.9.2. <u>Hydrolysis XI</u>: 2.5% potato amylopectin β-limit dextrin, 8.7 Boeh U/ml, 38 mM phosphate buffer and 25 mM imidazole-DCl containing chlorhexidene, p´H 6.25.

       a. G_4-amylase (DS43i). Partially purified and lyophilized G_4-amylase from Na Culture A, Purification IV, Step 4 (3.3.2.1, Table 3.6) was weighed (183.2 mg), 13.4 mg removed and added to 0.200 ml D_2O. Amylase was extracted from insoluble material by repetitious centrifugation, removal of some supernatant, and addition of 0.200 ml D_2O (twice) to the remaining supernatant-precipitate. The clear supernatant (0.400 ml) removed (0.100 ml from the first, 0.200 ml from the second, 0.100 ml last) was found to have 21.9 Boeh U/ml or 64% recovery of amylase, and calculated to contain 95 mM phosphate buffer. The 0.395 ml was lyophilized and stored at -20°C. The lyophilizate was diluted to 0.500 ml with 50 mM imidazole-DCl containing 0.001% chlorhexidene and the spectrum recorded.

The specificity of this G_4-amylase preparation was examined after storage at <9°C for 5 weeks. The product specificity from hydrolysis of 1% soluble starch in 20 mM Buffer D (imidazole with chlorhexidene) was examined by tlc (at 1.5h, 21h, 120h, 1 week) and by HPLC (at 1.5 h and 1 week) using the Synchropak column. The HPLC experimental and results are discussed in 4.3.2.1.2 and 4.2.2.1.2.

   b. *Hydrolysis (DS34)*. To the 0.5 ml of G_4-amylase was added 0.5 ml of 5% potato amylopectin β-limit dextrin (4.3.1.11, Spectrum DS30; p´H 5.85) and the spectra were recorded from 4 min after the addition of amylase through 34 min when the nmr tube was removed. The pH was measured and found to be p´H 6.25. At 140 min the spectrum (53 scans) was recorded, as well as at 1200 min (109 scans).

   After 6 days the hydrolysis mixture was stored at 4°C and further analyzed as described in 4.3.1.6.2, Hydrolysis VII. In addition, 0.050 ml of the hydrolysis mixture was added to 0.5 ml of 1% soluble starch in 20 mM Buffer D and the product specificity examined at 1.5h, 21h, and 120h.

   4.3.1.10. <u>G_4-amylolysis of β-Limit Dextrin from Wheat Amylopectin</u>. The β-limit dextrin prepared (4.3.2.1, Experiment I) from wheat amylopectin was examined by nmr (4.3.1.11) and hydrolyzed by G_4-amylase.

   <u>Hydrolysis XII</u>:   2.1% wheat amylopectin β-limit dextrin, 2.1 Boeh U/ml, 8 mM imidazole-DCl buffer containing chlorhexidene.

   a. *G_4-amylase*. G_4-amylase prepared for hydrolysis of potato amylopectin β-limit dextrin (Hydrolysis X, DS30B) was also used for Hydrolysis XII.

   b. *Hydrolysis (DS31A)*. G_4-amylase (0.100 ml) was added to 0.5 ml of 2.5% wheat amylopectin β-limit dextrin (4.3.1.11, Spectrum

DS31). Spectra were recorded from 6 min through 864 min.

After 6 days the hydrolysis mixture was stored at 4°C and further analyzed as described in 4.3.1.6.2, Hydrolysis VII.

4.3.1.11. <u>Spectra of Limit Dextrins</u>. Limit dextrins from the amylolysis preparations described in 4.3.2.1, Experiment I, Part 3 were twice exchanged with D_2O. Solutions were prepared (5.9.2) just before nmr analysis in D_2O containing CH_3CN. The following spectra were recorded:

DS30	potato amylopectin β-limit dextrin	2.5%
DS31	wheat amylopectin β-limit dextrin	2.5%
DS32	wheat amylopectin G_4-limit dextrin	5.0%
DS32S	wheat amylopectin G_4-Supernatant 2S	in 0.5 ml, % ND
DS32P	wheat amylopectin G_4-Supernatant 2P	in 0.5 ml, % ND
DS33	wheat amylopectin	2.5%
DS35	Sigma potato amylopectin* G_4-limit dextrin	2.6%
DS36	Sigma potato amylopectin* β-limit dextrin	2.5%
DS37	Sigma potato amylopectin*	2.5%

* Sigma potato amylopectin from Sigma A-8515 (all other potato amylopectin was a gift from E. Percival, B.V. 0.18); G_4-Supernatants 2S and 2P refer respectively to the supernatant and precipitate from reprecipitation of concentrated ethanol supernatant (see 4.3.2.1).

Further analysis of the nmr samples is described in 4.3.2.1, Experiment I, Part 4.

4.3.2. Hydrolysis of α-1,4-Glucans.

4.3.2.1. Preparation of Limit Dextrins.

4.3.2.1.1. Experiment I: Preparation
of Beta-Amylase and Maltotetraohydrolase Limit Dextrins from Wheat
Amylopectin and Two Potato Amylopectins.

All amylase activities are expressed as Boehringer Units.
Reducing sugar was measured by the alkaline ferricyanide method. The
total carbohydrate in the amylopectin samples was determined by the
phenol sulfuric acid method and the weight of substrate amylopectin
expressed in mg carbohydrate or % TC (Total Carbohydrate), see 5.8.2.
Methods and materials are described in Chapter 5.

Part 1. Amylolysis of Sigma Potato Amylopectin. A 5% w/v
sterile solution of Sigma potato amylopectin (3.65% TC) in 20 mM
Buffer D was prepared by mixing buffer with 2.5 g amylopectin to form
a paste, diluting with buffer to 50 ml, heating in a boiling water
bath to dissolution, and autoclaving for 15 min. Beta-amylase, 110 µl
suspension, (two dilutions of suspension in Buffer D averaged 5.4
Units/µl) was added to a cotton plugged boiling tube containing 25.0
ml sterile amylopectin solution giving 24 U amylase/ml. G_4-amylase,
1.0 ml, (7.0 Boeh U/ml) from Purification III, Step 8 (3.2.1.3) which
had been purified by affinity column chromatography was added to two
7.0 ml portions sterile solution giving 0.88 Boeh U/ml. The amylase-
amylopectin solutions were placed in a 37°C water bath. After 47
hours the solutions were analyzed by tlc and for residual amylase
activity. At 48h the β-amylase solution was precipitated with 2
volumes of ethanol (50 ml), see Procedures 5.4.2. The two G_4-amylase-
amylopectin solutions were combined and precipitated with 15 ml of

Table 4.13.   Procedures for Experiment I, Preparation of Limit Dextrins.

Part 1.   *Part 2.*

　　5% Sigma p.a.   2.5% p.a.   2.5% w.a.

+β-amylase	+G$_4$-amylase	+β-amylase	+β-amylase	+G$_4$-amylase
+ EtOH	+ EtOH	+ EtOH	+ EtOH	+ EtOH

Sup′nt 1   Sup′nt 1   Sup′nt 1   Sup′nt 1   Sup′nt 1

Part 3.

Ppt   Ppt   Ppt   Ppt   Ppt

+β-amylase	+G$_4$-amylase	+β-amylase	+β-amylase	+G$_4$-amylase
+ EtOH	+ EtOH	+ EtOH	+ EtOH	+ EtOH

　　　Sup′nt 2   Sup′nt 2   Sup′nt 2   Sup′nt 2   Sup′nt 2

Sigma β-LD   Sigma G$_4$-LD   p.a. β-LD   w.a. β-LD   w.a. G$_4$-LD

conc;

+ EtOH

Sup′nt 2S   Sup′nt 2P

p.a., potato amylopectin; w.a., wheat amylopectin; Sup′nt, supernatant; Ppt, precipitate; β-LD, β-limit dextrin; G$_4$-LD, G$_4$-limit dextrin.

ethanol. Both the β- and G_4-amylase ethanol hydrolysis mixtures were left at room temperature for 3 days; the precipitates removed by centrifugation for Part 3 and the supernatants designated Supernatant 1 of the respective digest.

Part 2. Amylolysis of Wheat Amylopectin and Potato Amylopectin. Wheat and potato amylopectin (gifts, see materials, referred to only as wheat amylopectin or potato amylopectin) were each solubilized as described in <u>Methods in Carbohydrate Chemistry</u> (47) (i.e., with addition of ethanol, 3 M NaOH, heating to a homogeneous solution, neutralization with HCl), except the solutions were diluted with buffer to give 2.5% amylopectin (1.25% TC wheat amylopectin, 1.53% TC potato amylopectin), 36 mM Imidazole-HCl buffer, pH 7.0 containing 0.001% chlorhexidene. To boiling tubes containing 16 ml each of potato and wheat amylopectin was added 110 μl beta-amylase suspension, to give 37 Units/ml. To 16 ml of wheat amylopectin was added 500 μl of G_4-amylase (20.1 Boeh U/ml from Purification III, Step 10; see 3.2.1.3) to give 0.61 Boeh U/ml. Control solutions of wheat amylopectin (4 ml) and potato amylopectin (2 ml), and the amylase-amylopectin solutions were each layered with several drops of toluene and placed in a 37°C water bath.

After 42 hours, 0.25 ml samples were removed from each boiling tube and precipitated with 2 volumes of ethanol. The supernatants were analyzed by tlc and for reducing sugar. The precipitates were resolubilized in 0.5 ml of 20 mM Buffer D (imidazole-HCl, pH 7, containing 0.002% chlorhexidene) by heating in a boiling waterbath, then cooled and again incubated, as previously, with the respective β-amylase (36 Boeh U/ml) or G_4-amylase (0.5 Boeh U/ml). After 2 hours incubation each mixture was analyzed by tlc and for reducing sugar.

After 47 hours, the remaining solutions in the boiling tubes

were precipitated with 2 volumes of ethanol and stored at room temper-
ature overnight. The ethanol mixtures from Parts 1 and 2 were each
centrifuged; the clear supernatants decanted and designated Superna-
tant 1 from the respective solution. The precipitates were used in
Part 3.

Part 3. Amylolysis of Dextrins from Parts 1 and 2. The preci-
pitates from the β-amylolysis of amylopectins in Parts 1 and 2 were
redissolved by heating in 50 mM acetate buffer, pH 4.9 (7.5 ml), 50 µl
β-amylase suspension was added to each to give 36 Boeh U/ml; then
incubated at 37°C. The precipitates from G_4-amylolysis of wheat and
Sigma potato amylopectins (Parts 1 and 2) were redissolved by heating
in 50 mM Buffer D (7.5 ml and 2.0 ml, respectively); G_4-amylase from
Purification III, Step 10 (200 µl and 50 µl) was added to each giving
0.5 Boeh U/ml, and then both incubated at 37°C. The wheat amylopec-
tin-no amylase precipitate was redissolved in 50 mM Buffer D and
incubated at 37°C.

After 19 hours, the digests were heated in boiling water for 8
min, 100 µl removed from each and frozen for later analysis. The
remaining digest was precipitated with 2 volumes of ethanol, then
centrifuged. (Part of the ethanol mixture from the G_4-amylolysis of
wheat amylopectin was lost). The clear supernatants were decanted and
designated Supernatant 2 from their respective mixture. Part of each
β-amylolysis precipitate and all of each G_4-amylolysis precipitate
were used for high field proton NMR studies described in Part 4. The
remainder of each β-amylolysis precipitate was dried over P_2O_5.

Supernatants 1 and 2 from each amylolysis were concentrated to
dryness under reduced pressure. Supernatant 2 from G_4-amylolysis of
wheat amylopectin was further fractionated by the addition of 8 ml

ethanol to dry Supernatant 2, then 4 ml of water, mixing until no further precipitate dissolved, then removal of the precipitate (Supernatant 2P) by centrifugation. The supernatant (Supernatant 2S) was concentrated under reduced pressure, and both 2P and 2S exchanged with D_2O for analysis by NMR as described in Part 4. After NMR analysis, the precipitate and Supernatant 2S were analyzed by tlc, for reducing sugar, and for total carbohydrate. The other supernatants (1.0 ml of water was added to each dry solid) were similarly analyzed.

Part 4. Further Analyses of Amylolysis Fractions.

4a. NMR Analysis. The 400 MHz nmr spectra of the limit dextrins from Part 3 were recorded as described in 4.3.1.11 using 32 scans, pulse interval 7.1 s, and 310°K. Also included were the low molecular weight limit dextrins, Supernatants 2P and 2S, from G4-amylolysis of wheat amylopectin. General procedures are described in Chapter 5. All samples were frozen after nmr analysis and later analyzed for total carbohydrate and by HPLC and tlc.

4b. HPLC Analysis. Sigma potato amylopectin and the Sigma β-limit and G_4-limit dextrins from Part 4a were heated in boiling water and then filtered for HPLC analysis, and the total carbohydrate of the aqueous solutions (2.5%, 5%, and 5% respectively) measured before and after filtration. The filtrates were examined on both the Synchropak and the Chrompak columns. Some HPLC analyses were repeated after removal of D_2O by dessication over P_2O_5 and redissolving in HPLC solvent. Attempts to filter either the wheat amylopectin or its β-limit dextrin were unsuccessful; even dilute solutions of 0.5% clogged the filter. Wheat amylopectin G_4-limit dextrin and the two supernatants, 2P and 2S, were filtered and analyzed on both the Synchropak

and the Chrompak HPLC columns, and the low molecular weight fraction, Supernatant 2S was also analyzed on the Spherisorb column.

4.3.2.1.2. Experiment II: HPLC Studies of Amylolysis by Maltotetraohydrolase.

a. The hydrolysis mixtures from nmr studies, Hydrolyses X-XII, were analyzed as described for each of the studies in Experimental Sections 4.3.1.9 and 4.3.1.10.

b. Freshly solubilized wheat amylopectin (1% w/v, 30.2 μmole ge/ml) was added to a vial containing a small amount of G_4-amylase (the D_2O-exchanged preparation from Purification III, Step 10 used for Hydrolysis X in 4.3.1.9.1) and a timer started. At 5 min a sample was removed and filtered through an 0.45 micron filter and immediately injected on the Synchropak HPLC column. A second sample was removed after 28 min, filtered, and chromatographed immediately. At 35 min, both the 5 min and the 28 min filtered samples were heated in boiling water. At 55 min the remaining sample was filtered, HPLC run immediately and again 4 days later. Total carbohydrate concentration was measured on each of the filtered 5, 28, and 55 min samples.

c. In a second amylolysis study, soluble starch was solubilized (1% w/v) in 20 mM Buffer D and 0.5 ml incubated at 37°C with 0.050 ml G_4-amylase (<2 Units of the D_2O-exchanged preparation for Hydrolysis XI in 4.3.1.9.2 from Purification IV, Step 4). After 1.5 hours, an 0.200 ml sample was removed and heated in boiling water for 3 min. The remaining 0.350 ml was layered with 1 drop of toluene, sealed, left overnight at 37° and then at room temperature. Samples were analyzed on tlc and by HPLC on the Synchropak column.

4.3.2.1.3. <u>Experiment III</u>: <u>Hydrolysis</u>
<u>of Wheat Amylopectin by G_4-Amylase</u>. Amylase activity was determined
by the Boehringer assay, reducing sugar by the alkaline ferricyanide
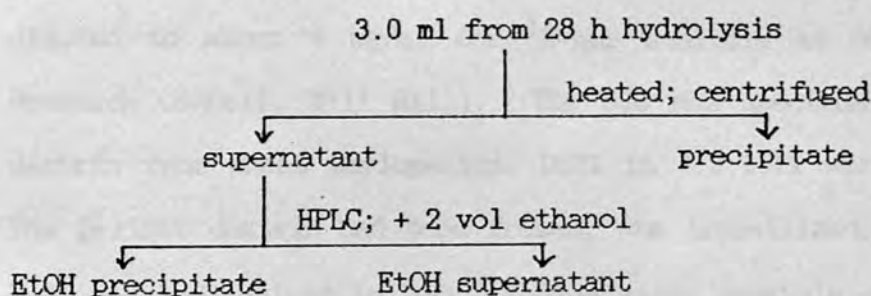method, total carbohydrate by the phenol sulfuric method and expressed
as μmole ge.

a. Hydrolysis. Wheat amylopectin (50 mg/ml, 2.5% TC, w/v) in
50 mM imidazole buffer containing 0.002% chlorhexidene, pH 7 (Buffer
D) was solubilized by heating at 90°C for 5 min. Total carbohydrate
in the wheat amylopectin solution and in two controls (one in H_2O
only, heated at 95°C) were determined and all were found to contain
50% ±1% TC, see 5.8.2. G_4-amylase (5 Boeh Units from Purification
III, Step 8 described in 3.2.1.3, 8.2 Boeh U/ml) was added to 20 ml of
amylopectin solution to give a final amylase concentration of 0.24
Boeh U/ml. The mixture was incubated in a waterbath for 50 min at
20°C; then transferred to a waterbath at 37°C. Samples were removed
from the reaction mixture (generally 0.2 ml, except at 28h when 3.0 ml
was removed; total volume removed = 6.8 ml) and reducing sugar deter-
mined immediately or else set in boiling waterbath for 1 min; then
analyzed later. Amylase activity and product specificity were deter-
mined at 22 and 24 h and the white precipitate present examined by
Gram's stain. At 30.8 h the remaining hydrolysis mixture (≈13.2 ml)
was set in a boiling waterbath for 10 min; then stored at -20°C.

b. Analysis of Hydrolyzate. The hydrolyzate from 28 h and from
30.8 h was partially purified. After each step the fraction(s) were
analyzed for reducing sugar (RS) and for total carbohydrate (TC).

The 3.0 ml sample removed at 28 h was analyzed immediately. The
sample was heated in a boiling waterbath for 15 min and the heterogen-
eous mixture containing white precipitate analyzed; then centrifuged.

The precipitate was washed with 2.0 ml water, recentrifuged and both the precipitate and wash analyzed for RS and TC and examined by tlc. Methanol, ethanol, and CH_2Cl_2 were added to the precipitate but there appeared to be no change. The wheat amylopectin control was likewise heated and centrifuged, no precipitate formed, the solution was analyzed for RS and TC; it was found to clog an HPLC filter.

The supernatant from the 28 h hydrolysis was analyzed for RS and TC, examined by tlc, then by HPLC on the Chrompak column. For HPLC analysis the supernatant was analyzed for RS and TC before and after (i) filtration through an 0.45 micron filter (as in 5.7.3) and (ii) chromatography on the Chrompak column. Fractions (1.0 ml) were also collected from the Chrompak column (RI and uv detection) and each analyzed for RS and TC, uv absorbance between 200-300 nm, and by tlc. The remaining supernatant (1.3 ml) was precipitated with 2.5 ml ethanol (see 5.4.2). The EtOH precipitate and supernatant were dried by rotovaporization; both clear glasses dissolved immediately in 0.5 ml water containing 0.02% azide and were analyzed for TC and by HPLC on the Chrompak column.

3.0 ml from 28 h hydrolysis

heated; centrifuged

supernatant   precipitate

HPLC; + 2 vol ethanol

EtOH precipitate   EtOH supernatant

The frozen hydrolysis mixture from 30.8 h was thawed, heated in a 95°C waterbath to slightly clarify the turbid solution, analyzed for TC and RS; then centrifuged. The precipitate was washed with 1.0 ml H_2O and dried for chemical analysis.

The supernatant was precipitated with 3 volumes of ethanol. The

EtOH precipitate was washed with ethanol and the wash-filtrate added to the ethanol supernatant.   The EtOH supernatant and wash-filtrate were concentrated by rotavaporization and analyzed by tlc and for TC and RS.

```
                    13.2 ml from 30.8 h hydrolysis
                              |
                  ┌───────────┤ heated; frozen; heated; centrifuged
                  ↓           ↓
            supernatant                    precipitate
                  |
                  | + 3 vol ethanol
          ┌───────┴────────┐
          ↓                ↓
    EtOH supernatant    EtOH precipitate
```
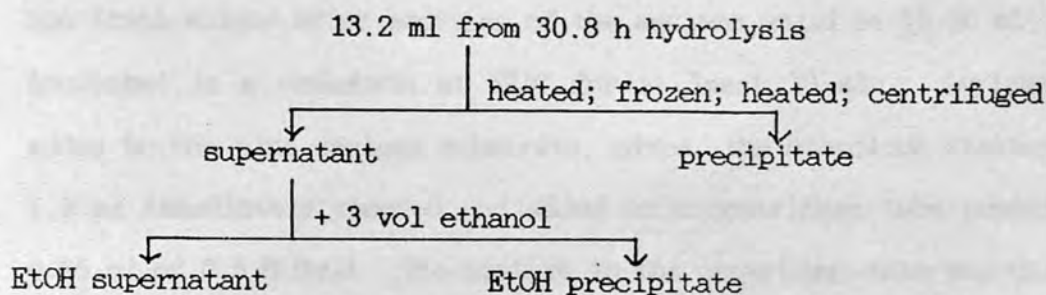
The EtOH precipitate was air dried on a fine sinta glass filter, removed from the filter by the addition of H_2O; then lyophilized.   Some EtOH precipitate was lost in the freezedrier.   The lyophilizate was weighed (150.6 mg) and a 2.55% solution prepared for analysis of TC and RS, by tlc, and by HPLC on the Chrompak column.   The remaining lyophilizate (131.9 mg) was dissolved in H_2O and reprecipitated with another 3 volumes of EtOH, dried and stored over P_2O_5.   Approximately 33 mg was twice exchanged with D_2O, dissolved in D_2O. and a portion diluted to about 4 mg/ml for ^{1}H-nmr analysis at 500 MHz (Medical Research Council, Mill Hill).   The 500 MHz spectrum of the β–limit dextrin from wheat amylopectin, DS31 in 4.3.1.11 was also recorded. The β–limit dextrin had been frozen, was lyophilized, exchanged with D_2O, and redissolved in D_2O.   Sodium azide crystals and acetone were added to the two samples which were stored at RT until the spectra were recorded at 500 MHz.

4.3.2.2.   Hydrolysis of Blue Amylose.

4.3.2.2.1.   General Procedures.   Blue amylose (100 mg Sigma A-3508, Amylose Azure, lot 33F-3880) was weighed

into each assay boiling tube, and in a blank. Amylase activity was
measured by the Boehringer assay and the volume containing 1.0 Boeh
Units or 0.1 Boeh Units (37°C) of amylase was calculated. Phosphate
buffer (100 mM, pH 7.0) was added to the assay tubes and blank so that
the final volume after addition of the amylase would be 10.00 ml; then
incubated in a waterbath at 37°C for at least 10 min. Amylase was
added to the blue amylose substrate, mixed, the stopclock started and
1.0 ml immediately removed and added to a centrifuge tube containing
0.25 ml of 0.5 M NaOH. The mixture in the centrifuge tube was diluted
to 3.75 ml with 2.50 ml of H_2O, mixed, and centrifuged. The superna-
tant was decanted and the absorbance read at 620 nm in a double beam
spectrometer with the blank supernatant in the reference cell.

4.3.2.2.2. Amylolysis I with 0.1 Boeh
U/ml, Hydrolyses A-C. Alpha-amylase from *B. amyloliquefaciens* (see
5.1.2) in 100 mM phosphate buffer (2.25 Boeh U/ml), G_4-amylase from Na
Culture B, Purification V, Step 4b (1/53 Boeh U/ml, Table 3.8,
3.2.2.2) and G_4-amylase from Trypticase I, Purification III, Step 4
(2.86 Boeh U/ml, Table 3.5, 3.2.2.3) were each added to 100 mg of Blue
Amylose in 100 mM phosphate buffer to give a final concentration of
0.1 Boeh U/ml. Samples were taken for analysis at 0, 30, 60, 95, and
120 min and at 24 h. The electricity, and hence the waterbath, went
off after 80 min, so that the temperature at 95 min was 35°C, at 120
min was 32°C, and at 24 h was 15°C.

The product specificity (see 5.6.1) of each of the amylase
preparations was examined using 1% potato starch in 33 mM phosphate
buffer.

4.3.2.2.3.   <u>Amylolysis with 0.01 Boeh</u>

<u>U/ml</u>.   The α-amylase and the two G_4-amylase preparations used in 4.3.2.2.2 were again used for hydrolysis of blue amylose, except at 0.01 Boeh U/ml, in order to determine the rate of amylolysis.   Procedures were the same except the waterbath was maintained at 37°C and samples were taken at 0, 30, 60, 100, 120, and 167 min.

4.3.2.2.4.   <u>Amylolysis II with 0.1 Boeh</u>

<u>U/ml, Hydrolyses D and E</u>.   Hydrolysis of blue amylose was repeated, the G_4-limit dextrin purified and further hydrolyzed by α-amylase.

a.   *Amylolysis*.   Alpha-amylase (1.13 Boeh U/ml, similar preparation to Amylolysis I) and G_4-amylase from Trypticase I, Purification III, Step 8 (8.2 Boeh U/ml, Table 3.5, 3.2.2.3) were added to blue amylose as described in Procedures and samples taken at 0, 10, 30, 61, 115, and 205 min from the G_4-amylolysis mixture, and at 0, 15, 35, 66, 120, and 210 min from the α-amylolysis mixture.

At 215 min, 0.442 ml (0.50 Boeh U) of α-amylase was added to the remaining 4.0 ml of α-amylase hydrolysis mixture to give an additional 0.125 Boeh U/ml of amylase and a 1.11 dilution of substrate.   Likewise, 0.061 ml (0.50 Boeh U) of G_4-amylase was added to the remaining 4.0 ml of G_4-amylase hydrolysis mixture, giving an additional 0.125 Boeh U/ml of G_4-amylase but a 1.015 dilution of substrate.   Samples were taken as usual at 264 min and the absorbance (A_{620}) corrected by multiplying by the respective dilution of substrate.   At 23.5 h 1.0 ml was removed for analysis and added to 0.25 ml of H_2O instead of 0.1N NaOH, and the precipitate and tube washed with 2.5 ml H_2O so that the amylase activity could be measured after determination of the absorbance, corrected as at 264 min.

Amylase activity in the 3.75 ml from 23.5 h was measured (using

0.5 ml + 0.6 ml Boehringer substrate) and the activity in the reaction mixtures calculated to be Boeh U/ml $*$ 3.75. The percent activity remaining was calculated from the total activity in the original mixture being 0.10 Boeh U/ml + 0.125 Boeh U/ml.

The supernatants from α- and G_4-amylolysis of blue amylose were examined by tlc.

The remaining precipitates (calculated: 2.44 ml in the α-amylase mixture, 2.06 ml in the G_4-amylase mixture, 2.00 ml in the control) were washed two times with H_2O, dried over P_2O_5, and weighed.

b. Alpha-amylolysis of G_4-limit dextrin. The precipitate from the 23.5 h sample from G_4-amylolysis was washed two times with H_2O; then 1.0 ml of 100 mM phosphate buffer added to the precipitate and 0.200 ml removed into each of three centrifuge tubes. The dextrin solutions were incubated at 37°C; then 0.012 ml of G_4-amylase (to give 0.46 Boeh U/ml), α-amylase (to give 13.0 Boeh U/ml), or H_2O was added to the respective tubes. After 1.5 h at 37°C, 1.30 ml of H_2O was added to each tube, centrifuged, and the absorbance of the supernatant measured at 620 nm. For comparison with previous absorbances the absorbance was corrected by (1.5/0.2) / (3.75/1), the dilution factor, since absorbance is linear below 20 mg/ml (91). Amylase activity in the G_4-amylase sample was measured (22% of the activity remained) an additional 0.012 ml G_4-amylase and 0.200 50 mM Buffer D was added to the G_4-limit dextrin which was incubated for 1 h a second time and it was confirmed that no hydrolysis occurred.

4.3.3. Preparation of Maltotetraose.

4.3.3.1.   General Procedures.   The substrate solution was placed in an Amicon ultrafiltration cell fitted with a PM-10 membrane (10000 dalton retention). G_4-amylase was added to the substrate solution and the hydrolysis mixture slowly stirred.

4.3.3.2. Experiment I.

4.3.3.2.1.   Hydrolysis. G_4-amylase (1.98 Units, DNS assay) from Trypticase I Culture, Purification I, Step 4 (3.2.1.1, Table 3.1) hydrolyzed cornstarch and potato amylose. Ultrafiltrate was collected periodically as summarized in 4.2.3, Table 4.9, stored at 9°C until analysis by tlc for identification of oligosaccharides, by the DNS method for reducing sugar, and by HPLC for quantification of oligosaccharides.

a. *Hydrolysis of Cornstarch*. A 2% solution (100 ml) of cornstarch (Safeway Foodstores) was prepared in 10 mM glycerophosphate buffer containing 5 mM $CaCl_2$, pH 7.0. G_4-amylase (1.75 ml of 1.13 DNS U/ml, DNS assay, 25°C) was added to 20.0 ml of solution in the UF cell. The hydrolysis mixture was layered with 4 drops of toluene and incubated at room temperature. After 24 h incubation an additional 30.0 ml of 2% cornstarch solution was added to the hydrolysis mixture. During the next 30 min, 36.5 ml of ultrafiltrate was collected (Filtrate I,1).   The remaining 15 ml of hydrolysis mixture was further incubated. At 94.5 h (after the initial addition of amylase) another 30 ml of cornstarch solution was added. At 117 h 29 ml of ultrafiltrate was collected (Filtrate I,2), leaving 16 ml hydrolyzate in the UF cell.

b. Hydrolysis of Cornstarch and Potato Amylose. A slurry of 5.03 g potato amylose (Sigma) in 35 ml H_2O was prepared. The pH was adjusted from pH 9 to pH 7 with dilute HCl. The neutralized amylose slurry was added to the 16 ml of hydrolyzate remaining in the UF cell at 119 h after the initial addition of G_4-amylase. At 143.5 h, 30.0 ml of filtrate was collected (Filtrate I,3) and at 167 h a final 3.0 ml was collected (Filtrate I,4).

4.3.3.2.2.   <u>Purification of Ultrafiltrate</u>. Filtrates I,1 - I,4 were combined and concentrated under reduced pressure to approximately 50 ml. The concentrate was examined by tlc and by HPLC on the APS-Hypersil column; then lyophilized. A solution of the lyophilizate (16 mg/ml H_2O) was analyzed for protein, for reducing sugar (AF method), and for total carbohydrate.

a. Ultrafiltration with PS membrane. Lyophilizate (376 mg) in 75 ml H_2O was placed in an ultrafiltration cell fitted with a Millipore Pellicon Type PS membrane (1000 dalton retention). Ultrafiltrate (60.6 ml) was collected, concentrated under reduced pressure, examined by tlc and HPLC, lyophilized and weighed (37.4 mg). The concentrate was washed four times with 25 ml of H_2O, and a total of 96 ml ultrafiltrate collected, lyophilized and weighed (22.0 mg). The turbid ultraconcentrate was lyophilized and weighed (321 mg). Solutions (4%, 1%, and 2% respectively) of the lyophilizates were examined by tlc, for reducing sugar (AF method) and for total carbohydrate.

b. Gel Filtration Chromatography. A 25% solution (0.500 g in 2.0 ml) of lyophilizate from Filtrates I,1 - I,4 was prepared in hot degassed H_2O. The solution was applied through a Pharmacia sample

applicator cup onto the Bio-Gel P-4 column described in 5.7.2. Colloidal gel remaining in the cup was washed with 1.0 ml H_2O (and the water soluble portion washed into the column). The cup containing the gel was removed and dried over Silica Gel. The dried gel was weighed and a cloudy 0.4 mg/ml solution examined by tlc and for protein content, reducing sugar, and total carbohydrate.

The first 150 ml of effluent from the column was collected in a flask; thereafter fractions of 9.6 ml (except fractions 1-5 contained ≈15 ml each) were collected (LKB fraction collector) and stored at 5°C. Even numbered fractions and fractions 23-29 were analyzed for total carbohydrate. Even numbered fractions and fractions 25 and 27 were examined by tlc. Fractions 24-27 were examined by HPLC using the APS-Hypersil column. Fractions 2, 3, 23, 25, and 27 were analyzed for protein. Fractions 2 and 3 were pooled and lyophilized. Fractions 26, 27, and pooled fractions 24-25 were dried under vacuum at 50°C for 4 h. Dried fractions were weighed and stored over Silica Gel. The conductance (WPA CMD400 Conductivity Meter) of fractions 21-23, 29-34, and even number fractions from 36-60 was measured.

4.3.3.3. <u>Experiment II</u>. G_4-amylase (125 Units) was used to hydrolyze potato amylose and the ultrafiltrate collected for analysis.

The ultrafiltration cell and PM-10 membrane were sterilized and connected to a sterile UF reservoir containing >2 liters of water. (The toluene used in Experiment I to inhibit microbial growth had etched the UF cell, consequently it was not used in this experiment and, where feasible, the system was sterilized.) The UF cell was incubated in a waterbath at 35-40°C. A similar system has been previously described (8).

Potato amylose (21.0 g) was added to sterile water already mixing in the ultrafiltration cell. G_4-amylase (3.0 ml of 41.7 U/ml, DNS assay at 37°C, from Trypticase I Culture, Purification II, Step 4 Buffer B described in 3.2.1.2 and shown in Table 3.2) in 10 mM glycerophosphate buffer (without $CaCl_2$), pH 7, was added to the amylose mixture. Ultrafiltrate was collected for 16 h at a flow rate of ≈ 60 ml/h and examined by HPLC on the APS-Hypersil column.

A portion (not measured) of the ultrafiltrate was concentrated under reduced pressure. During concentration tap water was drawn into the filtrate. The filtrate-tap water was removed and lyophilized and kept separate from the remaining ultrafiltrate which was also lyophilized. The lyophilizates were weighed (filtrate only, 1.98 g; filtrate containing tap water, 1.94 g) and stored over calcium chloride.

A 2% solution of the ultrafiltrate lyophilizate and a 4% solution of the filtrate-tap water lyophilizate were prepared. Each solution was analyzed by tlc, for reducing sugar (AF method), and for total carbohydrate. The weight of ultrafiltrate in the lyophilizate containing tap water impurities was calculated by proportion from the lyophilizate weight and the total carbohydrate content:

1.98 g/4.1 μmole ge/mg (filtrate only) = x/3.3 μmole ge/mg (containing tap water). Results are reported on the portion which did not contain tap water and the total filtrate weight calculated as x + 1.98 g.

4.3.3.4.   Experiment III. G_4-amylase was again used to hydrolyze potato amylose and the ultrafiltrate collected for analysis.

Distilled water (4 liters) was sterilized in the ultrafiltration reservoir and the apparatus set up as in Experiment II. Potato amylose (5.00 g) was added to sterile water in the UF cell and

equilibrated in the waterbath at 32°C. G_4-amylase (4.7 ml, 14.7 U/ml, DNS assay at 37°C) in 10 mM glycerophosphate buffer, pH 7, from Trypticase I Culture, Purification II, Step 4, from PIPES buffer (3.2.1.2, Table 3.2) was added to the amylose.

Ultrafiltrate was collected continuously for ≈20 h, until the system ran dry (1 liter of water was apparently lost when autoclaving the reservoir). Filtrate III,1 (160 ml) was collected continuously during the first 1.25 h, Filtrate III,2 (74 ml) during the next 0.5 h, III,3 (580 ml) during the next 3 h, III,4 (2100 ml) during the next 14 h, and III,5 (90 ml) during the last 0.5 h. Each fraction was examined by tlc and by HPLC using the APS-Hypersil column. The filtrates were combined, reexamined by HPLC and lyophilized. A 2% solution of lyophilizate in H_2O was prepared an analyzed for reducing sugar (AF method) and total carbohydrate.

Lyophilizate (700.0 mg) was added to 50.0 ml of 50 mM Imidazole-HCl buffer, pH 7 containing 0.002% chlorhexidene for removal of G_4-amylase from the affinity column described in 3.3.3.1, Step III,9 and shown in Fig. 3.2. The cloudy solution was filtered through Sinta Glass 4, and the slightly cloudy filtrate was next filtered through AG glass filter paper which gave a clear filtrate. The clear filtrate was then applied to the affinity column.

Chapter 5. Methods and Materials.

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5.9. High Field Proton NMR Spectroscopy.

CHAPTER 5.   METHODS AND MATERIALS.

5.1.   **Materials**.   Unless otherwise specified the following materials were used:

5.1.1.   **Carbohydrates**: soluble starch (BDH, AnalaR); potato amylopectin, a gift from E. Percival, B.V.= 0.18; Sigma potato amylopectin (Sigma A-8515, lot 102-3922); wheat amylopectin, prepared by P. A. Leonard (93) using the method of Schoch, B.V.= 0.054; amylose from potato type II (Sigma); potato starch (BDH, General Purpose 9083920C); maltotriose (Sigma, lot 113F0537); maltose monohydrate (BDH); α-D(+)-glucose, anhydrous (Sigma); D-glucose (AnalaR).

5.1.2.   **Amylases**: β-amylase from sweet potato, *Ipomoea batatas* (Boehringer, crystallized suspension in ammonium sulfate solution, 1367106/1.0 Oct 1978); α-amylase from *Bacillus amyloliquefaciens* (Sigma, alpha amylase Type II-A, lot 126B-0220).

5.1.3. **Miscellaneous**: Water refers to distilled water or purified water (Elga Water Purifier); $CaCl_2$ (AnalaR); NaCl (AnalaR); n-octanol (BDH); D_2O (see 4.3.1.3.2).

5.2. **Buffers**.

PIPES: 10 mM PIPES [Piperazine-N,N´-bis(2-ethane sulphonic acid)], (H & W), containing 5 mM $CaCl_2$, pH 7.0. The pH was adjusted to 7.0 with NaOH.

Phosphate: Prepared from potassium dihydrogen phosphate (BDH) for the specified concentration, pH adjusted with KOH to pH 7.0.

Acetate: sodium acetate-acetate, pH 4.6. Prepared from glacial

acetic acid (s.g. 1.05), pH adjusted with NaOH.

Buffer A: sodium   β-glycerophosphate (Koch-Light), pH 7, pH adjusted to 7.0 with HCl.

Buffer B: sodium glycerophosphate containing 5 mM CaCl$_2$, pH 7, pH adjusted to 7.0 with HCl after the addition of CaCl$_2$.

Buffer C: Imidazole (BDH)-HCl, pH 7.0.

Buffer D: Imidazole (BDH)-HCl containing 0.002% chlorhexidene (chlorhexidene diacetate hydrate, Sigma, lot 84F-0014), pH 7.0.

Buffer E: Imidazole (BDH)-HCl containing 0.002% Hibitane (ICI, chlorhexidene gluconate), pH 7.0.

D$_2$O-exchanged buffers are described in 5.9.

5.3. Definition of Terms.

5.3.1. As Related to Microbiology.

autoclave: sterilization at 121°C, 15 psi, for at least 15 min.

growth cycle: describes microbial growth by dividing the changes in cell mass into lag, exponential, and stationary phases.

lag: lag growth phase, the period during which little or no growth can be detected, preceeding exponential growth.

exponential: exponential or log phase, the period during which rapid cell division occurs and the logarithm of the culture density (measured as dry weight, see 5.5) increases linearly with time.

stationary: stationary growth phase, the period during which the medium has become unsuitable for growth and there is little change in cell mass.

specific growth rate, μ: the maximum rate of culture growth per unit of culture density, calculated as described in 5.5.5.3.

5.3.2. As Related to Enzymes.

Boehringer activity: glucosidase activity assayed by the coupled enzyme system used in the Boehringer α-Amylase PNP C-system Assay and described in 5.6.2.4.

G_4-amylase: maltotetraohydrolase, E.C. 3.2.1.60

specific amylase rate: The maximum rate of amylase induction in bacterial culture per unit of amylase concentration, calculated as described in 5.5.5.

5.3.3. As Related to Carbohydrates.

DP: number average degree of polymerization = number of glucose units per number of reducing sugar units.

ge: glucose equivalents, determined by the phenol sulfuric method for analysis of total carbohydrate, see 5.8.2.

CL: average chain length.

% Branching: number of α-1,6-linkages per 100 glycosidic bonds.

5.4. General Procedures.

5.4.1. Preparation of Polysaccharide Solutions. Unless otherwise indicated, solvent was added to form a cold paste, then diluted to the approximate volume, heated above 90°C to dissolution, cooled, and solvent added to make up the appropriate volume. If the solvent was phosphate buffer (see 5.5.3.3), the polysaccharide was first heated to dissolution in water (half the final volume), then concentrated buffer (at two times the molar concentration) was added to the cooled solution and the volume made up to the required final volume.

5.4.2. Fractionation by Alcohol Precipitation.

For final volumes greater than 15 ml, alcohol was added slowly by drops from a separatory funnel into the aqueous solution which was mixed with a magnetic stirrer. The mixture was left standing overnight at room temperature, then centrifuged on an MSE bench centrifuge for at least 8 min at 2730xg (4000 rpm) and the supernatant decanted. Alcohol was removed from the supernatant by concentration under reduced pressure.

For small volumes, alcohol was added by drops to the solution mixed by vortexing in the centrifuge tube, left standing, then centrifuged for 5 min in the CamLab bench centrifuge.

5.4.3. Concentration Under Reduced Pressure. Unless

otherwise specified, solutions were concentrated at <50°C on a Buchi rotavapor-R connected to a water pump.

5.4.3. Centrifugation.

CamLab: CamLab EBA III bench centrifuge fitted with either 3 ml or 10 ml conical tubes, centrifuged at a fixed angle. Between 5 to 10 min centrifugation at maximum speed gave a clear supernatant from bacterial cultures.

MSE 6L: MSE Mistral 6L was always centrifuged with 1.0 liter plastic jars with lids at 4°C at 2500 rpm (maximum speed), 2075xg. The jars were sterilized by autoclaving.

MSE bench: MSE Super Minor Bench centrifuge (head radius= 6 in) was generally fitted with 50 ml plastic tubes (x 8) and centrifuged for 25 min. Much of the centrifugation was done with the centrifuge stored in the cold room at 2-8°C.

MSE 18: MSE High Speed Centrifuge with angle head rotor 69179

(radius = 5.6 in) fitted with 250 ml autoclavable bottles; all centrifugation was done at 4°C.

5.5. **Microbiological Methods**.

    5.5.1.   Bacterial Strain.   Except where noted, the *Pseudomonas stutzeri* strain 17686, NCIB 11359, (freeze-dried 5 Sept 1977, National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland) was used in all cultures.   Ampoule I was revived October 1982 and Ampoule II in June 1984.   An agar slope of cells was also received from Aberdeen in July 1984 and is referred to as ´NCIB slope´.

    5.5.2.   Maintenance of Bacterial Strain.   Cell cultures were stored as McCartney slants at 7°C on 3% agar slopes of either Trypticase I or Soy media.   Cell viability and integrity were periodically checked by smears on petri plates and by Gram´s stain and subcultured onto fresh agar slopes.

    5.5.3.   Media.   All media except Trypticase I were prepared for sterilization as stock solutions with the starch (1.5% concentrate) separate from the remaining ingredients.   The stock solutions were then mixed aeseptically to give the final concentrations below.   For discussion see Sterlization, 5.5.3.3.

5.5.3.1. Complex Media.

Trypticase I: 1% trypticase peptone (BBL)

                0.5% yeast extract (Oxoid Powder L21)

                0.28% dipotassium hydrogen orthophosphate (BDH)

          0.1% potassium dihydrogen orthophosphate (BDH)

          1% soluble starch (Merck)

          (Trypticase peptone is a pancreatic digest of casein.)

Trypticase II: 1% trypticase peptone (BBL), lot GODHMY 7/81

          0.5% yeast extract (Oxoid Powder L21)

          0.28% dipotassium hydrogen phosphate (BDH)

          0.1% potassium dihydrogen phosphate (BDH)

          1% potato starch (BDH)

      Soy: as in Trypticase II, except

          1% trypticase soy broth (BBL lot 209610), replaced trypticase peptone. (Trypticase soy broth contains 57% trypticase peptone, 10% soy peptone, 17% NaCl, 8% dipotassium hydrogen phosphate, and 8% dextrose.)

    Bacto: as in Trypticase II, except

          1% bacto-tryptone (Difco) replaced trypticase peptone. (Bacto-tryptone is also a pancreatic digest of casein.)

    Yeast: as in Trypticase II, except

          yeast extract batch 2698902 replaced batch 02711693.

     YeCa: as in Defined Ca medium below, plus

          0.5% yeast extract (Oxoid, batch 26989027)

5.5.3.2. Defined Media.

       Ca: 0.28% dipotassium hydrogen phosphate (BDH)

          0.1% potassium dihydrogen phosphate (BDH)

0.1% ammonium sulfate (BDH Lab Reagent), 8mM

0.002% magnesium sulfate (BDH General Purpose), 16mM

0.0015% calcium chloride dihydrate (H&W), 0.1mM

1% potato starch (BBL)

Na: as in Ca, except

0.5% sodium chloride replaced calcium chloride.

Salts: as in Ca, plus

0.5% sodium chloride.

5.5.3.3. Sterilization. An aqueous concentrated
potato starch suspension, 1.5%, was prepared as described in 5.4.1.
and autoclaved separately from the remaining stock solution. In order
to minimize retrogradation occurring with time, the starch was prepar-
ed near the time of use. The starch and the stock solution were then
mixed, generally just prior to use in order in minimize starch retro-
gradation catalyzed by ions (94). The earliest microbial studies used
Trypticase I medium which was prepared by solubilizing all constitu-
ents together and then autoclaving. At that time the author did not
realize that autoclaving starch in the presence of phosphate can cause
cross-linking and resistance to amylolysis (66). Hence, after the
first cultures, starch and phosphate containing mixtures were heated
separately, cooled, and then mixed.

5.5.4. Culture Procedures and Apparatus.

5.5.4.1. Adaptation to Media. Incubations in
Boiling Tubes. Unless other details are specified, the following
procedure was followed: A loop of bacterial cells from a stock agar

slope (see Culture Maintenance 5.5.2) was used to inoculate 2-5 ml of complex medium, generally Trypticase or Soy, and incubated at least 24 h at 30°C. This was then transferred, initially as a 20-50% inoculum, into the new medium (5-15 ml in a boiling tube plugged with cotton wool) and incubated at 30°C with slow aeration (continuous minimal bubbling of air from the bottom of the testtube via a Pasteur pipette also loosely plugged with cotton wool). The boiling tubes were placed in a constant temperature waterbath at 30°C. Subsequent 5-20% subcultures into fresh medium were transferred from the previous subculture when subcultures became turbid, usually after 1 day, or at intervals as described in the experiment. Adaptation was most efficient with large inocula initially at ≥2 days, decreasing to a 5% inoculum and <1 day.

5.5.4.2. <u>Cultures in the Orbital Shaker</u>. In order to have sufficient inoculum for large scale cultures, an intermediate subculture was prepared in a Gallenkamp Cooled Orbital Incubator (1H-460) using 250 ml Erlenmeyer flasks containing 50 ml medium and 1.0 ml of inoculum from a boiling tube subculture. The flasks were agitated by swirling at 200 rpm (revolutions per minute) and the temperature thermostatically controlled at 30°C.

Each subculture's bacterial integrity was checked by petri smear incubated at least 24 h and/or by a Gram's stain and by confirmation of its amylase activity by assay and/or specifity for maltotetrao-hydrolase.

5.5.4.3. <u>Cultures in the Bio Flo Fermenter</u>. A New Brunswick Bio Flo Fermenter, Model C30 was used for batch cultures with Ca medium. The system contained a 2.0 liter glass fermenter cell

fitted with an integrated heater and thermostat, a jacketed thermometer, a condenser, a magnetic stirring bar set in a finned baffle
fitted to the bottom of a hollow aeration tube, an inlet port, and a
tube for the removal of samples. The cell unit was sterilized by
autoclaving. The system contained an integrated air pump, regulator
and airflow meter. The baffle speed could be regulated in revolutions
per minute. Medium was mixed in the fermenter by aeseptic addition of
1.5% starch and the remaining stock solution.

                         5.5.4.4. <u>Cultures in the Gallenkamp Fermenter</u>.
Batch cultures were prepared using a Gallenkamp Modular Fermenter
System consisting of a 1.0 liter glass fermenter cell containing
insert similar to the Bio Flo Fermenter (i.e., an integrated heater
and thermostat, a jacketed thermometer, a condenser, a magnetic
stirring bar set in a finned baffle fitted to the bottom of a hollow
aeration tube, an inlet port, and a tube for the removal of samples).
Unless otherwise mentioned, a few drops of antifoam agent, isopropylene glycol, were added before sterilization. The sealed cell unit
was sterilized by autoclaving. Sterile medium was mixed in the
fermenter by the aeseptic addition of 1.5% starch and stock medium
solution. After thorough mixing of the medium, a culture (= an
adapted subculture) from the orbital shaker was added to give a 10%
inoculum (usually 90ml in 900ml of medium). Agitation was adjusted to
give thorough, continuous mixing; which, in conjunction with a regulated airflow gave a mixture of small bubbles throughout the culture.
The culture temperature was regulated at 30°C. Samples were removed
through the sampling tube by suction with a syringe.

5.5.5. Measurement of Cell Growth. The cell concentration in the microbial cultures was indirectly measured by photometric determination of the culture turbidity. The turbidity was then calibrated to the dry weight of cells grown in Na medium. Cell growth in all culture media was measured turbidimetrically and the equivalent dry weight calculated from the one dry weight determination. Hence the absolute dry weights may not be accurate; still, the growth phases of the cells in various media should be relevant and is the main interest here. The specific growth rate in exponential growth was also determined for some of the cultures.

5.5.5.1. Turbidimetry. Cell concentration was estimated turbidimetrically by photometric measurement of the culture suspension in a Perkin Elmer double-beam spectrophotometer (1 cm path length, 650nm) using either medium or water in the reference cell. Absorbances >1.8 were diluted in order to give an absorbance <1.5; then multiplied by the appropriate factor. Both medium and water, when used as a diluent or as cell reference, introduce errors; in particular, the medium introduces errors because the starch gives a slightly turbid solution and therefore a false biomass. For example, when medium was used for the cell reference the bacterial growth in Ca medium initially appeared to be negative, an artifact caused by the amylolysis of the starch proceeding at a rate faster than the increase in turbidity from the growing cells. Generally it was found that a reference of, or dilution with water was more satisfactory than medium.

5.5.5.2. Bacterial Dry Weight Calibration. The dry weight of *Pseudomonas stutzeri* growing in Na medium was determined

at several times during the growth phases as well as in late exponential growth phase. The turbidities of the culture suspensions and appropriate dilutions were measured at the same times. As discussed below, only the dry weight of the cells in late exponential growth, those at 13.5 hours, were used for biomass calibration of the turbidimetric measurement to dry weight. This relationship, Biomass (mg/ml) = $0.299 \, A_{650} - 0.010$, was used for conversion of turbidimetric measurements in all culture media to dry weight.

The culture grown for the determination of dry weight was initiated from cells originating from the NCIB slope, revived in Soy medium, and then adapted to Na medium by subculturing four times in Na medium. Procedures were as described in 5.5.4. The fourth subculture was incubated 30 hours in dimpled flasks incubated in the orbital shaker, at which time turbidity had just begun to plateau. The fourth subculture was then used as a 10% inoculum in freshly prepared Na medium (900 ml) in the Gallenkamp fermenter and the culture incubated as described in 5.5.4. at 30°C with aeration of 0.5 liters/min. Sometime during the first 9 hours aeration stopped but was reinitiated at 9 h. Samples were taken for analysis and determination of biomass at 9.5h, 11.5h, 13.5h, and 17h after the initial inoculation.

The cells harvested at 13.5h were taken as standard cells. Gram's stain showed these to be gram-negative rods of the maximum size observed for *Pseudomonas stutzeri*, in late exponential growth (determined from a semilog graph of time vs turbidity).

Dry weight was determined on ten samples of standard cells (harvested at 13.5h) as follows: Using a clean volumetric pipette, 10.0 ml of culture was filtered under reduced pressure through a millipore filter (Oxoid Nuflow cellulose acetate, 0.22 micron N47/22G, which had been previously dried over P_2O_5 and weighed), washed with 25

ml sterile saline solution, rinsed with 10 ml sterile water, and dried at 96°C for four days to a constant weight. The mean dry weight was found to be 0.914 ± 0.029 mg/ml.

From the harvest at 13.5h a series of accurately prepared dilutions (2-, 2.5-, 5-, 10-, and 20-fold) was made by diluting with the filtrate from the dry weight determination. Absorbance was measured at 650 nm, 1 cm pathlength; the reference cell contained filtrate, absorbance 0.00. A biomass calibration curve was prepared as shown in the Graph "Standard Biomass Curve". As shown in the graph, absorbance is nonlinear at 1.5, consequently only the four data points at A<1.3 were used to determined by Least Squares Regression analysis the linear relationship, Biomass (mg/ml)= $0.299 \, A_{650} - 0.010$. This formula was used to calculate the equivalent dry weights from the experimentally measured absorbances.

Standardization by dry weight calibration is a conventional, practical method (95) but is arbitrary and does not reflect the dynamic processes occurring within a cell culture. It fixes a dry weight-absorbance relationship from one very specific time in a cell culture and erroneously implies a dry weight for an absorbance under very different conditions. Bacterial cell size, density and refractive index change during the growth cycle causing a change in absorbance properties. Cell size and shape are also influenced by the composition of the growth medium and pH (17), both of which change during batch culture.

The dry weight and turbidity of the culture removed at 9.5h, 11.5h, and 17h was also determined and the results summarized below. A graph of the dry weight against time showed the growth curve to be the same as that indicated by the graph of absorbance vs time, i.e. 9.5-13.5h in the exponential phase, and 17h in stationary phase.

'Standard' Biomass Curve
Figure 5.1
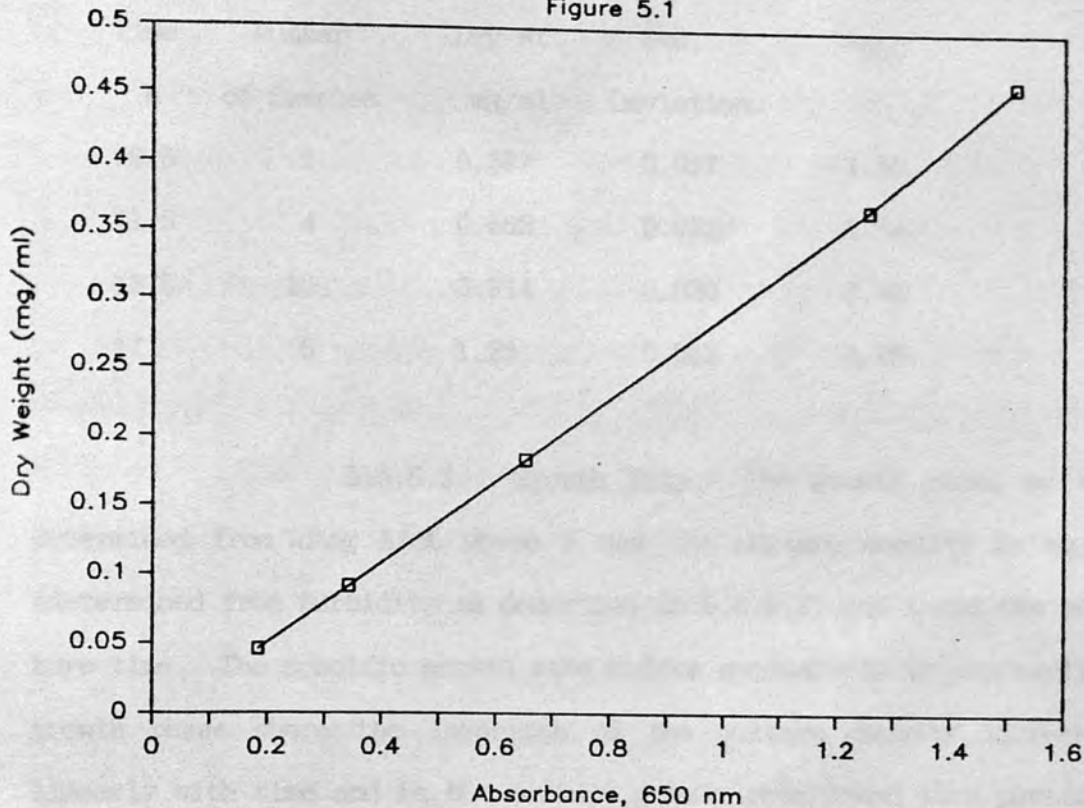
Fig. 5.1. Standard Biomass Curve for standardization of dry weight. *Pseudomonas stutzeri* cells from a Na Culture were harvested in late exponential growth, at 13.5 h. The turbidity and dry weight of these cells was measured and the relationship found to be Biomass (mg/ml)= $0.299 A_{650} - 0.010$.

However, the biomass curve of the dry weights vs absorbance was non-linear, with the actual absorbance measured varing between 6-46% higher than the absorbance predicted from the standard biomass curve determined using dilutions from 13.5h. This nonlinearity occurs in many systems (17) and may be in response to any number of variables (16, 95). The high absorbances are probably related to the consider-able slime the culture appeared to have as observed by distinct

aggregation of cells on Gram's stain.

Time h	Number of Samples	Dry Wt mg/ml	Std. Deviation	A_{650}
9.5	5	0.387	0.017	1.55
11.5	4	0.562	0.027	2.74
13.5	10	0.914	0.029	3.42
17	5	1.23	0.062	4.28

5.5.5.3. <u>Growth Rate</u>. The growth rate, μ, was determined from $d\log A/dt$ where A was the culture density in mg/ml (determined from turbidity as described in 5.5.5.2) and t was the culture time. The specific growth rate refers exclusively to exponential growth phase where the logarithm of the culture density increased linearly with time and is the maximum growth rate found in a particular culture.

5.5.6. Measurement of Amylase Activity.

5.5.6.1. <u>Amylase in Culture Samples</u>. Culture samples were removed and a portion centrifuged for at least 7 min on a CamLab bench centrifuge. The clear supernatant was stored at 0-4°C until analysis for amylase activity (by the Boehringer assay unless otherwise specified) and for product specificity as described in 5.6.1.

5.5.6.2. <u>Amylase Rate</u>. The amylase rate of a bacterial culture was calculated from the linear increase in the log of the amylase concentration with time; i.e. $d\log B/dt$ where B was the concentration of Boehringer amylase activity found in the culture supernatants and t was culture time. Specific amylase rate, μ_{am},

refers exclusively to a maximum rate of amylase induction and was determined only if the semilog plot of amylase concentration and time showed a linear relationship. Only the linear portion of the semilog plot was used for the calculation of the specific amylase rate.

5.6. Enzymatic Methods.

5.6.1. Product Specificity. The product specificity is that oligosaccharide produced by amylolysis of a 1-2% starch solution at 20-30°C. The oligosaccharide was identified by thin layer chromatography (5.7) which readily distinguished oligosaccharides up to G_6. Glucose syrup was run concurrently as a standard. When solutions were incubated for more than several hours all materials except the enzyme solution were kept sterile, and, if left overnight, were also layered with toluene. Starch solutions incubated with G4-amylase or β-amylase showed only G_4 or G_2, respectively, with high molecular weight polysaccharide remaining at the tlc origin. Incubations with α-amylase showed intermediate as well as low molecular weight oligosaccharides.

5.6.2. Amylase Assays. All enzyme solutions were kept at 0-9°C until addition to the appropriate substrate solution for determination of amylase activity. Most enzyme samples could be frozen without loss in amylase activity.

5.6.2.1. Turbidimetric Assay. The Turbidimetric Assay as described by Rungruansah and Panijpan (96) is continuous assay method which monitors the decrease in turbidity of a starch solution following amylolysis. Assays were done at room temperature in a Perkin Elmer 551 or 555 spectrophotometer at 650 nm. The amylase substrate, 2% soluble starch (or potato amylopectin, where noted) in

Buffer B was prepared as described in 5.4.1. Relative amylase activity is expressed in turbidimetric units of activity. One turbidimetric unit is that amount of enzyme which produces a decrease in absorbance of unity in 1 minute.

The turbidimetric assay is a rapid, convenient assay, particularly suited for detection of endo-amylase activity but is not readily standardized as is discussed by Rungruansah and Panijpan (96). Because soluble starch gave a low initial turbidity it was not an ideal substrate; potato amylopectin or potato starch were better substrates. Starch solutions in Buffer B gave turbid solutions whose initial absorbances varied from 0.17 to 0.36 and increased throughout the day, probably due to retrogradation/complexation catalyzed by the calcium ion in the buffer.

Even so, a linear relationship was found between the turbidimetric assay at R.T. and the DNS assay at 25°C assays (4 points from 3 stages in amylase purification from Trypticase I medium) and found to be: DNS Units = 2.87 Turbidimetric Unit - 0.10. (S.E. 0.26)

5.6.2.2.   Dinitrosalicylate Reducing Sugar Assay (DNS). The assay procedure described in *Methods in Enzymology, Vol. I* (65) was adapted using, unless otherwise mentioned, 1% potato amylopectin in 10 mM Buffer B. Solutions were incubated in a waterbath at 25°C (or at 37°C where indicated) and reducing sugar measured on samples taken at 0 min and after 5, 10 and 15 min incubation (or when amylase activities were higher, at 0, 3, 6, 9 min). Amylase activity was calculated from the initial linear increase in reducing sugar determined from the plot of reducing sugar vs time. One unit of amylase activity is that amount of enzyme which produces one μmole of reducing sugar/min (calibrated with maltose).

The DNS assay is reported to give an artificially high reducing value with increasing oligosaccharide chain length; in addition, calcium ion (as in Buffer B) interferes with determination of reducing sugar (35). The above modification in which the rate of increasing in reducing sugar was determined was expected to compensate for the errors in absolute measurement of reducing sugar. However, when the assay was determined at 30°C using 1% potato starch in 15 mM phosphate buffer and compared with the amylase activity determined at 30°C by the alkaline ferricyanide assay described below, the DNS assay was found to give less apparent activity than the AF assay. The relationship was found to be: DNS Units= 0.42 AF Units + 0.04. (Corr. coeff. 0.98, S.E. 0.01)

5.6.2.3.   Alkaline Ferricyanide Reducing Sugar Assay (AF). Amylase activity was determined by measuring the increase in reducing sugar in a solution containing 2.0 ml of 1.5% potato starch in 30 mM phosphate buffer to which 0.5 ml of enzyme sample was added. The solution was incubated at 37°C (or 25°C where indicated) and samples removed after 0, 1, 2, and 3 h (or more frequent intervals at higher activity) and reducing sugar measured by the alkaline ferricyanide method (5.8.3.2). Amylase activity was calculated from the initial linear increase in reducing sugar determined from the plot of reducing sugar vs time. One unit of amylase activity is that amount of enzyme which produces one μmole of reducing sugar/min (calibrated with maltose).

5.6.2.4.   Boehringer α-Amylase PNP C-system Assay (Boehringer). Amylase activity was determined by the Boehringer Mannheim colorimetric coupled enzyme system which uses p-nitrophenyl-α-D-

maltoheptaoside as substrate with α-glucosidase as a secondary enzyme. Solutions in a microcuvette with 1.0 cm pathlength were incubated in a thermostated cell (Perkin Elmer 551 or 555S spectrometer with Digital Controller) at 37°C, and activity (Units/liter) calculated from the increase in absorbance using the relationship:

$$U/l = 283.0 \times (V_T / V_e) \times A_{405}/min$$

where V_T is the total volume in the cuvette, V_e is the volume of the amylase sample, and 283.0 is a conversion factor. The relationship and the conversion factor ($10^6/\epsilon$) was determined from Beer's Law and the formula A= ϵcl. Thus the rate of amylolysis, $dc/dt = dA/dt \times 1/\epsilon l$, is produced by V_e of amylase in V_T of substrate solution. The molar absorptivity coefficient, ϵ, was given by Boehringer Mannheim as 3166. Where amylase activity was determined at 25°C, the conversion factor was 315.9, calculated from a molar absorptivity coefficient of 3533.

One unit of Boehringer activity is that amount of enzyme which hydrolyzes one μmole of p-nitrophenylmaltoheptaoside/min.

5.6.3. Purification Techniques.

5.6.3.1. Ammonium Sulfate Precipitation. Salt was added slowly with constant stirring. PH was read on a PTI-6 Universal Digital pH Meter, and adjusted with dilute ammonium hydroxide where necessary. Solutions were stored >24 h before centrifugation.

5.6.3.2. Acetone Precipitation. In the cold room, acetone (AnalaR) was added dropwise to a continuously mixing solution. The solution was stored at least 24 h before centrifugation.

5.6.3.3. <u>Ultrafiltration</u>. High pressure filtration utilized either Amicon cells or a ChemLab C-400 cell and flat membranes prepared and stored in 10% ethanol as recommended by the manufacturers. A nitrogen cylinder was used for a pressurehead of approximately 50 psi. In solutions where foaming occurred, one or two drops of n-octanol were added. Concentrates, when described as being washed, were reduced to a minimum volume and buffer or water of at least equal volume was added, the solution was reconcentrated and the process repeated at least once more. Unless otherwise stated, ultra-filtration was done in the cold room at 4°C. The following membranes were used:

> ChemLab G10T or Amicon PM-10 (10,000 dalton retention)
>
> Amicon Diaflo XM100A (100,000 dalton retention)

5.6.3.4.   <u>Affinity Chromatography on Sephadex G100</u>. A Sephadex G100 column (2.6 cm x 6.9 cm) was prepared as recommended by Pharmacia (97) in a jacketed Pharmacia K26/40 column fitted with adapters. The jacket temperature was maintained at 5°C using refrigerated 75% ethanol. The column void volume was found to be 15 ml using Blue Dextran 2000 (Pharmacia). The column was washed with >100 ml of 50 mM Buffer E. Samples were applied by gravity feed from a syringe through a junction adapter. Fractions (each 40 drops) were collected using the drop counter on an LKB fraction collector fitted with Unicord II uv detector (280 nm). Fractions were assayed by the Boehringer method.

5.6.3.5. <u>Hollow Fiber Filtration</u>. The procedures recommended by Amicon for the Amicon Hollow Fiber Filtration Apparatus and for use and storage of the Diaflo membrane cartridge were

followed. The hollow fiber cartridge (HIMP01-43, 0.1 micron retention) and apparatus were used at room temperature, however the flasks containing process solutions (starting material, retentate, and ultrafiltrate) were kept in an icebath throughout filtration.

5.6.3.6. <u>Microfiltration</u>. Solutions were filtered using the Amicon Centricon T30 (30,000 dalton retention) microfiltration apparatus. The Centricon tubes were centrifuged in the CamLab bench centrifuge and the concentrate washed by the addition of buffer and again centrifuging to concentrate the solution.

5.7. Chromatographic Methods.

5.7.1. Thin Layer Chromatography (tlc). Oligosaccharides were separated by tlc on Silica Gel (CamLab Cambridge MN-Polygram Sil G 0.25 mm) using the mobile phase proposed by Mansfield and McElroy (98) of n-butanol, ethanol, water (5:3:2) and detected by charring (5% H_2SO_4, >200°C). Glucose syrup, DE 50 was used as a standard, giving the following $R_{Glucose}$: maltose, 0.88; maltotriose, 0.76; maltotetraose, 0.67; maltopentaose, 0.53; maltohexaose, 0.42. Intermediate molecular weight polysaccharide such as that produced by G_4-amylolysis of 5000-12000 daltons (Chapter 4.2.2.1.) streaked between the origin and R_G 0.3.

5.7.2. Gel filtration. Bio-Gel P-4, 100-200 mesh, ≈90g, was hydrated in H_2O and prepared as recommended by Bio-Rad (Catalogue I, 1983). A jacketed Pharmacia column, 26 mm x 100 cm, fitted with an adapter was rinsed and dried three times with 1% dichlorodimethylsilane in benzene in order to reduce wall effects (99). The jacket was fitted to a Colora Ultrathermostat waterpump set at 34°C. The column

was prepared (100) and fitted with a peristaltic pump (LKB Varioperpex 12000) as recommended by Pharmacia. The column was washed at 53 ml/h with 1100 ml deionized, degassed H_2O. The jacket temperature was increased to 65°C and equilibrated overnight (101). The column (2.6 x 93 cm) void volume was found to be 210 ml, determined using Blue Dextran 2000 (Pharmacia).

5.7.3. High Performance Liquid Chromatography (HPLC).

Carbohydrates were detected by differential refractometery (Waters, M-6000) on a Waters Liquid Chromatograph after separation on one of the following columns:

APS-Hypersil (Shandon Hyperspheres, aminopropyl-silica gel), 250 x 46 mm; mobile phase: acetonitrile-water (65:35 v/v). Standard (flow rate, 1.0 ml/min): glucose syrup (DE 50) eluted glucose, 11.4 min; maltose, 13.2 min; maltotriose, 15.0 min; maltotetraose, 17.7 min. Peak areas were determined from (W x 0.5H).

Spherisorb 3 aminopropyl, S5NH-2722, 250 x 45 mm; mobile phase: acetonitrile-water (65:35 v/v). Standard (flow rate, 1.5 ml/min): glucose syrup eluted glucose, 4.8 min; maltose, 6.2 min; maltotriose, 8.4 min; maltotetraose, 11.6 min.

Synchropak GPC 100 (Anspec, silica support with a carbohydrate bonded layer), 250 mm x 46 mm ID; mobile phase: water. Standardized using 1% Dextrans (Pharmacia) at a flow rate of 0.2 ml/min: T2000, 9.8 min; T250, 10.0 min; T70, 10.6 min; T40, 12.0 min; T10, 15.4 min; and maltose, 18.4 min. The plot of time vs log of molecular weight was linear between 10000-70000 daltons (T10-T70).

Chrompak TSK G2000 SW, 300 mm x 7.5 mm ID, a gel permeation column; mobile phase: water containing 0.02% NaN_3. The column was standardized using Dextrans (Pharmacia) at 0.5 ml/min and also at 1.0

ml/min: T250, 6.5 ml; T70, 6.8 ml; T40, 7.5; T10 10.4; Dextran av MW 17900, av 9.2 ml; and maltose, 13.0 ml. The plot of time vs log of molecular weight was linear from 10000 to 40000 daltons (T10-T40) although the relationship is probably linear from 5000 daltons.

All samples were filtered through an HPLC 45 micron filter (ACRO L313, Gelman) before application to the Spherisorb, Synchropak or Chrompak columns. Some samples were detected by uv, 254 nm, as well as by RI. The lag volume between RI detection and the collection point was 0.5 ml, standardized with G_2, Chrompak column.

Peak areas, when reported as percentages, were integrated on a 308 Computing Integrator (Lab Data Control). Peak areas when reported as ratios were determined from A = peak width x ½peak height. Kainuma *et al* (102) reported a direct 1:1 relationship between RI peak area and peak weight of maltooligosaccharides G_1-G_7. In addition, a comparison of the specific refractive indices of glucose and maltose was found to be equal and linear between 0.1 and 20% w/v (103).

5.8. Other Analytical Methods.

5.8.1. Colorimetric Protein Assays.

5.8.1.1. Folin-Lowry. Protein was assayed using the procedure described by BDH with Folin and Ciocalteau's phenol reagent (BDH, lot 82135) and standardized using bovine serum albumin (Sigma A-4378).

5.8.1.2. Bradford Extramicro. The microprotein assay of Bradford (104) was adapted using 1-10 µg protein in 0.4 ml, to which 1.0 ml Bradford reagent was added. Bradford reagent was prepared by diluting to 0.5 liter instead of 1 liter, then filtered.

Standard bovine serum albumin (Sigma) gave a linear absorbance (595 nm, 1 ml cuvette, 1 cm pathlength) between 0.05-0.39.

5.8.2. Total Carbohydrate Concentration. Total carbohydrate was determined by the phenol-sulfuric acid method (105) as described in <u>Methods in Carbohydrate Chemistry</u> (106). Glucose was used as a standard, absorbance was measured at 490 nm, and carbohydrate expressed as μmole glucose equivalents (ge)/ml.

Analyses of the total carbohydrate (TC) of materials and of the dried glucans (vacuum dried, 22 hours at 92°C) are shown below; values are the averages of at least triplicates.

	Carbohydrate Content (w/w)	
	<u>Before Drying</u>	<u>After Drying</u>
maltose monohydrate	0.95	
maltotriose	0.99	
soluble starch (AnalaR)	0.85	0.95
potato starch (BDH)	0.64	0.97
potato amylopectin (Sigma)	0.73	0.86
potato amylopectin (Percival)	0.61	0.85
wheat amylopectin (Leonard)	0.50	0.65

Glucans were solubilized as described in 5.4.1. and mg carbohydrate/ mg weight calculated from:

$$TC/mg = \frac{(\mu mole\ ge/\ ml)\ (0.162)}{(mg/ml)}$$

For calculations with maltose and maltotriose the theoretical average molecular weight of glucose in each oligosaccharide, 171 and 168 respectively, was used instead of 162.

5.8.3. Colorimetric Assays for Reducing Sugar.

5.8.3.1. <u>Dinitrosalicylate (DNS)</u>. Reducing sugar was determined as described in *Methods in Carbohydrate Chemistry*, *Vol. 4* (36) using maltose monohydrate (Merck) as a standard.

5.8.3.2. <u>Alkaline Ferricyanide (AF)</u>. Reducing sugar was determined using an adaptation of Imoto and Yagishita's (107) modification of Scales' procedure. BRIJ-35 (Sigma), 0.5 ml, was included in the 1.0 liter alkaline ferricyanide (AnalaR reagent) solution and found to have no effect on the slope given by the standard, maltose monohydrate. The Reagent, 4.0 ml, was added to 1.0 ml solution containing 0.1 - 0.7 µmole reducing sugar, and heated in boiling water for 15 min, and the absorbance read at 420 nm. This Reagent has been reported to have an identical molar reducing value for maltodextrins (108) but when examined here maltotriose gave a higher molar reducing value than maltose. A comparison of the slopes gave glucose -1.05, maltose -1.70, and maltotriose -2.15; the intercepts being 1.22-1.26. All reducing sugar values reported in Results are given using maltose as standard; therefore, when longer oligosaccharides were contained in solutions the actual amount of reducing sugar would have been higher.

5.8.4. **Mutarotation of Oligosaccharides.**

5.8.4.1. <u>Measurement of Optical Rotation</u>. Optical rotation was measured using a Perkin Elmer 241 polarimeter fitted with a glass-jacketed microcell (≈2 ml), path length 1 decimeter and the optical rotation measured continuously at 589 nm (Na, D line) or 436 nm (Hg). Procedures described in *Methods in Carbohydrate Chemistry, Vol. I* (109) were followed. The temperature was maintained at

37°C in the jacketed cell using a circulating waterbath (Colora Ultra-thermostat). Both solvent and the cell were equilibrated at 37°C for at least 15 min before initiation of the experiment.

The carbohydrate was accurately weighed in a 2 ml or 20 ml volumetric flask to give a 4% solution on the addition of solvent. At zero time solvent was added to the crystalline sugar contained in a stoppered volumetric flask, the solution was rapidly mixed to dissolve the sugar, then quickly added to the polarimeter cell. The optical rotation was recorded continuously on a Shandon Southern Auto-graph S Recorder.

5.8.4.2. Calculations for Mutarotation Constants and Specific Rotations.

The mutarotation of glucose and its oligomers is almost entirely an α-β pyranose interconversion (69) and the rate constant for this anomerization reaction may be determined using the conventional formula:

$$k = (k_1 + k_2) = 1/t \ \ln \left[(r_0 - r_e) / (r_t - r_e) \right]$$

where $(k_1 + k_2)$ is the mutarotation constant for the balanced reaction, r_0 is the rotation at time t= 0, r_t is the rotation at time t, and r_e is the equilibrium rotation at t= ∞. The initial optical rotation, the optical rotation at infinity and the rate constant for each sugar solution were calculated using an iterative computer program provided by Dr. P. Finch.

5.9. High Field Proton NMR Spectroscopy.

[1]H-Nmr spectra (400 MHz) were recorded on a Bruker spectrometer using a 35° pulse with an accumulation time of 3.408 s. Spectra were recorded on samples after equilibration at 37°C.

5.9.1. Terminology.

p´H: pH meter reading.

pD = pH meter reading + 0.4 (68)

α-H1,4: H1 of α-1,4-glycosidic bond

α-H1 red: H1 of α-anomer, reducing sugar

α-H1,6: H1 of α-1,6-glycosidic bond

β-H1 red: H1 of β-anomer, reducing sugar

H4 term: H4 of non-reducing terminal glucosyl unit

β-H2: H2 of β-anomer, reducing sugar

5.9.2. Sample Preparation.

5.9.2.1. D$_2$O-Exchange. Unless otherwise descri-bed, samples were exchanged in deuterium oxide (Spectrosol BDH, 99.75% min isotopic purity) by lyophilization of the aqueous solution, addi-tion of D$_2$O (0.5 ml) and lyophilization again. Usually the exchange in D$_2$O and lyophilization were repeated a second time. The exchanged sample was stored over Silica Gel under vacuum, until the final addition of D$_2$O (P & S, 99.96%), just prior to use in spectroscopy studies.

5.9.2.2. Buffers for ^{1}H-nmr Spectroscopy. Buffers were exchanged with D$_2$O. Those buffers which were kept for later use were sealed in vials and stored at <9°C over Silica Gel.

56 mM phosphate buffer: The aqueous phosphate buffers described in 4.3.1.2.1 for mutarotation studies (56 mM, pH 6.6 and 7.0) were lyophilized and twice exchanged with D$_2$O.

200 mM imidazole-DCl buffer, p´H 7.35: 20 ml of 200 mM imida-zole-HCl buffer, pH 7 was freeze-dried. After addition of 17 ml D$_2$O to the lyophilizate the p´H was adjusted from 10.1 to 7.35 with 1 M

HCl, then diluted to 20 ml with D_2O.

Imidazole-DCl buffer containing CH_3CN and 0.002% chlorhexidene diacetate: Chlorhexidene diacetate was twice exchanged with D_2O and a stock D_2O solution of 0.53% chlorhexidene prepared; imidazole-HCl buffer, pH 6.5 was exchanged with D_2O and diluted to give an 0.5M stock solution; solutions at the specified concentrations were prepared by diluting the stock solutions with D_2O. For nmr studies, solutions were diluted with D_2O containing CH_3CN to give the final concentrations specified.

5.9.2.3.   Carbohydrates.   Polysaccharides were twice exchanged with D_2O (5.9.2.1) by addition of D_2O to carbohydrate and ultrasonication before lyophilization. It was found that soluble starch which had been heated in D_2O before lyophilization could not be resolubilized for nmr studies; therefore polysaccharides were not solubilized prior to exchange. Just before use in nmr studies the D_2O-exchanged polysaccharide was solubilized in D_2O (P & S, 99.96%) by heating and ultrasonication in a hot waterbath. Solutions containing D_2O with CH_3CN were prepared from 5.0 ml D_2O containing $\approx 2.5\mu l$ redistilled CH_3CN.

5.9.2.4.   Amylase.   Amylase solutions which were D_2O-exchanged by microfiltration (5.6.3.6) were assayed by the Boehringer Assay (5.6) after D_2O-exchange and stored at 0-9°C about 24 h before use in nmr studies. Amylase solutions which were lyophilized, were assayed by the Boehringer Assay before lyophilization and D_2O-exchange. As discussed in Chapter 3, lyophilized solutions of G_4-amylase in which pH was controlled (believed to be the case in nmr studies) showed little loss in amylase activity and frequently showed

an increase in activity (probably to be due to removal of inhibitory polysaccharide by retrogradation of the polysaccharide).

5.9.3. Hydrolysis Procedures. Starch substrate was solubilized and 0.45 ml equilibrated at 37°C in the spectrometer before the spectrum was recorded. The nmr tube was removed and amylase was immediately pipetted into the starch solution, zero time noted, the tube was inverted several times in order to mix the solutions, and replaced into the spectrometer for equilibration and accumulation of spectra.

5.9.4. NMR Spectra and Calculations. NMR spectra DS12-DS24 were run at pulse intervals of 3.4 s, spectra DS25-DS27 at pulse intervals of 5.1 s (i.e. 35° pulse, accumulate 3.4 s, delay 1.7 s), and DS28-DS43 at 7.1 s. In kinetic experiments (4.3.1, Hydrolyses I-XII) spectra were recorded after an accumulation of 32 scans/spectra unless otherwise specified. The signals at 5.4 ppm (α-H1,4), 5.2 ppm (α-H1 red), 3.4 ppm (H4 term), and 3.3 ppm (β-H2) were integrated, as well that of the internal standard, CH_3CN, when included in the solution. (See 5.9 for definition of terms.)

The spectra were analyzed as follows:

1.   In solutions containing CH_3CN (Hydrolyses IV-XII) the integrals from proton resonances were each divided by the CH_3CN integral. This permitted comparison of the integrals from spectra recorded at different times during a given hydrolysis.

2.   The integrals, including spectra which did not contain CH_3CN, were then corrected for partial saturation as described in 5.9.4.1.

3.   In Hydrolyses I-III, which did not contain CH_3CN, the

corrected integrals were divided by the α-H1,6 integral in order to compare spectra.

4. The relative integrals from Steps 2 and 3 were used in calculations described in 5.9.4.2-5.9.4.6 for quantitative determination of product, % β-anomer, % hydrolysis, and chain length.

5.9.4.1.   Correction Factors for Partial Saturation. For full relaxation times between nmr pulses, the time between pulses should be $5T_1$; however, if $5T_1$s had been used between pulses with an accumulation of 32 pulses for each spectrum, the number of spectra recorded would have been limited and the kinetic data restricted.   Instead, the peak integrals were corrected for partial saturation. Correction factors were calculated by Dr. Chris J. Groombridge and are given in Table 5.1.   Nmr experiments were run at pulse intervals of 3.4s (DS12-DS24), 5.1s (DS25-DS27) and 7.1s (DS28-DS43) and the integrals for each signal corrected by multiplying by the correction factor T_p shown in Table 5.1.

Table 5.1. Correction Factors (T_p) for Partial Saturation at Pulse Intervals of 3.4s, 5.1s, and 7.1s.

		T_p		
Peak	T_1	(3.4 s)	(5.1 s)	(7.1 s)
5.4 ppm (α-H1,4)	0.860	1.0081	1.0011	1.0001
5.2 ppm (α-H1 red)	2.077	1.0990	1.0387	1.0140
3.4 ppm (H4 term)	1.566	1.0530	1.0165	1.0045
3.3 ppm (β-H2 red)	2.410	1.1319	1.0563	1.0229

5.9.4.2.   Product (P_a and P_t).   Product was calculated and compared from two sets of integrals. i.) Product as the sum of the α-anomer and the β-anomer: P_a = α-H1 red + β-H2. ii.)   Product as new non-reducing termini (see discussion in 4.2.1.3.2): P_t = H4 term – α-H1,6.   Both product integrals were plotted against time.   The product produced per branch, P_a/α-H1,6, was also calculated and plotted against time.

5.9.4.3.   Percent β-anomer. The proportion of β-anomer to total anomeric product was determined and plotted against time. % β-anomer = β-H2/(α-H1 red + β-H2).

5.9.4.4.   Total Glycosidic Protons (Total H1). The integrals of the α- and β-anomers (P_a), and the α-1,4- and α-1,6-linkages were summed and the average and standard deviation of the spectra in a given hydrolysis calculated: Total H1 = α-H1 red + β-H2 + α-H1,4 + α-H1,6.   For Hydrolyses I-III (in which integrals were standardized relative to the internal α-H1,6 integral) and Hydrolyses IV, IX, and X (in which the HOD signal partially obscured the α-H1,6 integral) the Total H1 = α-H1,4 + H4 term.

5.9.4.5.   Percent Hydrolysis.   Percent total glycosidic bonds hydrolyzed was calculated from: % Hydrolysis = 100 * (α-H1 red + β-H2) / (α-H1 red + β-H2 + α-H1,4 + α-H1,6) = 100 * P_a / Total H1.   The percent α-1,4-bonds hydrolyzed was also calculated from: % 1,4 Hydrolysis = 100 * P_a/ (P_a + α-H1,4), and used for those spectra in which the HOD signal partially obscured the α-H1,6 signal.

5.9.4.6. <u>Average Chain Length (CL)</u>.

a.   Of Dextrins in Hydrolysis Mixtures. If a single oligomeric product is produced, or is in a known proportion, and if the dextrin is sufficiently large that the proportion of reducing sugar from the dextrin is small, then the average chain length of the dextrin can be determined from the glycosidic linkages remaining (α-1,4 and α-1,6) per branch:

$$\text{Dextrin CL} = \{(\alpha\text{-H1,4} - nP_a) + \alpha\text{-H1,6}\} / \alpha\text{-H1,6}$$

where n = the number of α-1,4-linkages in the oligomeric product, i.e. n = 3 for G_4, or 1 for G_2. The change in average chain length of the dextrin was followed during hydrolysis by plotting CL against time.

b.   Of Purified Products. The average chain length was determined from the Total H1/Branches.

$$\text{CL} = (\alpha\text{-H1,4} + P_a + \alpha\text{-H1,6}) / \alpha\text{-H1,6}$$

Chapter 6.   **Summary and Recommendations for Future Studies.**

6.1.   **Glycosidase Induction in** *Pseudomonas stutzeri*.

6.2.   **Purification of Maltotetraohydrolase.**

6.3.   **Studies with Maltotetraohydrolase.**

   6.3.1.   Characterization of G_4-Limit Dextrins.

   6.3.2.   Characterization of Amylopectins.

6.4.   **Significance.**

CHAPTER 6. SUMMARY AND RECOMMENDATIONS FOR FUTURE STUDIES.

6.1. Glycosidase Induction in *Pseudomonas stutzeri*. *P. stutzeri* was adapted to and grown in eight different media. During the initial stages of adaptation to various media, G_4-amylase was produced in each of the media. The culture supernatants were not examined for other glycosidases but each could be shown to contain G_4 product specificity. With adaptation to the various media the ability to secrete G_4-amylase was suppressed in all but one of the complex media and in two of the defined media.

High levels of G_4-amylase activity were produced in only one of the five complex media studied. Several of the media used here, in which G_4-amylase was suppressed, were very similar to the complex media used by Robyt and Ackerman (7) and by others (9, 12). Whereas they obtained high or intermediate levels of G_4-amylase induction, cultures adapted here produced none or very low levels of G_4-amylase. The complex media used by others and also used here, differed in the source or batch number of the protein digest and/or the yeast extract but otherwise were similar, as were the culture conditions. There appears to be subtle differences between the complex media which make it difficult to study G_4-amylase induction or at least to compare G_4-amylase induction when different batches of complex media are prepared. In studies here, high levels of G_4-amylase activity were only induced in the complex Soy medium, and the level of activity was transient. Dellweg *et al* (9) similarly found a rapid loss in activity in stationary phase, whereas Robyt and Ackerman (7) did not. All of these the media contained 1% starch as amylase inducer, potassium

phosphate buffer at pH 7; and the cultures were incubated at the same temperature and at a high rate of aeration.

Complex media generally induce higher levels of extracellular enzymes (25) and, in the eight media studied here, the Soy medium did produce the highest level of G_4-amylase. However the uncertainties in G_4-amylase induction warrants use of a defined mineral medium in which the maximum level of G_4-amylase may be lower, but there is more control over the contents of the medium.

In a defined medium of known composition, changes in cell morphology, amylase induction, and cell growth can be directly related to changes in the medium or in growth conditions. One of the defined media, the Na medium, in which G_4-amylase was secreted throughout exponential growth and remained at an intermediate concentration into stationary phase, could be used as a standard for studies.

In Na medium, changes in G_4-amylase induction could be related to changes in the rate of aeration, cell growth, pH, or the addition or deletion of various ions including calcium, magnesium, and phosphate. The effect of several of these changes have already been observed in Na medium but require further investigation. Differences in G_4-amylase induction were observed in three Na batch cultures. The highest G_4-amylase activity was induced in Na Culture B, after a period of low aeration, when rate of growth was slow (G_4-amylase activity also increased in cultures grown at 30°C and then stored at 9°C). In addition, throughout G_4-amylase induction in Na Culture B, the pH remained between pH 7.1 and 6.5 (as in Culture A but not Culture C). The pH of the culture appears to be critical since in Wober's complex

starch medium at pH 6, G_4-amylase was not secreted but remained cell-bound (29).

There have been reports of another exoenzyme, alkaline phosphatase, being released by *Pseudomonas* species, the release of cell-bound enzyme being dependent on the pH of the growing bacteria (60). In addition, magnesium ion has been shown to release some enzymes from the periplasmic membrane (60), and phosphate has been reported (7) to be absolutely essential for G_4-amylase induction in *P. stutzeri*. G_4-amylase secretion and exopolysaccharide production as related to starch or maltodextrin concentration could also be examined in defined medium. The production of exopolysaccharide has been related to an excess of carbohydrate, particularly when other nutrients are limiting (22), as well the ionic environment and the rate of aeration (23, 110). It has been shown that the cotransport of H^+ and/or Na^+ is linked to the active transport of amino acids and some sugars across the cell membrane (32, 33), which in turn may affect to G_4-amylase secretion. *P. stutzeri* secreted G_4-amylase in the three media (Soy, Na, and Salts), all contained Na^+, whereas the one defined medium which did not contain sodium (Ca medium) did not induce secretion of G_4-amylase.

In cultures in the Ca medium, the cell morphology was very irregular and no G_4-amylase was secreted, but what appeared to be a classical, G_2-producing endo-α-amylase was produced, just before or during early exponential growth. The specificity and properties of this amylase should be confirmed. The amylase was produced at an unusual period in the growth cycle and was present for a very short time. When Na^+ was added to Ca medium (= Salts medium), then this

amylase could not be detected. Even when cells were being adapted to Ca medium, the induction of the two amylases (G_4-amylase and the classical α-amylase) appeared to be an either/or induction. If the classical α-amylase was produced, no G_4-amylase activity was detected; and in G_4-amylase preparations at different stages of purification and from different cultures only G_4-amylase appeared to be present. Several aspects could be examined in the Ca medium. The cell morphology and induction of different amylases could be examined during cell adaptation to the Ca medium. After adaptation, the properties of the classical α-amylase could be examined.

Since G_4-amylase is produced during exponential growth, a ready means of production could be growth of *P. stutzeri* in continuous culture. By this method cells are maintained in exponential growth as cells are removed (with culture supernatant containing G_4-amylase) and fresh medium added to the remaining bacterial cells. Since G_4-amylase was found to hydrolyze unsolubilized amylose, it may be possible to grow bacterial cells on unsolubilized amylose thus obviating solubilization of the polysaccharide substrate. Perhaps G_4-amylase induction with slow growth in an ultrafiltration system (with cells retained but amylase filtered) would be possible. The bacterium and the induction of G_4-amylase lends itself to investigation.

6.2. Purification of Maltotetraohydrolase. G_4-amylase has been purified to a high level of purity by others. Robyt and Ackerman (7) purified G_4-amylase to a specific activity of 2590 U/mg (alkaline ferricyanide assay, 40°C), Dellweg *et al* (9, 10) to 287 U/mg (Phaedebas assay, 37°C), and Sakano *et al* (12, 13) to >100 U/mg (Nelson-Somogyi assay, 30°C). New methods of purification of G_4-amylase were not

developed here.

In G_4-amylase studies here, both partially purified and preparations of high specific activity were used and found to give similar results without regard to specific activity. Studies of endo-amylolysis using blue amylose and G_4-amylase preparations with specific activities of 0.1 U/mg and 2000 U/mg (Boehringer assay, 37°C; Hydrolyses C and E, respectively, shown in Fig. 4.19) similarly, only partially hydrolyzed blue amylose. In the nmr studies described in 4.2.1, different G_4-amylase preparations with different specific activities produced similar results, consistent with the studies. For example, G_4-amylolysis of potato amylopectin β-limit dextrin, by G_4-amylase from an affinity column (III,10; specific activity not determined but probably >2000 U/mg), and partially purified G_4-amylase (IV,4; 44 Boeh U/mg) both gave similar hydrolysis products, with the G_4-amylase from the affinity column giving a higher percent hydrolysis (the reverse would be expected if contaminating carbohydrase were present in the partially purified preparation).

Contaminating carbohydrase(s) did not appear to be present in, or if present, not in sufficient concentration to interfere with the G_4-amylase studies reported here. These included the nmr studies of anomeric and product specificity, degree of hydrolysis, hydrolysis of β-limit dextrin, the preparation of G_4-limit dextrin, the endo-amylolysis of blue amylose, and the preparation of maltotetraose. For preparation of G_4, the crude culture supernatant, with substrate in the ultrafiltration cell, is probably quite satisfactory. The crude culture supernatant, or perhaps partially purified G_4-amylase, is also probably sufficient for studies of the fine structure of starch or

glycogen. If a limit dextrin is produced (even from G_4-amylase containing contaminating carbohydrase) and can be characterized, then a piece of the structure of amylopectin is also characterized.

Although contaminating glycosidases did not appear to be a problem, slime and/or G_4-amylase inhibitor was a persistent problem. Slime or other insoluble material could sometimes be removed by freeze-drying G_4-amylase preparations, high speed centrifugation, or precipitation with calcium hydroxide. G_4-amylase is reversibly adsorbed to slime, insoluble material, and α-1,6-glucans (probably also to G_4-limit dextrin). This property has been used for purification of G_4-amylase, for example by adsorption to cornstarch (12) and by affinity chromatography on Sephadex (reference 9 and in Chapter 3.2.1.3). This property can also interfere with purification, as shown in most of the purifications in Chapter 3. Affinity chromatography was successful only on G_4-amylase preparations which retained little gel-forming and/or inhibitory material. Lyophilization or freezing may make the material insoluble and G_4-amylase easily removed from it.

6.3. Studies with Maltotetraohydrolase. Endo-activity has been shown to be an integral part G_4-amylase activity. G_4-amylase is not able, however, to hydrolyze α-glucans to the extent of most α-amylases, possibly because of steric factors or a requirement for a high number of binding sites along a glucosyl chain. This requires investigation; knowledge of the binding specificity would give details of the structure of α-glucans. In addition, further studies on the fine structure of α-glucans are required, for which G_4-amylase can be a valuable tool.

6.3.1. Characterization of G_4-Limit Dextrins. G_4-limit dextrins of narrow weight distribution were produced from wheat amylopectin, wheat amylopectin β-limit dextrin, and potato amylopectin β-limit dextrin. These differed in their average molecular weights and average chain length, and require further characterization. Glycogen phosphorylase and β-amylase could be used, since they should not hydrolyze G_4-limit dextrin if the outer chain stubs are shorter than G_4 or G_2 respectively. The extent of these hydrolyses, if they occur, could be determined by nmr studies similar to the G_4-amylolysis nmr studies. Debranching enzymes and chromatography would show the distribution of chains in the G_4-limit dextrin.

G_4-limit dextrins were precipitated from hydrolysis mixtures by the addition of 2-3 volumes of ethanol. Alternatively the G_4-limit dextrin could be fractionated by gel filtration so that there would be little doubt of the average dextrin properties.

An efficient method for preparation of G_4-limit dextrin and of maltotetraose should be G_4-amylolysis of amylopectin (or glycogen) in an ultrafiltration cell. A membrane with a molecular weight retention of 5000 should ensure that all G_4-limit dextrin was retained and concurrently produce partially purified G_4. If sterile conditions were maintained, then hydrolysis mixture could contain only substrate and amylase in water, and could continue for several days. G_4-amylase was found to remain active at room temperature, for >24 hours. There was little loss of activity in the presence of substrate (or probably dextrin) and activity was retained in water or a minimum of salts or buffer. This stability permits extensive hydrolysis of substrate, and the production of high yields of product molecules with minimal

requirements for purification.

6.3.2. Characterization of Amylopectins. The percent hydrolysis of different amylopectins and the different G_4-limit dextrins produced indicates that amylopectins differ not only in the outer chain length, but in the size of the clusters within an amylopectin molecule, and in the length of the chains connecting the clusters. G_4-amylolysis of β-limit dextrins, and differences in the oligosaccharide products produced from G_4-amylolysis, supports a model of amylopectin containing different intercluster chain lengths for different amylopectins.

Similar modifications of the cluster model have recently been suggested. Thurn and Burchard (56) concluded that amylopectin contained densely branched clusters interconnected by longer chains of about 22 units in length. They suggested that the clusters contained 3-22 branching units in a cluster and that the interconnecting B chains carried on average 1-4 clusters. Bertoft (111) reported a shift in the molecular weight of superclusters from waxy-maize starch, as α-amylase produced macrodextrins. He never observed a peak between 14,000-30,000 and found that the macrodextrins with molecular weights between 30,000-65,000 were more resistant to α-amylolysis than other intermediates. From studies with cyclodextrin glycosyltransferase, Bender *et al* (84) suggested that there are three types of clusters in potato and waxy maize amylopectin. The DP of the β-limit dextrins was 40-140 with an average DP 0f 75 and 80 respectively. They also concluded that potato amylopectin clusters were not as densely packed as maize clusters and that potato amylopectin contained more interchain branches throughout the molecule, so that potato amylopectin was not

as readily broken into cluster units.

G_4-amylolysis produced one type of cluster from wheat amylopectin, of narrow weight distribution with a molecular weight of between 10000-15000, a DP of $\approx$88. Potato amylopectin may contain two types of clusters, one similar to that of wheat amylopectin and one about 2.5 times larger. The clusters from potato amylopectin were less densely packed than those from wheat amylopectin.

6.4. **Significance.** *Pseudomonas stutzeri* can be adapted to a variety of media, including very minimal media in which marked changes in cellular metabolism occur. Cell morphology, cell aggregation, and glycosidase induction or suppression are are among the changes which are pronounced. Such distinct changes could be used for studies of the effects of ions, molecules, or culture conditions on the growth of *P. stutzeri* and the induction of exoenzymes.

G_4-amylolysis has shown that amylopectin contains clusters and that these clusters are fairly uniform in molecular weight. The proportion of clusters (G_4-limit dextrin) and the size of the clusters differs in different types of amylopectin. This is an important characteristic of different sources of amylopectin and G_4-amylase is able to differentiate these characteristics. G_4-amylase can thus be used to characterize and distinguish between many types of amylopectin.

^{1}H-nmr spectroscopy was demonstrated as a powerful analytical method for kinetic and mechanistic analysis of the enzymatic hydrolysis of α-glucans. In a simple, one step procedure the enzymatic hydrolysis of polysaccharide was followed by recording spectra at

regular intervals. Several parameters were determined including the substrate and product specificity of several amylases, the percent hydrolysis of glycosidic linkages, and the number of product molecules produced per branch. It is thus possible to determine the rates of enzyme hydrolyses in order to provide data for fitting to alternative kinetic models.

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