

https://dspace.org/

# The degradation of starch by the oral streptococci.

**Coogan, Maeve Mary.**

**1990**
http://hdl.handle.net/10539/22946

Downloaded from a DSpace repository. https://dspace.org/

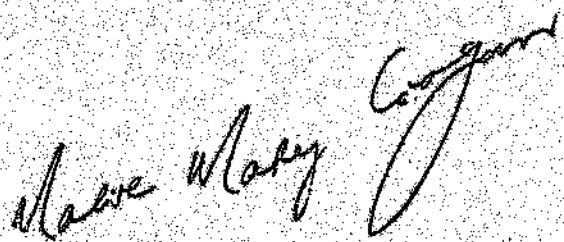
# The degradation of starch by the oral streptococci

Maeve ; Coogan

A thesis submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Doctor of Philosophy.

Johannesburg, 1990

I hereby declare that this thesis is my own work and has not been submitted or incorporated in another dissertation or thesis for any other degree.



Maeve Mary Coogan

DEDICATION

TO MY HUSBAND GIL

ACKNOWLEDGEMENTS

I wish to thank my supervisor Dr. R.L. Jones for his continued advice, guidance and assistance throughout this study and to Professor K L Manchester for his invaluable criticism. I also wish to thank my co-supervisor Prof. H Koornhof for his practical advice and for the use of the gas chromatograph.

I thank Dr G. Colman for his friendly advice and for providing the starch hydrolysing isolate FW 213 from the culture collection at Colindale, England. A special word of thanks to Mrs Fiona Ringdahl without whose tireless assistance and encouragement this study would not have been possible. I also wish to thank Professor J.F. van Reenen for his continued encouragement, support and advice and Dr P.J. Greaven for his support during the final stages of the preparation of this thesis.

I wish to thank Dr D. Cuff of Astrochem for allowing me the use of the ion chromatograph. To Professor V Pretorius, Dr H W Viljoen and Mr A Hasset of the Institute of Chromatography, University of Pretoria for their help with the gas chromatography-mass spectroscopy analyses. I am also grateful to Dr P. Bow of the Department of Conservative Dentistry for undertaking the clinical examinations. A special word of thanks to Mrs Denny Meyer of the Department of Statictics for her statistical assistance and friendly advice.

I also wish to thank Mrs Carol Cordosa of Central Graphic Services for the preparation of the figures and histograms and Mr P. Stickler of the Department of Geography for the preparation of the diagrams of starch and the chromatograms. A special word of thanks to Miss Colleen O'Connor for the many hours spent in the typographical preparation of this manuscript and Mrs Gwen Maclachlan for additional typographic assistance.

I wish to thank the South African Sugar Association for their generous financial assistance which enabled me to undertake this study. A further word of thanks to the Research and Education Fund of the Dental Association of South Africa and the Anonymous Dental Fund for additional financial support.

Finally I wish to thank my husband Gil for his patient understanding and encouragement during the preparation of this thesis.

ABSTRACT

Two carbohydrates predominate in modern diets namely sucrose and starch. They are metabolized by oral microorganisms to products implicated in dental caries. The influence of sucrose and its metabolites is well documented but starches have received less attention. This study investigated the secretion of starch hydrolysing enzymes by oral streptococci and the production of sugars, acids and polysaccharides from the two components of starch, amylose and amylopectin. A stock strain FD 213 and a further 20 strains of starch hydrolysing streptococci from 7 subjects were studied. Eight isolates were obtained from carious debris and 12 from dental plaque.

Most cultures adapted well to growth on amylose and amylopectin but required time for enzyme induction. This was seen as diauxic growth in some cultures. The remaining strains possessed constitutive enzymes and grew almost immediately. All isolates released small amounts of glucose and glucose oligomers and produced lactic and acetic acids. However, isolates from healthy teeth did not grow more vigorously, produce a lower pH and more acids than strains from carious lesions. This shows that organisms from carious lesions and healthy teeth are similar and suggests that diet and not a characteristic flora may be primarily implicated in the carious process.

Fifteen strains produced a water insoluble polysaccharide from amylopectin. The most active strain S. sanguis 1 204 produced two polysaccharides. Both polysaccharides consisted of glucose units joined by alpha-1,4-linkages with alpha-1,4,6-branches. The first polymer, designated amyloglucan was adherent, highly insoluble and resistant to enzyme attack which implies that it will accumulate on teeth. Methylation analysis, infra-red spectroscopy and enzymic analysis showed it was chemically similar to amylopectin but it was less branched with longer interior and exterior chains. The second polymer was a non-adherent soluble glucan similar to amyloglucan with long exterior chain lengths. Infra-red spectroscopy, methylation and enzymic analysis show amyloglucan and the soluble glucan are not synthesized de novo but are the products of the modification of the amylopectin molecule.

All isolates produced enzymes with alpha-amylase like properties. Further investigation of S. sanguis MC 204 and S. mitis 101 showed they produce two extracellular enzymes. The ability of these enzymes to degrade amylose and amylopectin and to produce dextran from sucrose was determined. Their action was similar to alpha-amylase and glucosyltransferase.

This investigation proves that acids and polysaccharides are produced from starch. The production of these metabolites from starch and sucrose supports the view that these carbohydrates are complementary and additive in their cariogenic activities.

LIST OF PUBLICATIONS

The following publications have arisen from material included in this thesis.

Coogan, M M (1977) Relationship between dental caries, sex and starch hydolysing organisms. Journal of Dental Research, 56, D177 (No. 14).

Coogan, M.M. & Jones, R.L. (1983) The action of oral streptococci on starch. Journal of Dental Research, 62, 500.

Coogan, M.M. & Jones, R.L. (1984) The production of acids from starch by the oral streptococci. Journal of Dental Research, 63, 773.

Coogan, Maeve M. (1985) A note on a code for the identification of the 'viridans' streptococci. Journal of Applied Bacteriology, 58, 513 - 516.

Coogan, Maeve M. (1986) A code for the identification of the 'viridans' streptococci. Journal of the Dental Association of South Africa, 41, 129 - 133.

Coogan, M M, Jones, R L, Meyer, D H & Viljoen, H W (1988) Starch and dental caries. Journal of the Dental Association of South Africa, 43, 515 - 519.

Coogan, M M, Jones, R L & Meyer, D H (1989) Starch and dental caries. Journal of Dental Research, 68, 720 (No. 22).

Coogan, M M, Jones, R L & Langenegger, S (1990) The production of water insoluble polysaccharide from amylopectin by Streptococcus sanguis I and II. Proceedings of the International Symposium on Streptococci and Streptococcal Diseases. Reed Books Limited: Berkshire (in Press).

# CONTENTS


| | |
|---|---|
| Declaration | ii |
| Dedication | iii |
| Acknowledgements | iv |
| Abstract | v |
| List of Publications | vi |
| Contents | vii |
| Introduction | viii |

STUDIES ON THE DEGRADATION OF STARCH BY THE ORAL STREPTOCOCCI

| | |
|---|---|
| 1. Introduction | 1 |
| 2. Materials and methods | 80 |
| 3. Results | 118 |
| 4. Discussion | 173 |
| 5. References | 194 |
| 6. Appendix | 226 |


* * * * * * *

THE DEGRADATION OF STARCH BY THE ORAL STREPTOCOCCI.

Research into the role of carbohydrates in dental caries dates back to the last century with the stulation of the acidogenic theory of Miller (1895). He proposed that microorganisms produce acids which cause the destruction of teeth. He produced artificial dental caries by incubating a tooth in bread, sugar and saliva and was able to soften and decalcify enamel and dentine.

Most studies have implicated sucrose in this process but there is no clear quantitative relationship between sucrose consumption and the establishment of dental caries. This suggests that a number of fermentable carbohydrates besides sucrose may be implicated in the carious process. Starches have been implicated in the carious process for various reasons. Under the influence of bacteria they produce acids, stimulate the formation of acidogenic plaque and produce carious lesions in animals. Furthermore, after a starch rinse the pH of plaque drops well below the critical level for decalcification of enamel. None of these studies however, have determined the type of acids that were produced and this indicated that there was a need to investigate acid production from starch.

It is well known that there are 2 components found in starch namely, amylose and amylopectin. Different amounts of these constituents occur in different starches. For example, wheat starch contains more amylopectin than potato or rice starch. This

difference may be the reason why wheat starch appears to be more cariogenic than potato or rice starch (Lanke, 1957). No studies have been undertaken on the caries potential of purified amylose or amylopectin for this reason these starches were investigated.

The cariogenicity of carbohydrates does not depend entirely upon the amount of titratable acids produced, but on the exposure of the tooth surface to these acids. This can be influenced by the retention and clearance of foodstuffs, for example, foods that adhere readily to teeth are able to decalcify enamel more readily than non-adherent foods because acids are held in intimate contact with enamel by these foods. One of the reasons why sucrose is retentive is that it is metabolized to form extracellular polysaccharides which becomes part of the plaque matrix.

The ability to synthesize these polysaccharides may determine whether oral organisms are cariogenic because they aid attachment to teeth and promote plaque formation. In addition plaque polysaccharides lead to the creation of an anaerobic environment which stimulates the production of lactic acid, the most caries promoting of the acids found in plaque. Plaque polysaccharides also trap acids against the tooth and aid decalcification.

Starch may contribute more actively to the carious process after it has been metabolized into polysaccharides. In this regard a number of starch branching enzymes have been described (Greenwood and Milne, 1968; French, 1975). If starches are metabolized to

polysaccharides in the mouth they would have a similar action to sucrose because they could be metabolized to acids by bacteria and they could create an environment which would contribute to the carious process.

The main enzymes that degrade amylose and amylopectin are the alpha-amylases. There are 2 possible sources of these enzymes in the mouth namely, saliva and the oral flora. Although salivary alpha-amylases have been extensively investigated, little work has been undertaken on the starch degrading enzymes produced by plaque microorganisms. These enzymes may be more important than salivary enzymes because they occur in plaque and have intimate contact with sites on the tooth where caries attack occurs.

A variety of starch hydrolysing organisms occur in the mouth, particularly the streptococci (Buchanan and Gibbons, 1974; Facklam, 1977). These cocci predominate in the mouth and have been implicated in the carious process. Starch hydrolysing streptococci were studied 20 years ago but the degradation of amylose and amylopectin by these organisms was not investigated. An aim of the present study was to investigate the degradation of amylose and amylopectin by the oral streptococci.

It is not known whether caries promoting organisms differ from the same organisms found in healthy subjects. The metabolites they produce may differ. Particular attention was paid to the production of acids and polysaccharides because these metabolites may be important in the carious process.

# Studies on the degradation of starch by the oral streptococci

# CHAPTER 1

# INTRODUCTION

## CONTENTS

INTRODUCTION..... 4

1 CARBOHYDRATES AND DENTAL CARIES..... 5

1.1 THE ROLE OF SUCROSE..... 5

1.1.1 Acid production from sucrose..... 5
1.1.2 Polysaccharide production from sucrose..... 6

1.2 THE ROLE OF STARCH..... 8

1.2.1 Modification of starch..... 8
1.2.2 Acid production from starch..... 9
1.2.3 Polysaccharide production from starch..... 10

2 PROBLEMS ASSOCIATED WITH CHEMICAL ANALYSES OF STARCH..... 11

2.1 SOLUBILITY..... 12

2.1.1 Solubility of starch granule..... 12
2.1.2 Solubility of unrefined starch..... 13
2.1.3 Solubility of the components of starch..... 13
2.1.3.1 Solubility of amylose..... 14
2.1.3.2 Solubility of amylopectin..... 15

2.2 RETROGRADATION..... 16

2.3 VARIABILITY..... 18

2.3.1 Variability of starch granule..... 19
2.3.1.1 Size and shape of starch granules..... 19
2.3.1.2 Amylose and amylopectin content of starch granules.. 19
2.3.2 Variability of starch..... 20
2.3.2.1 Fractions of starch..... 21

2.4 STRUCTURE AND CHEMICAL PROPERTIES OF STARCH..... 23

2.4.1 Amylose..... 23
2.4.1.1 Chain length of amylose..... 23
2.4.1.2 Molecular weight of amylose..... 23
2.4.1.3 Fine structure of amylose..... 24
2.4.2 Amylopectin..... 29
2.4.2.1 Chain length of amylopectin..... 29
2.4.2.2 Molecular weight of amylopectin..... 30
2.4.2.3 Fine structure of amylopectin..... 31
2.4.3 Comparison of amylose and amylopectin..... 35

2.5 STRUCTURAL ANALYSES OF STARCH..... 36

2.5.1 Methylation analysis..... 36
2.5.2 Infra-red spectroscopy..... 38
2.5.3 Periodate oxidation..... 39
2.5.4 Enzymic analysis..... 40
2.5.5 Iodine reaction..... 44

3 BACTERIAL METABOLISM..... 46

3.1 METABOLIC PATHWAYS..... 46

4 ENZYMES..... 47

4.1 ENZYMES THAT REGULATE SUCROSE METABOLISM..... 47

4.1.1 The production of acid from sucrose..... 47
4.1.1.1 Sucrose-phosphotransferase system..... 47
4.1.1.2 Lactate dehydrogenase..... 49
4.1.1.3 Pyruvate kinase..... 50

4.1.2 The production of polysaccharides from sucrose..... 52
4.1.2.1 Glucosyltransferases (EC 2.4.1.5)..... 52
4.1.2.1.1 Physical properties..... 54
4.1.2.1.2 Mechanisms of action..... 55
4.1.2.2 Fructosyltransferase (EC 2.4.1.10)..... 55

4.2 ENZYMES THAT REGULATE STARCH METABOLISM..... 57

4.2.1 Enzymes that degrade starch..... 57
4.2.1.1 Alpha-amylase (EC 3.2.1.1)..... 57
4.2.1.1.1 Physical properties..... 60
4.2.1.1.2 Effect of pH..... 61
4.2.1.1.3 Effect of temperature..... 62
4.2.1.1.4 Kinetics of action..... 62
4.2.1.1.5 Measurement of kinetics..... 64
4.2.1.1.6 Mechanisms of action..... 64
4.2.1.2 Beta-amylase (EC 3.2.1.2)..... 65
4.2.1.2.1 Physical properties..... 66
4.2.1.2.2 Kinetics of action..... 66
4.2.1.2.3 Mechanisms of action..... 67
4.2.1.3 Amyloglucosidase (EC 3.2.1.33)..... 68
4.2.1.4 Pullulanase..... 69
4.2.1.5 Isoamylase (EC 3.2.1.65)..... 70
4.2.1.6 R-enzyme (EC 3.2.1.9)..... 71
4.2.1.7 Schardinger enzyme (EC 2.4.1.19)..... 72
4.2.1.8 Newer bacterial enzymes..... 73
4.2.1.8.1 Bacillus polymyxa amylase..... 73
4.2.1.8.2 Pseudomonas stutzeri amylase..... 73
4.2.1.8.3 G6 enzyme..... 73

4.2.2 Enzymes that regulate acid production from starch..... 74

4.2.3 Enzymes that produce starch..... 75
4.2.3.1 Phosphorylase (EC 2.4.1.1)..... 75
4.2.3.2 Starch synthetase (EC 2.4.1.21)..... 76
4.2.3.3 Q-enzyme (EC 2.4.1.18)..... 77

5 AIMS OF THE INVESTIGATION..... 78

LIST OF ILLUSTRATIONS

Table 1.1 The beta-amylolysis limit and chain..... 30
length of amylopectins.

Table 1.2 The degree of polymerization of amylopectin..... 31
isolated from naturally occuring starches.

Table 1.3 Comparison of the properties of amylose and..... 35
amylopectin.

Table 1.4 Physical constants of several alpha-amylases.... 61

Figure 1.1 Diagramatic representation of the structure..... 26
of amylose and the action of enzymes that
catalyse the degradation of this starch.

Figure 1.2 Diagramatic representation of the structure..... 33
of amylopectin and the action of enzymes that
catalyse the degradation of this starch.

Figure 1.3 Suggested mechanism of the bond-breaking step....63
in the hydrolysis of an alpha-1,4-glucosidic
bond by alpha-amylase.

# INTRODUCTION

Dental caries is one of the oldest diseases known to mankind. A great deal of research has been undertaken into the aetiology of dental caries and in particular into the role of carbohydrates such as sucrose. Starch has also been implicated as one of the causative agents but has not received the same attention as sucrose.

A major step forward in the understanding of this disease was the discovery 25 years ago of the production of polysaccharide from sucrose by Streptococcus mutans (Gibbons et al, 1966). This polysaccharide was considered important in the development of dental caries because it is sticky, accumulates in dental plaque and enhances the ability of the cariogenic species S. mutans to adhere to teeth (Gibbons et al, 1966; deStoppelaar, van Houte and Backer-Dirks 1970; Staat et al, 1975; Gibbons and van Houte, 1980). The possibility of S. mutans and other microorganisms utilizing carbohydrates other than sucrose to produce polysaccharides in dental plaque has been suggested by Bowen et al, (1983) but has not been explored. This thesis describes such a study. It reports on the secretion of starch hydrolysing enzymes by oral streptococci and the production of polysaccharides and acids from the two components of starch namely amylose and amylopectin. This investigation shows the oral streptococci can produce similar end products from both sucrose and starch.

Difficulties encountered during the microbiological and biochemical studies were associated with problems with the chemical analysis of starch and its products. These problems and their solutions are described in detail because they were responsible for the major obstacles in the progress of this study and in research undertaken by other workers in this field.

# 1 CARBOHYDRATES AND DENTAL CARIES

Research into the role of carbohydrates in dental caries dates back to the last century when Miller (1890) postulated his acidogenic theory. He was the first researcher to propose microorganisms are involved in the production of acids and the destruction of teeth. He produced artificial dental caries by incubating a tooth in bread, sugar and saliva and found he was able to stimulate enough acid production in 48 hours to decalcify sound enamel. In 3 months he produced softening of dentine and enamel similar to dental caries. Thus the ingestion of fermentable carbohydrate is followed by the production of acid (Bowen 1969; Edgar et al, 1975) and the decalcification of teeth.

## 1.1 THE ROLE OF SUCROSE

### 1.1.1 Acid production from sucrose

Sucrose has been implicated in the carious process for two reasons. Firstly, the cariogenic bacteria S. mutans produce lactic, formic and acetic acid from this carbohydrate (Duguid, 1985). Secondly, lactic acid which is considered the most cariogenic acid

predominates in plaque formed in association with high sucrose consumption and increases in significant quantity in plaque challenged with a rinse of this carbohydrate (Bowen, 1976; Distler and Kroncke, 1983).

### 1.1.2 Polysaccharide production from sucrose

Dental plaque is the soft, tenaceous bacterial deposit which forms on the surface of the teeth (McHugh, 1970). Some plaque bacteria such as, for example, *S. mutans*, *S sanguis*, *S. mitis* and *S. salivarius* form plaque deposits in the form of polysaccharides *in vivo* and *in vitro* (Walker and Jacques, 1987). These polysaccharides have been identified as glucans and fructans and are synthesized by the enzymes glucosyltransferases and fructosyltransferases (Critchley *et al*, 1967; Guggenheim, 1970).

Many studies have shown sucrose derived polysaccharides contribute to plaque formation and enable microorganisms to adhere to teeth (Gibbons *et al*, 1966; Wood and Critchley, 1966; Gibbons and Banghart, 1967; Guggenheim and Schroeder, 1967; Mc Cabe, Keyes and Howell, 1967; Keyes, 1968; Gibbons and Fitzgerald, 1969). Apparently glucosyltransferase in saliva is adsorbed onto hydroxyapatite where it retains activity and produces glucans in the presence of sucrose (Rolla *et al*, 1983). Glucosyltransferase is not essential for the attachment of *S. mutans* to teeth but glucan synthesis appears to promote the accumulation of *S. mutans* cells (Gibbons, 1983).

To date some of the most studied glucans are the extracellular dextrans produced by S. mutans. They can be divided into water soluble and insoluble fractions and may be recognised as a gelatinous residue which attaches readily to glass (Gibbons and Nygaard, 1968; Sidebotham, Weigel and Bowen, 1971; Tosiki et al, 1976). Methylation studies have shown there are two types of chains in glucans with a predominance of either alpha-1,3 or 1,6 linkages. (Walker et al, 1983).

Water soluble glucans have many branches and contain a high per centage of alpha-1,6-linkages (Trautner, Birkhed and Svensson, 1982). In contrast water insoluble glucans are mainly linear consisting of an alpha-1,6 backbone with side chains containing a high proportion alpha-1,3-linkages (Nisizawa et al, 1976; Hare, Svensson and Walker, 1978; Brooker, 1979; Trautner and Fulgenhauer, 1979; Chassy, 1983; Ciardi, 1983; Walker et al, 1983). The chains pack into sheets with maximal intra- and intersheet hydrogen bonding which makes the glucan insoluble (Marchessault and Deslandes, 1981).

There are at least two reasons to support the belief that water insoluble glucans with alpha-1,3-linkages are important in the carious process. Firstly, cariogenic strains of S. mutans produce water insoluble glucans with a high proportion of these linkages (Trautner, Gehring and Lohman, 1978; Walker, Pulkownik and Morrey-Jones, 1981). Secondly, the suppression of these bonds results in a decrease in viscosity of the polysaccharide and no adherence to glass (Takehara and Inoue, 1981).

Most of the microorganisms investigated for insoluble glucan synthesis belong to the S. mutans group. There are other species including S. sanguis, S. salivarius and S. mitis capable of producing insoluble dextrans but their role in dental caries is not clear (Gibbons and Banghart, 1968; Ceska et al, 1972; Dewar and Walker, 1975; Hare, Svenson and Walker, 1978; Freedman et al, 1979; Tsumuraya and Misaki, 1979; Bowen et al, 1983).

## 1.2 THE ROLE OF STARCH

Changes in starches brought about by chemical and enzymic methods may contribute to the carious process.

### 1.2.1 Modification of starch

The modification of starches, for example, hydrogenation and the removal of barriers may change their susceptibility to enzymic degradation. In a study in 1978 Toors and Herczog showed hydrogenated potato starch is readily degraded by S. mutans . If this starch is hydrogenated further it loses its potential to produce acid and can be used as a sugar substitute (Frostell et al, 1974; Edwardson, Birkhed and Mejare, 1977; Frostell and Birkhed, 1978; Birkhed et al, 1979).

Another example of starch modification is given by Snow and O'Dea (1981) who isolated an alpha-amylase inhibitor from unrefined starch. This substance is destroyed when starch granules are passed through a roller mill to produce refined flour. This supports the ideas of Fiehn and Moe (1983) who proposed substances

in unrefined starch may inhibit alpha-amylase in plaque. The barrier to alpha-amylase activity may be fibre because Snow and O'Dea (1981) found fibre forms a physical barrier to enzyme hydrolysis. This system may operate in the mouth because Mormann, Schmid and Muhlemann (1983) have found alpha-amylase inhibitors are successful in reducing the acidogenicity of plaque in rats and humans fed a starch diet.

### 1.2.2 Acid production from starch

The production of acids from starch by microorganisms is a basic requirement for implicating starch in the carious process. Tatevossian (1982) observed starches are degraded to sugars in the mouth. These sugars will be further degraded to acids and for this reason starch is able to lower the pH of debris in carious lesions (Caldwell and Bibby, 1958).

Several workers have investigated the production of acids from starch. Birkhed and Skude (1978) found plaque suspensions produced as much acid from soluble starch as glucose. Furthermore, caries prone patients produced acid more readily than caries free subjects (Vratnos et al, 1979). Studies have shown the critical pH for decalcification of teeth lies between 5,2 and 5,7 (van Houte, 1980). Mormann and Muhlemann (1981) measured the pH of interdental plaque after the consumption of wheat starch and found it dropped to 4,75. Similar results were obtained when Distler and his coworkers (1982) used a starch solution as a rinse whereas pretreatment of starch with amylase enhanced acid production (Buehrer and Miller, 1983).

The production of acid from starch by oral bacteria has also been investigated. Steinkraus, Bibby and Gilmour, (1969) found S. mutans grown on wheat, corn, rice and rye starch produced titratable acids. Later in 1972 Thatchet used salivary microorganisms and obtained titratable acids readily from starch.

1.2.3 Production of polysaccharide from starch

As pointed out above, sucrose derived polysaccharides are considered a major contributing factor in the carious process. However, Bowen in a discussio. on the carious process said 'I think we need to know what other polysaccharides are found in dental plaque' (Bowen at al, 1983). From a biochemical point of view there seems no reason why starch cannot be modified to form a new polysaccharide, but this has yet to be shown. The observation that some branching enzymes are capable of producing a branched · lymer from starch (French, 1975) supports this contention.

## 2 PROBLEMS ASSOCIATED WITH CHEMICAL ANALYSES OF STARCH

Most problems associated with chemical analyses of starch are related to its variable and insoluble nature. The basic reason is starches consist of a variety of components. Some have been extensively studied whereas others remain ill defined.

Starches occur in plants as granules of different complexities (Lehninger, 1975). Early reports indicated granules are heterogenous but only 50 years ago researchers realized they contained at least two components. The first was a linear polymer called amylose and the second a highly branched polymer was designated amylopectin (Pacsu and Mullen, 1941). Later work has shown five different components may be isolated from starch (Lansky, Kooi and Schoch, 1949; Perlin, 1958; Banks and Greenwood, 1959; Whistler and Doane, 1961; Montgomery, Senti and Sexton, 1961). These components may combine in different proportions in starch to give a heterogenous mixture.

Chemical analyses of starch are difficult to undertake for at least 4 reasons. Firstly, starch is insoluble. Secondly, the polymer has the tendency to retrograde. Thirdly, it is heterogenous and therefore variable. Fourthly, starch possesses complex chemical characteristics that require a variety of sophisticated methods for analysis.

## 2.1 SOLUBILITY

Starch is insoluble in both the granular and refined forms. The development of satisfactory methods to dissolve starch granules and the components of starch was the first step in the study of these polymers.

### 2.1.1 Solubility of starch granules

Starch granules are insoluble in cold water but swell when heated. Initially the swelling is reversible but becomes irreversible when the gelatinization temperature is reached. With gelatinization crystallinity is destroyed and material from the granules diffuses into the water. Many factors may influence gelatinization including the degree of crystallinity, the amount of amylose and the size of the granule. Large granules gelatinize at a lower temperature than small granules. Granules may be dispersed by autoclaving, mechanical grinding, treatment with liquid ammonia and dissolving in either cold alkali or dimethyl sulfoxide (Alsberg, 1938; Greenwood, 1970, 1976; French, 1984).

X-ray diffraction studies have shown granules contain crystalline and amorphous regions (Ahmed and Lelievre, 1978). The crystalline regions are more resistant to acid attack and enzyme hydrolysis than the amorphous areas (French, 1984). Early workers believed these regions were rich in amylose but more recent studies suggests they are caused by the formation of compact double helices between chains in adjacent amylose molecules or between clusters of chains from single or neighbouring amylopectin molecules (Manners, 1985a).

### 2.1.2 Solubility of unrefined starch

The behaviour of unrefined starch in solution does not follow the simple pattern of crystalloids because the degree of molecular aggregation profoundly influences solubility. There is no sharp demarcation between insoluble and dissolved states. Instead a continuously varying spectrum of solubility levels exists (Radley, 1968).

Starch dissolves in water by hydration with the transfer of interpolysaccharide binding to polysaccharide-water binding. Initially water molecules of absorption occupy hydrogen bonding positions not involved in intermolecular bonding. Water penetrates amorphous regions and competes for intermolecular bonds which eventually become solvated (Whistler, 1973). Starch in solution is highly unstable and undergoes spontaneous aggregation to a less soluble state. This change in solubility can be detected by increased resistance to enzyme action (Radley, 1968).

### 2.1.3 Solubility of the components of starch

As was mentioned previously unrefined starch is a mixture of at least two components, amylose and amylopectin. When these components are separated they differ in their solubility characteristics.

2.1.3.1 Solubility of amylose

Pure amylose is insoluble in cold water (Meyer, Bernfeld and Wolff, 1940) but dissolves when heated because temperatures above 124°C destroy hydrogen bonding (Protzman, Wagoner and Young, 1967). On cooling below 65°C partial crystallization or retrogradation occurs (Whistler and Johnson, 1948; Whistler, 1953). If a neutral aqueous solution is required the amylose is dissolved in aqueous alkali and then neutralized (McCready and Hassid, 1943). When this solution is allowed to stand it becomes turbid and the amylose precipitates (Paschall and Foster, 1952).

The tendency for amylose to precipitate from neutral aqueous solutions has led to the search for alternative solvents. Meyer and coworkers (1950) used chloral hydrate, hydrazine hydrate and ethylenediamine hydrate to dissolve amylose. Foster and Hixon (1943, 1944) found anhydrous ethylenediamine was a good solvent, but it is difficult to handle. Dimethyl sulphoxide (DMSO) is the most recent solvent to receive attention. It dissolves amylose readily and is inert. The solution may remain stable for several weeks, but precautions must be made to exclude water (Foster, 1965). Despite the development of several methods none is completely satisfactory.

Amylose is insoluble because it is a linear molecule with a uniform structure that probably forms strong intermolecular bonding. Insoluble molecules usually form perfectly ordered arrays to develop crystalline regions that are firmly cross-linked. They are easily associated, form crystals, have extended ribbon-like

structures and contain either 1,3-linkages or 1,4-linkages and few 1,6-linkages (Whistler, 1973).

2.1.3.2 Solubility of amylopectin

Unlike amylose, amylopectin forms reasonably stable aqueous solution. Solubility depends upon structural characteristics and the presence of certain bonds. For example, amylopectin does not form ribbon-like structures and contains 1,4-linked main chains and flexible 1,6-linked branches. Branching in the molecule greatly reduces the possibility of intermolecular association and leads to ready solubility. The presence of irregularities and either carboxyl, sulphate or phosphate groups also increases the solubility of a polysaccharide (Whistler, 1973).

Amylopectin dissolves readily in sodium or potassium hydroxide, chloral hydrate, hydrazine hydrate, ethylenediamine hydrate and dimethyl sulphoxide (Foster, 1965) Degradation of the polymer occurs in the presence of hydrogen and hydroxyl ions but neutral aqueous solutions are stable for long periods (Young, 1984).

## 2.2 RETROGRADATION

The tendency of starch to retrograde has complicated studies into the chemical stucture and enzymatic degradation of these polysaccharides. Retrogradation is the process by which starch in the dissolved or hydrated state reverts to a water-insoluble form (Whistler, 1953). Insoluble amylose is microcrystalline and consists of extended parallel chains which interact by strong secondary forces, probably hydrogen bonds. Retrogradation involves interaction between neighbouring molecules, mutual alignment, expulsion of water and formation of new intermolecular forces (Foster, 1965).

Studies on the hydrolysis of retrograded amylose by acid and alpha-amylase yielded resistant fragments with a degree of polymerization (D.P.) between 32 and 50. The production and size of these fragments indicate retrograded amylose consists of double-helical crystalline regions 10 nm long interspersed with amorphous regions. Miles et al (1985) proposed amylose solutions separate into polymer rich and deficient regions and crystallization occurs in the polymer rich regions. The amorphous regions are hydrolyzed by acid and alpha-amylase whereas the crystalline region remains intact (Jane and Robyt, 1984).

Apparently, retrogradation is an intermolecular process with the rate depending upon the amylose concentration. High molecular weight amylose retrogrades less readily than lower weight polymers. Possibly high molecular weight preparations have intramolecular and not intermolecular polymer-polymer interaction. The reaction is

characterised by a lag phase with the formation of nuclei of suitable size and conformation and slow structural transformations in the amylose molecules. After this phase the reaction rate increases rapidly in an autocatalytic fashion until precipitation occurs. The rate is slow for high molecular weight amylose provided the concentration is low. During the initial lag phase no increase in light scattering is observed, often for a period of weeks. Throughout this period physical investigations can be conducted (Foster, 1965).

Many factors can affect the retrogradation of amylose. Amylose from corn starch retrogrades more rapidly than similar fractions prepared from potato starch. Corn amylose preparations have a lower molecular weight than potato amylose (Loewus and Briggs, 1957). When either of these amyloses are partially hydrolysed by acid, the retrogradation rate is further increased. There appears to be an optimal size for retrogradation and the rate decreases markedly at high molecular weights (Whistler and Johnson, 1948).

Various salts and additives can affect the rate of retrogradation. Salts of monovalent anions and cations retard the rate. The most effective anion is iodide whereas potassium is the most effective cation. A solution of 0,1M potassium iodide can decrease the rate of retrogradation 10 fold. In contrast, polyvalent cations such as sulphate have an accelerating effect on retrogradation (Loewus and Briggs, 1957; Collison, 1968).

The pH also affects the rate of retrogradation. At pH 4 a solution of amylose remains relatively constant for a number of days whereas

at pH 6,5 retrogradation is more rapid (Paschall and Foster, 1952). An explanation is amylose may retain part of its helical character when it is dissolved in acids and alkali. Disruption of this helical conformation which occurs at a neutral pH may be a slow and essential step in the retrogradation process (Foster and Sterman, 1956).

Researchers believed retrograded solutions are not suitable for quantitative physicochemical studies, but Bernfeld and Gurtler demonstrated in 1948 that enzymic degradation, which is inhibited by retrogradation, is more complete if an alkaline solution of amylose is added to a strongly buffered enzyme solution. In many cases amylose remains dispersed for a long period in neutral aqueous solution so physical studies can be undertaken (Foster, 1965).

Amylopectin has little tendency to retrograde from neutral aqueous solutions. However, retrogradation may occur in highly concentrated solutions and at low temperatures (Greenwood, 1970; Young, 1984).

## 2.3 VARIABILITY

As was mentioned previously starches are heterogenous and variable. Starch granules as well as the components of starch show variability which has made the study of their structure and behaviour difficult (Williams, 1968; Greenwood, 1970).

## 2.3.1 Variability of starch granule

### 2.3.1.1 Size and shape of starch granules

The size and shape of starch granules vary considerably. Leaf starch is stored temporarily as tiny granules 1 μm in diameter (French, 1975). These increase during photosynthesis and decrease when they are converted to sugars for metabolism or translocation to other parts of the plant (Badenhuizen, 1969; Alexander, 1973). By contrast granules formed in storage organs are more than 100 times larger than leaf granules and are not subjected to daily changes.

Furthermore, the shape and size of starch granules is influenced by the plant origin. For example, potato starch granules are larger than wheat or tapioca granules (Greenwood and Thomson, 1962). On the one hand, both potato and wheat granules are of different shapes and sizes. On the other, granules in maize starch are almost uniform in size (Griffin, 1985). However, granular size may be influenced by environmental conditons such as the site of development (Sandstedt et al, 1962) or by genetic factors. Waxy maize, for example, can produce granules which are either small and compound or long and bulbous (Sandstedt and Abbott, 1965).

### 2.3.1.2 Amylose and amylopectin content of starch granules

The starch components present in a granule may vary. There are at least 3 factors responsible for this variability, the sources of the starch, the maturity of the plant and the presence of different

starch components. Firstly, seed and fruit starch in unripe apples, mangoes, green tomatoes, bananas, waxy maize, glutinous rice and genetic variants of barley contain no amylose. In contrast genetic variants of the pea and maize contain more than 50 percent amylose whereas maize known as amylomaize may contain 70 percent amylose. Secondly, researchers have established the amylose content of starch granules increases with maturity of the plant (Greenwood, 1956; French, 1975; Whistler and Daniel, 1984). Thirdly some starch granules contain material which has properties of neither amylose nor amylopectin but a compound with properties between these two components (Greenwood, 1956).

More recent work has shown this third fraction consists of chains of glucose units with alpha-1,4 linkages. The chain lengths differ from those of both amylose and amylopectin (Manners, 1985a). This starch appears to be a less branched amylopectin or a slightly branched amylose (Whistler and Daniel, 1984). The different fractions could be present in the starch granule simultaneously which suggests starch formation is a dynamic process in which the component are being continually formed and degraded.

### 2.3.2 Variability of starch

Researchers have found starch is not uniform but consists of at least 5 variable components (Lansky, Kooi and Schoch, 1949; Perlin, 1958; Banks and Greenwood, 1959; Whistler and Doane, 1961; Montgomery, Sexson and Senti, 1961).

2.3.2.1 Fractions of starch

Early studies undertaken in 1915 by Tanret separated the fractions of starch by gelatinization, sedementation and re-extraction in boiling water. He removed traces of amylose with cotton and concluded starch contained two separate entities. Pacsu and Mullen (1941) improved on Tanret's fractionation methods and isolated a blue staining component called alpha-amylose and a purple component beta-amylose which later became known as amylopectin (Radley, 1968).

Research performed by Haworth and co-workers in 1928 yielded di- and trimethyl ethers of D-glucose which suggested starch contained highly branched molecules. Methylation analysis of the fractions by Meyer, Wertheim and Bernfeld (1940) yielded 3,7 percent 2,3,4,6-tetra-O-methyl-D-glucopyranose from amylopectin and 0,32 percent from amylose. The tetramethyl sugar was derived from the non-reducing chain ends which showed amylose contains long linear chains whereas amylopectin is highly branched with many non-reducing ends.

Further evidence that starch consisted of two polymers was put forward by Meyer and co-workers in 1940 who showed methylated amylose formed pliable and amylopectin brittle films. Later in 1943 Hassid and McCready performed an analysis on potato starch and obtained 4,67 percent 2,3,4,6-tetra-O-methyl-D-glucopyranose from amylopectin and 0,32 percent from amylose. They found the average length of chains in the amylopectin molecule was 25 and amylose 350 D-glucopyranose units.

Starch may contain more than two components. A small yield of a third fraction has been isolated from certain starches (Lansky, Kooi and Schoch, 1949; Perlin, 1958; Banks and Greenwood, 1959). It has a shorter chain length and is degraded less readily by alpha-amylase than amylopectin which suggests it is a highly branched molecule. It is possible all starches contain this third constituent (Banks and Greenwood, 1959). Whistler and Doane (1961) have described a fourth fraction which they isolated from high amylose corn starches. It has a molecular weight between amylose and amylopectin, a chain length of 30 to 47 glucose units and is less branched than amylopectin. Furthermore, amylopectins with less branching than normal have been isolated from corn starch (Montgomery, Sexson and Senti, 1961). They occur with a short-chain amylose and can be separated from normal amylopectin by differential ultracentrifugation (Greenwood and Thomson; 1960, 1962). Most starches probably contain of a mixture of these fractions.

## 2.4 STRUCTURE AND CHEMICAL PROPERTIES OF STARCH

Despite nearly 60 years research into amylose and amylopectin uncertainties about their structures remain because both polysaccharides are complex. This complexity has made it difficult to study the chemical properties of starch.

### 2.4.1 Amylose

#### 2.4.1.1 Chain length of amylose

Early work on amylose showed the molecule consists of a long unbranched chain of 1,4 linked alpha-D-glucopyranose units with a D.P. of 200 to 350 (Meyer, Wertheim and Bernfeld, 1940, 1941; Hassid and McCready, 1943). Later studies indicated the molecule has a D.P. of thousands of glucose units (Banks and Greenwood, 1959b, 1967).

The size of the molecule may vary according to the plant source. Takeda and co-workers (1984) found potato amylose has a D.P. of 4920, 8 chains and branches with lengths from 4 to more than 100 glucose units, whereas wheat amylose is a short-chained molecule with a D.P. of 570 and 2 chains on average. These average values probably represent a mixture of chains of different lengths.

#### 2.4.1.2 Molecular weight

Researchers do not agree about the molecular weight of amylose because it is difficult to measure the number of glucose units in this starch.

Chemical end-group assay methods cannot be used directly on amylose because of the large molecular weight (Greenwood, 1970). Alternative methods include osmotic pressure measurements, enzymic methods and the use of triacetate and tricarbanilate derivatives (Greenwood and Robertson, 1954; Jorgensen and Jorgensen, 1960; Burchard and Husemann, 1961; Banks and Greenwood, 1968). However, measurement of the limiting viscosity number provides the most convenient and accurate method (Greenwood, 1970).

The cereal starches, corn, wheat and barley have the lowest molecular weights, whereas potato and tapioca starch have the highest weights (Foster, 1965). Values for molecular weights however, may vary according to the method of analysis. Meyer (1950) on the one hand measured the molecular weight of corn and potato starch by aqueous leaching and obtained values between 10,000 and 60,000. Foster (1965) on the other hand quotes weights from osmotic pressure measurement ranging from 50,000 to 200,000, with a D.P. from 300 to 1300 (Foster, 1965). These figures underestimate the size of amylose because it is degraded in the presence of air but not under anaerobic conditions (Baum and Gilbert, 1954). Amylose prepared in the absence of oxygen has a range of molecular weights between 160,000 to 700,000 (Greenwood, 1960) which is about four times larger than the values obtained under aerobic conditions.

2.4.1.3 Fine structure of amylose

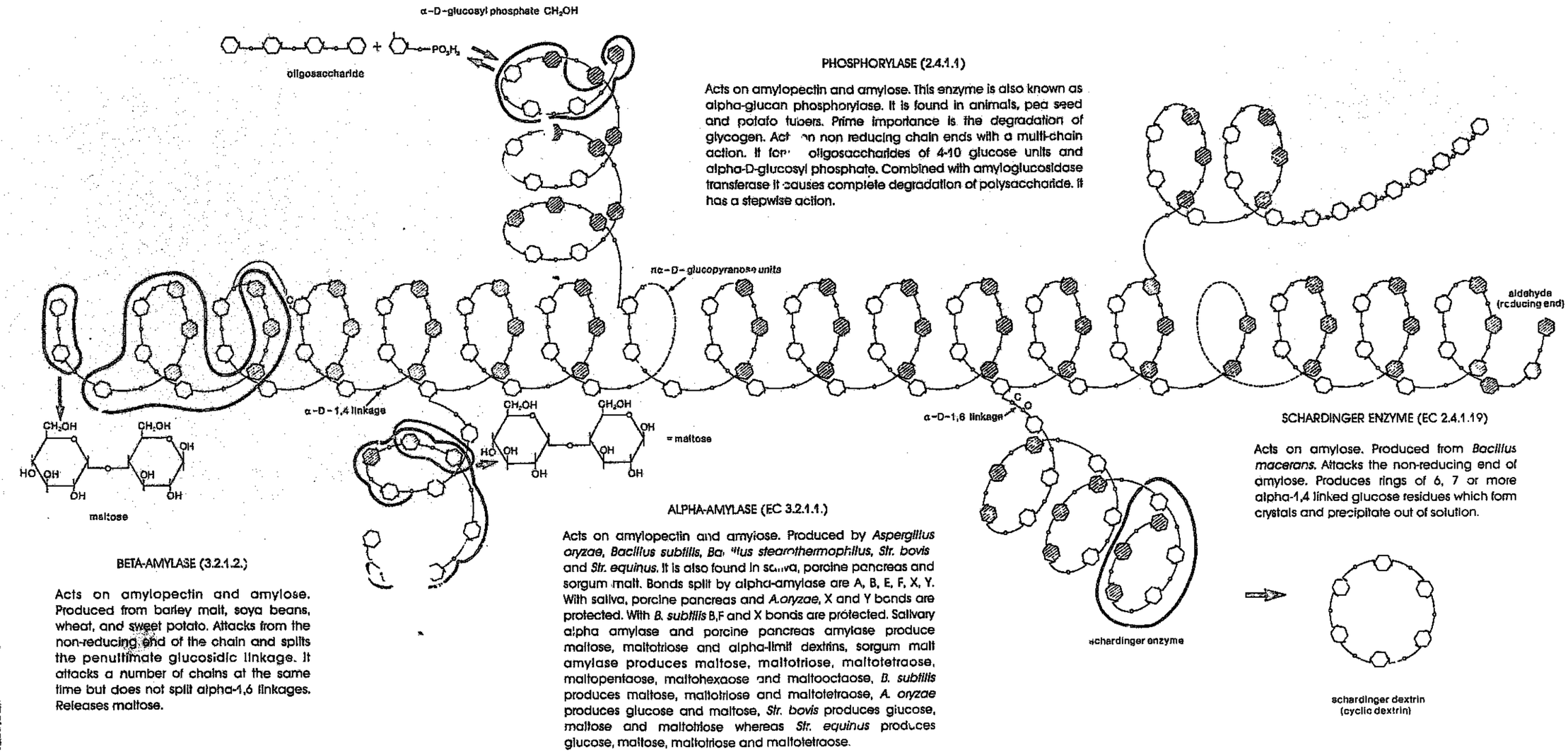
The linear nature of amylose also complicates investigation into the fine structure of the molecule. Manners (1989) believes most chains

are interlinked to form a lightly branched structure. However, evidence is accumulating that some amyloses contain a few long branches. The first evidence of limited branching was the observation amylose is not converted completely to maltose by beta-amylase (Cowie and Greenwood, 1957). The barrier to beta-amylase action however has been disputed but artificial barriers can be introduced by oxidation at either the C-2, C-3 or C-6 position (Cowie and Greenwood, 1957; Banks, Greenwood and Thomson, 1959a and 1959b). The presence of the natural barrier was confirmed by Greenwood in 1960 who studied a number of amylose fractions with various molecular weights and susceptibility to hydrolysis by beta-amylase. In a later study the action of pullulanase on amylose suggested an alpha-D-1,6 linkage was involved (Banks and Greenwood, 1966).

More than 50 years ago researchers found linear amylose had further structural features. Hanes (1937) proposed a helical structure with each turn containing 6 glucose units (Figure 1,1). The helices are characterised by a hydrogen bond between the C-2 hydroxyl group of one alpha-D-glucopyranosyl unit and the hydroxyl at C-3 of the following sugar unit (Sarko and Marchessault, 1967). The formation of these bonds was confirmed by St-Jacques and coworkers (1976) who undertook nuclear magnetic resonance studies on amylose chains in dimethyl sulphoxide. According to Foster (1965) this bonding may help to preserve the helical structure and lend rigidity to the coil. Potential energy calculations indicate more than 6 units per turn is impossible (Rao et al, 1967).

## AMYLOS.

The molecule normally contains 200-350 glucose units with a limit of approximately 1000. The size of the molecule can double during the growth of the plant. It also varies from plant to plant. The glucose units are joined by alpha-1,4 linkages while limited branching occurs f ·ed by alpha-1,6 linkages. In solution it forms a helix with 6 _ ose units for each turn of the helix.




### PHOSPHORYLASE (2.4.1.1)

Acts on amylopectin and amylose. This enzyme is also known as alpha-glucan phosphorylase. It is found in animals, pea seed and potato tubers. Prime importance is the degradation of glycogen. Act on non reducing chain ends with a multi-chain action. It for· oligosaccharides of 4-10 glucose units and alpha-D-glucosyl phosphate. Combined with amyloglucosidase transferase it causes complete degradation of polysaccharide. It has a stepwise action.

### BETA-AMYLASE (3.2.1.2.)

Acts on amylopectin and amylose. Produced from barley malt, soya beans, wheat, and sweet potato. Attacks from the non-reducing end of the chain and splits the penultimate glucosidic linkage. It attacks a number of chains at the same time but does not split alpha-1,6 linkages. Releases maltose.

### ALPHA-AMYLASE (EC 3.2.1.1.)

Acts on amylopectin and amylose. Produced by *Aspergillus oryzae, Bacillus subtilis, Ba· illus stearothermophilus, Str. bovis* and *Str. equinus*. It is also found in sa..iva, porcine pancreas and sorgum malt. Bonds split by alpha-amylase are A, B, E, F, X, Y. With saliva, porcine pancreas and *A.oryzae*, X and Y bonds are protected. With *B. subtilis* B,F and X bonds are protected. Salivary alpha amylase and porcine pancreas amylase produce maltose, maltotriose and alpha-limit dextrins, sorgum malt amylase produces maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose and maltooctaose, *B. subtilis* produces maltose, maltotriose and maltotetraose, *A. oryzae* produces glucose and maltose, *Str. bovis* produces glucose, maltose and maltotriose whereas *Str. equinus* produces glucose, maltose, maltotriose and maltotetraose.

### SCHARDINGER ENZYME (EC 2.4.1.19)

Acts on amylose. Produced from *Bacillus macerans*. Attacks the non-reducing end of amylose. Produces rings of 6, 7 or more alpha-1,4 linked glucose residues which form crystals and precipitate out of solution.

Figure 1.1 Diagramatic representation of the structure of amylose and the action of enzymes that catalyse the degradation of this starch. Information adapted from Hanes (1937); Meyer, Wertheim and Bernfeld (1940, 1941); Hassid and McCready (1943); Cowie and Greenwood (1957); Robyt and Whelan (1968a, 1968b); French, (1975); Robyt (1984).

X-ray diffraction studies show the helices in amylose form complexes to produce A, B, C and V forms of this starch (Wu and Sarko, 1978a). A-starches are found in grains, B-starches in tuberous plants, whereas C-starch is rare and occurs in peas and beans. The crystal structure of A-amylose is based on parallel-stranded, right-handed double helices which are packed into hexagonal units. The B polymorph forms double-stranded right-handed six-fold helices. The helices are wound parallel around each other and are filled with water molecules. A-amylose contains less water than B-amylose but similarities between these two types suggest interconversion is possible (Blackwell, Sarko and Marchessault, 1969; Wu and Sarko, 1978a, 1978b). The V structure is found when amylose complexes with organic molecules (Whistler and Daniel, 1984).

Theoretic calculations show V-amylose is more stable in a left-handed than a right-handed helix (Rao et al, 1967). The easy conversion of B to V-amylose implies little molecular reorganization is accompanied by the reversal of chain chirality. Apparently amylose exists as a left-hand helix in the solid state with six alpha-D-glucopyranosyl units per turn (Senti and Witnauer, 1948). The amylose-KOH complex is a distorted left-hand helix with six alpha-D-glucopyranosyl units per turn (Sarko and Biloski, 1980).

Measurements of the physical properties of amylose are not consistent, which suggests random coils and aggregated helices are formed in aqueous solutions (Paschall and Foster, 1952; Everett and Foster, 1959; Greenwood, 1960). Solutions of amylose probably contain an equilibrium mixture of several conformations (Greenwood, 1956). A

deformed helix has the lowest energy and would predominate (Hollo and Szejtli, 1958). A helix-coil transformation could not be found in neutral and alkaline aqueous solutions of amylose by optical rotation dispersion (Neely, 1961) but transformation was obtained with amylose-iodine complexes in aqueous dimethyl-sulphoxide solution (Peticolas, 1963).

The conformation of amylose in solution is also influenced by the pH. In acid solution the polymer exists in a helical form (Doty et al, 1957). As the pH is raised the carboxyl groups ionize and the polymer undergoes a transition from a helical to a randomly coiled form. In neutral solution the amylose molecule probably exists as a stiff coil with a helical backbone. Near pH 12 the helix starts to break down as the hydroxyl groups ionize. This results in an increase in chain flexibility and a shrinking of the molecular domain. At a higher pH there is a net increase of the negative charge on the polymer molecule. This is associated with an increased degree of ionization and expansion of the coil due to charge repulsion (Rao and Foster, 1963).

### 2.4.2 Amylopectin

Amylopectin is the second component of starch that was discovered nearly 50 years ago by Pacsu and Mullen (1941). They isolated a purple staining compound beta-amylose which later became known as amylopectin.

#### 2.4.2.1 Chain length of amylopectin

Methylation analysis has shown that amylopectin is a highly branched molecule which contains chains of about 27 glucose units each terminated by a non-reducing glucose unit. Approximately 4 percent of the glucose units are end groups and the same proportion are linked through positions 1, 4 and 6 (see Section 2.5.1). There is no clarity about the length of the branches which may alter with the maturity of the starch (Greenwood, 1970).

The susceptibility of amylopectin to hydrolysis by beta-amylase is used widely to analyse the molecule (see Section 2.5.4). This enzyme removes chains exterior to the branch point and leaves a beta-limit dextrin. The following table, adapted from Greenwood (1970), gives the average chain length and beta-amylolysis limit of a few amylopectins.

### 2.4.2 Amylopectin

Amylopectin is the second component of starch that was discovered nearly 50 years ago by Pacsu and Mullen (1941). They isolated a purple staining compound beta-amylose which later became known as amylopectin.

#### 2.4.2.1 Chain length of amylopectin

Methylation analysis has shown that amylopectin is a highly branched molecule which contains chains of about 27 glucose units each terminated by a non-reducing glucose unit. Approximately 4 percent of the glucose units are end groups and the same proportion are linked through positions 1, 4 and 6 (see Section 2.5.1). There is no clarity about the length of the branches which may alter with the maturity of the starch (Greenwood, 1970).

The susceptibility of amylopectin to hydrolysis by beta-amylase is used widely to analyse the molecule (see Section 2.5.4). This enzyme removes chains exterior to the branch point and leaves a beta-limit dextrin. The following table, adapted from Greenwood (1970), gives the average chain length and beta-amylolysis limit of a few amylopectins.

Table 1.1 The beta-amylolysis limit and chain length in glucose units of amylopectins

| Starch | beta - limit | chain length |
|---|---|---|
| Barley | 58 | 26 |
| Oat | 67 | 20 |
| Wheat | 57 | 19 |
| Maize | 58 | 23 |
| Potato | 56 | 24 |
| Waxy maize | 58 | 20 |

2.4.2.2 Molecular weight of amylopectin

Amylopectin is heterogenous and can be separated into subfractions with a range of molecular weights. Subfractions from potato amylopectin have molecular weights of between $7 \times 10^6$ and $73 \times 10^6$. Therefore, molecular weight depends on whether subfractionation occurs during isolation. Early studies with osmotic pressure show that the molecular weight of amylopectin fractions are higher than amylose and range between 50,000 to 100,000 (Meyer, 1950). These values are probably low because the swollen starch granules do not dissolve completely without some molecular degradation (Foster, 1965).

Apparently the average molecular weight of amylopectin lies between $10^5$ to $10^6$. These figures are not precise and indicate the inability of either chemical or osmotic pressure methods to analyse large polymers in detail. Light scattering studies however have yielded results 10 fold higher than the former methods (Foster, 1965).

The following table adapted from Greenwood (1970) gives the degree of polymerization of a few naturally occurring amylopectins.

Table 1.2 The degree of polymerization of amylopectin isolated from naturally occurring starches

| Starch | Polymerization |
|---|---|
| Barley | 190 |
| Wheat | 140 |
| Maize | 100 |
| Potato | 160 |
| Waxy maize | 145 |

2.4.2.3 Fine structure of amylopectin

Most researchers agree that amylopectin has a chain length of 20 to 25 glucose units and a D.P. of $10^2$ but they do not agree about the arrangement of the constituents in the molecule (Manners, 1985a). Three kinds of chains are found in amylopectin - A, B and C chains. A chains are connected to the remainder of the macromolecule only through the reducing chain end, whereas B chains are joined similarly but may carry one or more A and B chains at one of their primary hydroxyl groups. Every molecule contains a single C chain, unsubstituted at its reducing end (Gunja-Smith et al, 1970a; Manners, 1985a).

Starches may contain different numbers of A and B chains. Maize amylopectin has an A to B chain ratio of 1:1,22, potato of 1:1,06,

wheat of 1:7,5 and banana of 1:6. A comparison of the distribution patterns suggest that the length of B-chains from different sources may vary according to the plant source (Akai et al, 1971; Mercier, 1973). Maize amylopectin has chains with a length of 14 glucose units which form tightly packed clusters connected by long unbranched B chains, whereas potato amylopectin has longer chains of 18 units that are not tightly coiled and possess interchain branching (Bender, Siebert and Stadler-Szoke, 1982).

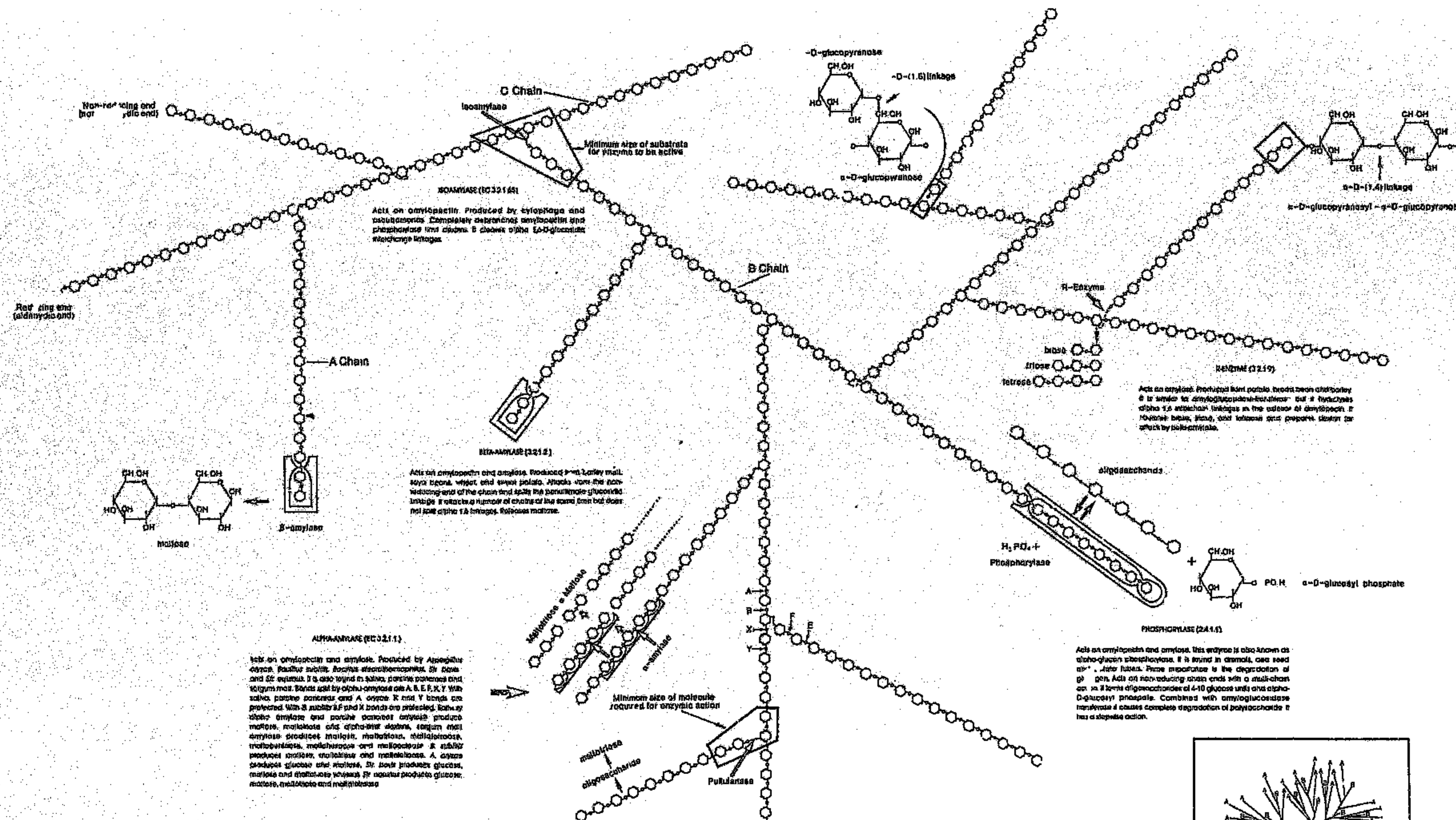
Several models have been proposed for the structure of amylopectin. The earliest models were the laminated structure proposed by Haworth, Hirst and Isherwood (1937) and the herring-bone structure of Staudinger and Husemann (1937). Later Meyer and Bernfeld (1940, 1941) proposed a randomly branched formula which contains equal numbers of A and B chains and one C chain, whereas the molecular structure of Marshall (1975) contains twice as many A as B chains. A diagramatic representation of the amylopectin molecule proposed by Meyer and Bernfeld (1940) and Marshall (1975) can be found in Figure 1.2.

No chemical methods exist to distinguishing between the different proposed structures. However, enzymic experiments support the irregular branched structure of Meyer and Bernfeld (1940). Bathgate and Manners (1966) investigated the action of pullulanase on beta-limit dextrins and obtained results consistent with the features of the Meyer and Bernfeld model. Lee and Whelan (1966) used pullulanase and beta-amylase and obtained results that agreed with periodate oxidation studies.

Figure 1.2 Diagramatic representation of the structure of amylopectin and the action of enzymes that catalyse the degradation of this starch. Information adapted from Meyer and Bernfeld (1940, 1941); Robyt and Whelan (1968a,1968b); French (1975); Marshall (1975); Robyt (1984).

## AMYLOPECTIN

The molecule usually consists of more than 200,000 glucose units. It has a highly ramified structure of individual chains of 27 units of glucose joined by alpha 1,4 linkages and terminated by a non reducing glucose unit. Interchain linkages are formed by 1,6 linkages. Three chains can be distinguished i.e. A, B and C. The chain length from the end of the chain to an alpha 1,6 branch may be 12 to 17 units. The chain length between branches may be 5-8 units but not less than 4.




**ISOAMYLASE (EC 3.2.1.68)**

Acts on amylopectin. Produced by cytophaga and pseudomonas. Completely debranches amylopectin and phosphorylase limit dextrins. It cleaves alpha 1,6-D-glucosidic interchange linkages.

**BETA-AMYLASE (3.2.1.2)**

Acts on amylopectin and amylose. Produced from barley malt, soya beans, wheat, and sweet potato. Attacks from the non-reducing end of the chain and splits the penultimate glucosidic linkage. It attacks a number of chains at the same time but does not split alpha 1,6 linkages. Releases maltose.

**ALPHA-AMYLASE (EC 3.2.1.1)**

Acts on amylopectin and amylose. Produced by *Aspergillus oryzae*, *Bacillus subtilis*, *Bacillus stearothermophilus*, *St. bovis* and *St. equinus*. It is also found in saliva, porcine pancreas and sorghum malt. Bonds split by alpha-amylase are A, B, E, F, X, Y. With saliva, porcine pancreas and A. oryzae X and Y bonds are protected. With B. subtilis B,F and X bonds are protected. Salivary alpha amylase and porcine pancreas amylase produce maltose, maltotriose and alpha-limit dextrins, sorghum malt amylase produces maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose and maltoheptaose. *B. subtilis* produces maltose, maltotriose and maltotetraose. *A. oryzae* produces glucose and maltose. *St. bovis* produces glucose, maltose and maltotriose whereas *St. equinus* produces glucose, maltose, maltotriose and maltotetraose.

**PULLULANASE (EC 3.2.1.41)**

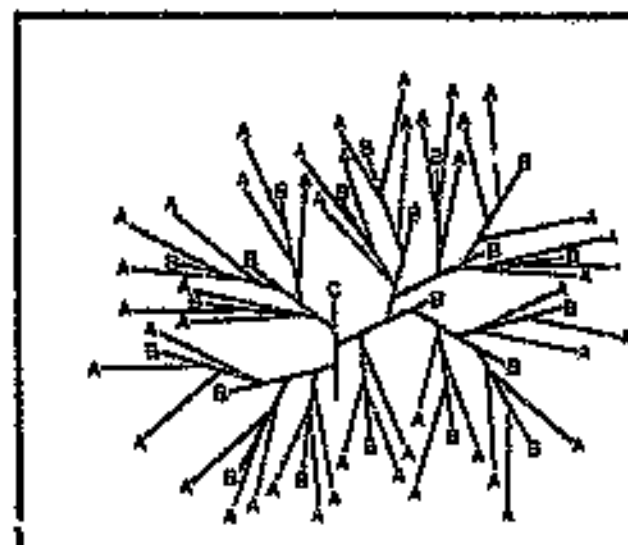
Acts on amylopectin. Produced by *Aerobacter aerogenes* and *Bacillus macerans*. It hydrolyses specific alpha 1,6 bonds provided that the 1,6 bond is joined by 1,4-linkages on both sides of the bond. D-glucose is never produced. Side chains (A chains) containing up to 6-10 units are susceptible to pullulanase action. Produces maltotriose.

**R-ENZYME (3.2.1.9)**

Acts on amylose. Produced from potato, broad bean and barley. It is similar to amylo-glucosidase-transferase but it hydrolyses alpha 1,6 interchain linkages in the exterior of amylopectin. It releases biose, triose, and tetraose and prepares dextrin for attack by beta-amylase.

**PHOSPHORYLASE (2.4.1.1)**

Acts on amylopectin and amylose. This enzyme is also known as alpha-glucan phosphorylase. It is found in animals, cereal seed and potato tubers. Prime importance is the degradation of glycogen. Acts on non-reducing chain ends with a multi-chain action. It forms oligosaccharides of 4-10 glucose units and alpha-D-glucosyl phosphate. Combined with amyloglucosidase transferase it causes complete degradation of polysaccharide. It has a stepwise action.




Diagramatic representation of the structure of amylopectin according to Manners (1972). Solid lines represent 1,4 linked alpha-D-glucose chains. Three types of chains can be distinguished A, B and C. A chains do not carry any substituent chains, B chains carry other chains linked to primary hydroxyl groups. The single C chain contains the only free reducing glucose unit in the molecule. [illegible]

French (1973) proposed the cluster model of amylopectin to account for the regions of dense branching and high viscosity. In 1974 Robin and his co-workers suggested a similar model based on studies with enzymes. More recently this model has been supported by the work of Manners and Matheson (1981), French (1984) and Manners (1985a). According to these workers amylopectin consists of clusters of chains arranged in tassels. The tassels occur on a string and are linked together by branch points. These branch points are arranged in tiers or clusters and are randomly distributed throughout the molecule. This work has led to the general acceptance of the cluster-type model which explains the properties of amylopectin more accurately than any other (Manners, 1985a).

The presence of other branches in amylopectin, for example, alpha-1,2 or alpha-1,3 linkages has been sought (Wolfrom and Thompson, 1956). Small amounts of glucose have been found in periodate oxidation studies (Halsall *et al*, 1947; Hirst, Jones and Roudier, 1948; Gibbons and Boissonas, 1950; MacWilliam and Percival, 1951; Greenwood and Robertson, 1954). This has been interpreted as evidence for the presence of alpha-1,3 linked glucose. However, a later study by Bahl and Smith (1966) does not support these results. According to Whistler and Daniel (1984), if these bonds exist they probably form a small percentage of the total number of branch points and have not been found.

French (1973) proposed the cluster model of amylopectin to account for the regions of dense branching and high viscosity. In 1974 Robin and his co-workers suggested a similar model based on studies with enzymes. More recently this model has been supported by the work of Manners and Matheson (1981), French (1984) and Manners (1985a). According to these workers amylopectin consists of clusters of chains arranged in tassels. The tassels occur on a string and are linked together by branch points. These branch points are arranged in tiers or clusters and are randomly distributed throughout the molecule. This work has led to the general acceptance of the cluster-type model which explains the properties of amylopectin more accurately than any other (Manners, 1985a).

The presence of other branches in amylopectin, for example, alpha-1,2 or alpha-1,3 linkages has been sought (Wolfrom and Thompson, 1956). Small amounts of glucose have been found in periodate oxidation studies (Halsall et al, 1947; Hirst, Jones and Roudier, 1948; Gibbons and Boissonas, 1950; MacWilliam and Percival, 1951; Greenwood and Robertson, 1954). This has been interpreted as evidence for the presence of alpha-1,3 linked glucose. However, a later study by Bahl and Smith (1966) does not support these results. According to Whistler and Daniel (1984), if these bonds exist they probably form a small percentage of the total number of branch points and have not been found.

### 2.4.3 Comparison of amylose and amylopectin

Table 1.3 summarizes some of the properties of amylose and amylopectin. This information was adapted from Williams (1968).

Table 1.3 Comparison of the properties of amylose and amylopectin

| | Amylose | Amylopectin |
|---|---|---|
| % conversion to maltose β-amylase + Z enzyme | 95 - 100 | 50 - 60 |
| Crystalline β-amylase | 70 - 96 | 50 - 60 |
| Molecule weight | $10^5$ - $10^6$ | $10^7$ - $10^8$ |
| Chain length | [illegible] | 19 - 28 |
| X-ray analysis | [illegible] | Amorphous |
| Solubility in water | [illegible] | Soluble |
| Stability in water | Retrogrades | Stable |

## 2.5 STRUCTURAL ANALYSES OF STARCH

As mentioned previously, the components of starch are complex and require complementary methods for complete analysis. Thus methylation analysis provides an indication of sugar linkages and infra-red spectroscopy gives information about the anomeric configuration of sugars in the molecule. Enzymic analyses and periodate oxidation yield information about the length of branches and the fine structure of starches. Finally, iodine staining indicates the presence of helices and branching in starches.

### 2.5.1 Methylation analysis

Evidence of the chemical structure of starch was first obtained from early methylation studies. Analysis involved repeated reaction of starch with dimethyl sulphate and sodium hydroxide. This resulted in partially methylated products that were fully methylated by repeated treament with silver oxide in boiling iodomethane (Purdie and Irvine, 1903; Denham and Woodhouse, 1913; Haworth, 1915; Irvine and Macdonald, 1926; Haworth, Hirst and Webb, 1928).

The products of the methylation were a large group later identified as 2,3,6-tri-methyl-D-glucose. This compound indicated starch consists of D-glucopyranose joined by 1,4-linkages. The production of a second small group 2,3,4,6-tetra-O-methyl-D-glucose proved the chains were of finite length (Haworth, 1929; Hirst, Plant and Wilkinson, 1932). Later in 1940 Freudenberg and Boppel established

2,3-di-O-methyl-D-glucose was also produced from hydrolysed starch which showed starch contained branch points involving 1,6-linkages.

The ratio of the reducing end group 2,3,6-tri-O-methyl-D-glucopyranose to the non-reducing end 2,3,4,6-tetra-O-methyl-D-glucopyranose gives an indication of the chain length of starch and is referred to as end-group assay. End-group analysis of amylose is difficult because there is a low yield of tetramethyl glucose. However, Hassid and McCready (1941) and Haworth, Heath and Peat (1942) have shown amylose has an average chain length of 100 glucose residues. Methylation of amylopectin followed by methanolysis yields about 4 per cent 2,3,4,6-tetra-O-methyl-D-glucopyranoside which proves the chains contain 27 glucose units. Each is terminated by a reducing end (Meyer, Wertheim and Bernfeld, 1940; Hassid and McCready, 1943).

Early methylation methods have been improved by the one step procedure devised by Hakomori (1964) and Sandford and Conrad (1971). In this method starch is methylated with sodium methylsulphinylmethanide in dimethylsulphoxide followed by iodomethane. Thereafter the methylated product is hydrolysed to a mixture of methylated monosaccharides are identified by chromatographic techniques.

There are disadvantages with the above methods because the analysis requires 100 mg of sample and takes 2 to 3 days to complete. For these reasons Harris and co-workers (1984) examined each step and optimized experimental conditions. They treated the carbohydrate

with methylsulphinyl carbanion to form polyalkoxide ions. This product was methylated, hydrolysed, reduced and finally acetylated. The resultant alditol acetates were separated by gas chromatography and identified by retention times and mass spectra (Dutton, 1974; Lindberg, 1981). An advantage of this method is only 5 mg of sample is required and the analysis can be completed in 1 working day.

### 2.5.2 Infra-red spectroscopy

The absorbance of infra-red radiation by carbohydrates can be used to identify specific structural features. This is achieved by comparing unknown spectra with spectra obtained from standard reference compounds (White and Kennedy, 1986). A number of authors including Samec (1953, 1954, 1957), Casu and Reggiani (1964, 1966, 1971) and Nisizawa et al, (1976) have studied the infra-red spectrum of amylose, d-glucose oligomers and dextrans. The intensity of the band near 1150 to 1160 $cm^{-1}$ involves the glucosidic bridge. Absorption below 650 $cm^{-1}$ has been assigned to the ring vibration whereas the spectrum from 750 $cm^{-1}$ to 400 $cm^{-1}$ maintains characteristics of the anomeric linkages but appears to be specific for each carbohydrate (Casu and Reggiani, 1971).

The fingerprint region for sugars lies between 1000 and 650 $cm^{-1}$ (Nisizawa et el , 1976). Absorption near 850 $cm^{-1}$ with peaks at 843 and 844 $cm^{-1}$ is characteristic of alpha-anomers and bands near 890 $cm^{-1}$ with peaks at 890 and 891 $cm^{-1}$ with beta-linked glucose units (Barker et al, 1954). The intensity of the bands near 930,

758, 605, 570 and 525 cm-' appears to be characteristic of alpha-1,4-linked polysaccharides. The absorption band at 793 cm-' originates from alpha-1,3-linkages whereas absorption at the wavelengths of 917, 844 and 768 cm-' suggests the presence of alpha-1,6-linkages (Nisizawa et al, 1976).

The spectrum of starches indicates hydroxyl groups are extensively hydrogen-bonded (Samec, 1953). Absorption for oxygen-containing groups in maize starch is stronger at 1680, 1055 to 952 and 855 cm-' and for potato starch at 926 cm-' (Samec, 1957). When the water content of starch is altered the band at 3335 cm-' undergoes change which is characteristic of a particular starch (Yovanovitch, 1961).

### 2.5.3 Periodate oxidation

The use of periodate oxidation for the analysis of carbohydrates was the first practical alternative to methylation analysis developed by Malaprada in 1934. Jackson and coworkers (J. 7, 1938) applied this method to the analysis of corn starch and found each D-glucosyl unit consumed one mole of periodate. Hydrolysis of the oxidized starch yielded glyoxal and D-erythrose. Periodate oxidized starches are unstable but they can be reduced to erythritol and glycerol and separated by chromatography (Jackson and Hudson, 1936; Jeannes and Hudson, 1955; Hamilton and Smith, 1956a, 1956b; Whistler and Daniel, 1984).

Periodate oxidized starches may also be oxidized to acids. Brown et al. (1945) suggested the formic acid produced from the reducing and non-reducing terminals could be used to determine the molecular weight. The observation that most of the formic acid produced by the oxidation of amylopectin would come from the non-reducing end enabled Anderson, Greenwood and Hirst (1955) to measure the branch lengths. This method gave values for amylose comparable to osmotic pressure values (Potter and Hassid, 1948a, 1948b). Periodate nonreducing end group measurements agreed with results obtained with methylation analysis (Bates, French and Rundle, 1943; Halsall, Hirst and Jones, 1947; Brown et al. 1948).

2.6.4 Enzymic analysis

Enzymic analysis of polysaccharides can be used to supplement chemical techniques. This method provides quantitative data and gives information about the fine structure of polysaccharides after gross features have been determined by nonenzymatic analysis. Recently its use has become more widespread with the availability of highly purified enzyme preparations (Marshall, 1974).

As was mentioned previously there are 3 types of chains in amylopectin, namely A, B and C-chains (Peat, Whelan and Thomas, 1956). The glucose units in the A-chain are linked through positions 1 and 4 and do not carry any substituent chain, the B-chains may have units linked through positions 4 and 6 and carry other chains linked to primary hydroxyl groups, whereas the C-chain carries the only free non-reducing group in the molecule (Figure

1,2). A property of amylopectin which is usually determined is the ratio of A:B-chains. This is sometimes known as the degree of multiple branching (Manners, 1985a).

Beta-amylase is the most widely used enzyme for the analysis of amylopectin. The action of beta-amylase on branched amylopectin stops in the region of the 1,6-D-glucosidic linkages. This feature is invaluable because the enzyme can be used to remove all parts of the molecule exterior to the branch-points and leaves the inner portion intact (Marshall, 1974). The intact portion is known as either beta-amylase limit dextrin or beta-limit dextrin. Greenwood (1970) suggests beta-limit dextrins form about 40 percent of the original amylopectin (See Section 2.4.2.1, Table 1,1).

According to Manners (1985b, 1989) beta-amylase leaves a beta-limit dextrin with 'stubs' containing an average of 2 glucose units per chain. The A-chain stubs contain either two or three residues per chain whereas there are one to two glucose units in the B-chain stubs. If equal numbers of A and B-chains are present, the exterior chain length (ECL) is the number of glucose units removed by beta-amylase plus two and can be calculated by the formula

$$ECL=(CL\times\beta\text{-limit})+2$$

The calculation for the interior chain length (ICL) is

$$ICL=CL-ECL-1$$

The chain length (CL) may be determined by either methylation analysis or periodate oxidation.

Debranching enzymes may also be used for the investigation of amylopectin. These enzymes are specific for 1,6-D-glucosidic interchain linkages and increase the beta-amylolysis limit of amylopectin and beta-limit dextrin (Hobson, Whelan and Peat, 1951). R-enzyme was one of the earliest enzymes used for this purpose (Hobson, Whelan and Peat, 1951). It occurs in cereals but is difficult to purify. For this reason debranching enzymes from microorganisms have been used in structural studies. Pullulanase was one of the first successful microbial debranching enzymes. It was isolated from Klebsiella aerogenes and converts pullulan into maltotriose but only partially debranches amylopectin (Manners, 1989).

Isoamylase, a newer debranching enzyme was first isolated from Pseudomonas amylodera by Yokobayashi, Misaki and Harada in 1969. It is more useful than pullulanase because it debranches amylopectin completely (Akai et al, 1971). It hydrolyses all the alpha-1,6-linkages and provides a simple method for the determination of chain length. This is achieved by the accurate measurement of an increase in reducing power after the action of the enzyme on the substrate (Gunja-Smith et al, 1970b).

A combination of beta-amylase and debranching enzyme can be used to determine the average chain length and the A to B chain ratio in amylopectin. The initial treatment with beta-amylase will degrade the exterior A-chains (Manners, 1985c). The oligosaccharides can be estimated by column chromatography (Peat, Whelan and Thomas, 1956), paper chromatography (Bathgate and Manners, 1966), gel

filtration chromatography (Enevoldsen, 1980) and high performance liquid chromatography (Bender, Siebert and Stadler-Szoke, 1982).

In the second stage, the debranching enzyme is allowed to react with the beta-limit dextrin. The A:B chain ratio is determined by measuring the amount of maltose and maltotriose released by the action of the enzyme (Peat, Whelan and Thomas, 1956). Originally a plant debranching enzyme was used but more recently this enzyme has been replaced by either bacterial pullulanase or isoamylase (Bathgate and Manners, 1966; Enevoldsen, 1980; Bender, Siebert and Stadler-Szoke, 1982).

An alternative method with the debranching enzyme involves the digestion of beta-dextrin with isoamylase. The liberated reducing groups are equivalent to all the B-chains plus half the A-chains. Thereafter the mixture is treated with a mixture of isoamylase and pullanase which will release all the A and B-chains. The difference in reducing power represents half the A-chains (Marshall and Whelan, 1974). Manners (1985a; 1985c; 1989) has reservations about this procedure because it is sensitive to experimental errors.

In a further modification of these methods bacterial isoamylase can be used to examine the chain length of A and B-chains in amylopectin. Complete debranching of the molecule is followed by fractionation of the mixture of linear chains using gel techniques (Lee, Mercier and Whelan, 1968; Hizukuri, 1985; 1986). This method results in 2 peaks representing chain lengths of 20 and 50. The

first peak consists of A chains plus some shorter B-chains whereas the second peak is the longer B-chains (Manners, 1985a; 1985c; 1989).

According to Manners (1985c) the method of choice for investigating the fine structure of amylopectin is the complete debranching of the molecule with a bacterial isoamylase followed by measuring the release of reducing groups. In the method described by Akai and coworkers in 1971 any microbial isoamylase can be used. After isoamylase is inactivated, beta-amylase is added and the released chains are converted to maltose and small amounts of glucose.

### 2.5.5 Iodine reaction

The reaction of starch with iodine was discovered during the early part of the last century by Colin and de Claubry in 1814 and Stromeyer in 1815 (Hollo and Szejtli, 1968). Starch granules become dichroic and absorb different amounts of light when they are stained. The nature of iodine binding was explained in 1937 when Hanes proposed amylose has a helical structure with each turn containing six glucose units. In the formation of amylose-iodine complexes one molecule of iodine is bound for every six molecules of glucose (Baldwin, Bear and Rundle, 1944). X ray diffraction studies show iodine molecules are bound in the centre of the helix (Rundle, 1947).

In contrast amylopectin binds iodine by adsorption and the helix plays a subordinate role because most side chains in this molecule

are not long enough to form helices (Greenwood, 1970). The presence of a few long chains may account for the low binding capacity of amylopectin which is between 0 and 0,7 per cent (Larson, Gilles and Jenness, 1953). Some authors dispute this figure and suggest binding is caused by residual amylose (Hollo and Szeitli, 1968).

Optical, potentiometric and amperometric methods have been developed to determine the amount of iodine binding (Hollo and Szeitli, 1968). The optical method involves measuring aqueous starch solutions containing potassium iodide and iodine. The triiodide complex ion formed from iodine and iodide exhibits a broad peak at 450 nm. Upon complex formation with amylose the peak shifts to a longer wave-length between 600-625 nm. Amylopectin gives a smaller shift with a peak near 550 nm. The $I_3^-$ and various complexes show two broad bands in the ultraviolet with maxima near 290 and 355 nm.

The colour of iodine complexes may vary. Amylose-iodine complexes have an intense blue colour, amylopectin a purple colour, glycogen a reddish brown colour, whereas glycogen beta-dextrin which has few external chains stains slightly ( (Bates, French and Rundle, 1943; Hassid and McCready, 1943; Meyer and Mark, 1950; Meyer, 1952; Grebel, 1968). No iodine staining is obtained with dextrins up to 8 glucose units, dextrins with 9 to 12 glucose units stain yellow brown to brown whereas from 12 to 15 glucose units the colour changes from brown to reddish purple (Thoma and French, 1960).

# 3 BACTERIAL METABOLISM

Metabolism can be divided into two mechanisms, the energy generating or degrading pathways called catabolism and the energy consuming or biosynthetic pathways known as anabolism. Growth is an intimate linkage between catabolism and anabolism whereby part of the energy derived from degradation is used to drive the process of synthesis (Stanier, Doudoroff and Adelberg, 1971).

## 3.1 METABOLIC PATHWAYS

Glycolysis is the major metabolic pathway for the generation of energy by the streptococci. The fermentation of g e is always initiated by a phosphorylation at the expense of ATP to yield glucose-6-phosphate (Stanier et al , 1971). Bacteria can utilize 4 pathways to degrade this product, the Embden-Meyerhof pathway, the hexose monophosphate pathway, the Entner-Doudoroff pathway and the phosphoketolase pathway (Doelle, 1975). Many streptococci are not able to utilize the hexose monophosphate pathway because they do not possess glucose-6-phosphate dehydrogenase (Doelle, 1975). They derive their energy and regenerate ATP from the Embden-Meyerhof pathway (Doelle, 1969).

Str. salivarius and Str. mutans utilize the Embden-Meyerhof pathway but lack the oxidative portion of the hexose monophosphate pathway and produce reduced nicotinamide adenine dinucleotide phosphate (NADPH) via a glyceraldehyde-3-phosphate dehydrogenase (Burchall, Niederman and Wolin, 1964; Brown and Wittenberger,

1971a,b; Yamada and Carlsson, 1973). The lactate dehydrogenase of these streptococci depends upon an intermediate of the Embden-Meyerhof pathway, fructose-1,6-bisphosphate for activity (Wolin, 1964; Brown and Wittenberger, 1972).

In contrast Str. sanguis utilizes the Embden-Meyerhof pathway and the hexose monophosphate pathway (Brown and Wittenberger, 1971a; Yamada and Carlsson, 1973). NADPH is generated via the hexose monophosphate pathway which also provides pentoses for nucleotide synthesis and a mechanism for their oxidation to the glycolytic sequence (Brown and Wittenberger, 1971a,b; Jocklik, Willett and Amos, 1984).

## 4 ENZYMES

All metabolic pathways are catalized by enzymes. Enzymes may be defined as biocatalysts of organic origin which have specific powers for accelerating or initiating chemical transformations without themselves being altered. The present nomenclature gives either an indication of the substrate and of the reaction catalysed by an enzyme.

## 4.1 ENZYMES THAT REGULATE SUCROSE METABOLISM

### 4.1.1 The production of acids from sucrose

In the streptococci sugar transport is one of the most important sites regulating the rate of glycolysis. The oral streptococci possess the sucrose-phosphotransferase system (sucrose-PTS) which transports this sugar into the cell. However, studies have revealed the glycolytic step catalysed by pyruvate kinase is amoung the most important regulatory steps of the overall rate of the Embden-Meyerhof pathway. In addition the fermentation pattern may shift from lactic to mixed acid fermentation from the regulation of the activities of lactate dehydrogenase and pyruvate formate lyase (Yamada, 1987).

#### 4.1.1.1 Sucrose-phosphotransferase system (Sucrose-PTS)

The first step in the fermentation of sucrose is transport into the cell by sucrose-PTS. This enzyme system reacts with the dissacharide to form sucrose-6-phosphate which is hydrolysed intracellularly by an inducible sucrose-6-phosphate hydrolase to glucose-6-phosphate and fructose. Glucose-6-phosphate is metabolized via the Embden-Meyerhof pathway, whereas fructose is phosphorylated by an ATP-dependent fructokinase to fructose-6-phosphate an intermediate of the same pathway (St martin and Wittenberger, 1979; Thompson and Chassy, 1981; Chassy and Porter, 1982). Sucrose-PTS has been observed in S. mutans and S. lactis (Slee and Tanzer, 1979; St Martin and Wittenberg, 1979; LeBlanc, Crow and Lee, 1980; Thompson and Chassy, 1981).

4.1.1.2 Lactate dehydrogenase

Lactate dehydrogenase is an enzyme that influences the products of fermentation. The streptococci are characteristically homofermentative organisms that produce mainly lactate and smaller amounts of acetate, formate and ethyl alcohol under anaerobic conditions. However, the same organisms grown under aerobic conditions convert approximately 35 percent of glucose to lactate, 10 percent to acetate and a further 10 percent to carbon dioxide (Mickelson, 1967,1972; Thomas, Ellwood and Longyear, 1979). This change in fermentation pattern suggests lactate dehydrogenase which is responsible for lactate production is inhibited by oxygen in the sense that oxygen reoxidises NADH and the system takes advantage of energy obtained from the conversion of pyruvate to actetate.

The control mechanisms for the different end-product formation appears to centre around fructose-1,6-bisphosphate which is necessary for the activation of lactate dehydrogenase (Wittenberger and Angelo, 1970; Brown and Wittenberger, 1972). In the presence of excess glucose, the intracellular level of fructose 1,6-bisphosphate is high and lactate dehydrogenase is fully activated. Pyruvate formate-lyase is strongly inhibited by a minute amount of glyceraldehyde 3-phosphate or dihydroxyacetone phosphate. Levels of those glycolytic intermediates are high in the presence of excess glucose (Yamada and Carlsson, 1976; Takahashi, Abbe and Yamada, 1982).

Under glucose limitation, the reduced level of fructose 1,6-bisphosphate, glyceraldehyde 3-phosphate and dihydroxyacetone phosphate deactivate lactate dehydrogenase and release the inhibition of pyruvate formate-lyase. Thus the fermentation pattern shifts from lactic to the mixed acid type. To keep the oxidation-reduction balance during anaerobic metabolism of glucose, streptococci have to produce formate, acetate and ethanol (Yamada, 1987). The influence of fructose-1,6-bisphosphate appears to be a feature common to all streptococci (Doelle, 1975).

Not all oral streptococci are equally sensitive to oxygen. Tanzer, Krichevsky and Keyes (1969) studied aerated cells of Str. mutans and found they produced mainly lactate and smaller quantities of formate, acetate, propionate and carbon dioxide. Later in 1975 Iwami, Yamada and Araya observed the intracellular concentration of fructose-1,6-bisphosphate of Str. mutans was lower under aerobic than anaerobic conditions, but equal amounts of lactate were formed.

4.1.1.3 Pyruvate kinase

Pyruvate kinase can regulate the Embden-Meyerhof glycolytic pathway (Iwami and Yamada, 1980). The enzyme from S. salivarius is activated by glucose-6-phosphate and that from S. mitis and S. sanguis by fructose-1,6-bisphosphate (Iwami and Yamada, 1980; Abbe, Takahashi and and Yamada, 1983). Furthermore, pyruvate kinase of S. mutans is regulated by both glucose-6-phosphate and

ribose-5-phosphate (Yamada and Carlsson, 1975b; Abbe and Yamada, 1982). Phosphate stabilizes pyruvate kinase and reactivates the inactive enzyme. In addition the enzyme has a requirement for the cations $K^+$ or $NH_4^+$ and $Mg^{2+}$ or $Mn^{2+}$ (Crow and Pritchard, 1977; Abbe and Yamada, 1982; Abbe et al, 1983).

The regulation of pyruvate kinase can be observed when the supply of glucose is limited. Low levels of glucose-6-phosphate in S. mutans and fructose-1,6-bisphosphate in S. sanguis restrict the activity of pyruvate kinase which results in the accumulation of phosphoenolpyruvate. The increased levels of this product can be used to transport other sugars into the cell. In the presence of excess glucose the high intracellular levels of glucose-6-phosphate and fructose-1,6-bisphosphate activate pyruvate kinase and the intracellular levels of phosphoenolphyruvate decreases. The shortage of this product suppresses the transport of other sugars into streptococcal cell (Yamada and Carlsson, 1975a, 1976; Abbe et al, 1983).

4.1.2 The production of polysaccharides from sucrose

Two streptococal enzymes are responsible for the production of polysaccharide from the disaccharide sucrose. Glucosyltransferases catalyse the synthesis of glucose polymers known as glucans, whereas fructosyltransferases are responsible for the production of fructans from the fructose moiety of the molecule.

4.1.2.1 Glucosyltransferases (EC 2.4.1.5)

Glucosyltransferases are enzymes that act on sucrose to produce glucose polymers. One of the major problems with research into glucosyltransferases is their ability to form large aggregates which can be divided into fractions that produce at least 4 types of glucans (Nisizawa et al, 1976; Kuramitsu, 1974; Mukasa and Slade, 1974; Russell, 1979; Grahame and Mayer, 1984; Tsumori, Shimamura and Mukasa, 1985). One fraction catalyses the synthesis of a water-soluble 1,6-alpha-dextran which may contain 1,3,-alpha-branches. Another fraction is involved in the production of a water-insoluble glucans with a 1,6-alpha-dextran backbone and branched 1,3-alpha-glucan side chains (Hare, Svenson and Walker, 1978; Montville, Cooney and Sinskey, 1978; Chassy, 1983; Ciardi, 1983; Grahame and Mayer, 1984; Tsumori, Shimamura and Mukasa 1983).

A third fraction was reported from cultures of S sobrinus grown in the presence of Tween 80. This cell-associated glucosyltransferase catalysed the formation of a polymer that was water-insoluble. In addition an enzyme from the culture filtrate produced a glucan with alpha-1,6-linkages that stained strongly

with periodic acid Schiff stain. The latter enzyme appears to be similar to the crude glucosyltransferase produced by S. salivarius (Shimamura, Tsumori and Mukasa, 1983; Wittenberger, Beaman and Lee, 1978). More recently Yamashita, Hanada and Takehara (1989) reported the purification of a fourth glucosyltransferase produced by S. sobrinus which synthesizes a highly-branched water-soluble glucan (Shimamura, Tsumori and Mukasa, 1982).

The activities of each of these fractions can be further resolved into multiple forms which differ in size, net charge and isoelectric points (Guggenheim and Newbrun, 1969; Ciardi, Hageage and Wittenberger, 1976). The major enzyme fractions cannot account for the multiple activity bands observed when electrophoresis analyses are performed on crude enzyme extracts (Tsumori, Shimamura and Mukasa, 1983; McCabe et al, 1983). In addition they are neither strictly specific for the synthesis of dextran nor is sucrose their sole substrate which suggests these bands may have other activities besides glucan formation.

McCabe and Hamelik (1983) described an enzyme from S. mutans catalyses the transfer of glucopyranoside from isomaltosaccharides to dextran to form a branched molecule, whereas Yamashita, Hanada and Takehara (1988) found an enzyme from S. sobrinus produces oligomaltosaccharides from sucrose consisting of 15 glucose units. Furthermore, in the absence of dextran, glucosyltransferases can catalyse exchange reactions between fructose and glucose and transfer glucosyl residues from sucrose to either water or oligosaccharide acceptors (Bourne, Peters and Weigel, 1964; Koepsell et al, 1953).

4.1.2.1.1 Physical properties

Studies on the mutiple glucosyltransferases of the S. mutans group have yielded conflicting results. Ciardi (1983) summarized the findings of earlier workers who reported they had isolated enzymes with molecular weights which varied from 2,000,000 to 45,000 Da. Later in 1986 Mukasa described glucosyltransferases that produced both water-soluble and insoluble glucans. He found these enzymes had molecular weights that varied between 156,000 and 161,000 Da. In the same year Asem, Kenney and Cole isolated two glucosyltransferases. The first had a molecular weight of 153,000 Da and produced an insoluble glucan with predominantly alpha-1,3-linkages and the second had a molecular weight of 162,000 Da and formed a soluble glucan with predominantly alpha-1,6-linkages.

More recently in 1989 Hamada and his coworkers purified a cell-associated and an extracellular glucosyltransferases with molecular weight of 156,000 Da. They found these enzymes produced a water-insoluble and a water-soluble glucan respectively. In the same year Yamashita, Hanada and Takehara (1989) described a fourth type with a molecular weight of 152,000 Da degraded to 137,000 Da on proteolysis. This enzyme catalysed the production of a highly branched water-soluble glucan. Most of the above glucosyltransferases have a pH optimum of between 5,5 and 6 and an optimum temperature of 37$^{o}$C.

The glucosyltransferases produced by S. sanguis have also been investigated. Luzio, Graham and Mayer (1982) reported in a study on S. sanguis ATCC 10558 they could resolve the dextransucrase

into 5 major bands with molecular weights of about 780,000, 682,000, 485,000, 339,000 and 231,000 Da and two minor bands of 260,000 and 280,000 Da. However, Grahame and Mayer (1984) showed the mutiple forms consist of only 1 protein with a molecular weight of 174,000 Da. They found the organism produces a protease that converts the native enzyme to a proteolysed form with a molecular weight of 156,000. Convertion did not proceed in the presence of SDS. The optimum pH of these enzymes was 6,5 and the optimum temperature was 37°C.

4.1.2.1.2 Mechanisms of action

The biosynthesis of dextran is the simplest form of saccharide synthesis in bacteria. The enzymes responsible for their synthesis are extracellular and do not require sugar nucleotides or lipid-phosphate sugars (Sutherland, 1979; Stoddart, 1984). The basic mechanism is disproportionation in which fructose is released from sucrose and glucose is formed into a polymer by transglucosylation. The reaction is reversible and branching in the molecule is common (Stoddart, 1984). The dextransucrase of S. mutans is constitutive because it is present in cultures supplemented by sugars other than sucrose (Brooker, 1979).

Insoluble alpha-1,3-glucan is synthesized by inserting glucosyl units from sucrose. During synthesis two nucleophilic sites at the active site of the enzyme are bound covalently through C1 to glucosyl and dextranosyl units. The glucosyl unit is added to the reducing end of the dextranosyl chain where the C3-OH of the incoming glucosyl unit makes a nucleophilic attack onto C1 of the dextranosyl chain forming an alpha-1,3-linkage. The dextranosyl

chain is thereby transferred to the glucosyl unit and its chain is lengthened by one glucose unit. A new glucosyl unit is added from sucrose to the free nucleophilic site on the enzyme and the process repeats itself with glucosyl and dextranosyl units alternating between the two enzyme sites (Ro t, 1983).

The de novo synthesis occurs at the reducing end of a growing chain during autopolymerization (Ditson and Mayer, 1984). Low levels of dextranase, maltose and low molecular weight dextrans suopress the formation of water insoluble glucans, whereas maltose reduces the proportion of cells that adhere to glass (Gibbons and Keyes, 1969; Koga and Inoue, 1979; Miyasaki and Newbrun, 1983).

4.1.2.2 Fructosyltransferase (EC 2.4.1.10)

Fructosyltransferase is a constitutive enzyme that transfers the fructosyl moiety of sucrose to a 2,1-beta-fructan chain and releases glucose (Carlsson, 1970). This is a hydrolysis reaction in which free energy is derived from splitting the hemi-acetal:hemi-ketal bond in sucrose. Soluble and insoluble fructans are formed but there is no evidence they contribute to colonization of the teeth or the carious process (Chassy, 1983). Many strains of S. mutans produce fructans but some strains of S. salivarius are the most abundant producers of this polysaccharide (Niven, Smiley and Sherman, 1941; Garszczynski and Edward., 1973; Ehrlich et al, 1975; Hancock, Marshall and Weigel, 1976; Yakushiji, Inoue and Koga, 1984).

## 4.2 ENZYMES THAT REGULATE STARCH METABOLISM

Researchers have discovered several enzymes involved in starch metabolism. They can be divided into 2 groups. Firstly, enzymes that catalyse the degradation of starch and secondly those involved in starch synthesis. Most of the starch degrading enzymes yield products that may contribute to the carious process.

### 4.2.1 Enzymes that degrade starch

Starch degrading enzymes are known as amylases because they degrade 'amylum', the latin name for starch (Kneen, 1950; Severson, 1950). Amylases are defined as extracellular enzymes which catalyse the hydrolysis of starch molecules to yield dextrins and progressively smaller polymers composed of glucose units. They may be divided into 2 classes, endoamylases and exoamylases. Endoamylases act randomly on the alpha-1,4-linkage but do not attack the alpha-1,6-bonds at the branching points. Exoamylases hydrolyse the polysaccharide from the non-reducing terminal bond and act by successive removal of small units. One class, the amyloglucosidases, cleave each bond to produce alpha-glucose. Another, beta-amylase, breaks each alternate bond to yield beta-maltose (Windish and Mhatre, 1965).

#### 4.2.1.1 Alpha-amylase

Alpha-amylases hydrolyse the non-terminal 1,4-alpha-D-glucosidic linkages of amylose and amylopectin by a partly random action to yield a mixture of glucose, maltose and higher glucose oligomers (Manners, 1989). They are designated alpha because they form

reducing groups with an alpha-anomeric configuration. All alpha-amylases are endoases because they are able to attack the interior of starch chains as opposed to beta-amylase which is an exoase with an endwise form of attack (French, 1975). Studies using highly purified alpha-amylase suggest linear amylose and the "linear" portion of amylopectin are degraded by this enzyme (Hopkins and Bird, 1954; Whelan, 1960; Robyt and French, 1963).

Salivary alpha-amylase produces maltose, maltotriose and maltotetraose in the early stages of the degradation of amylose, whereas amylopectin is degraded to maltose, maltotriose, maltopentaose and larger oligosaccharides. In the later stages the main products of both starches are maltose and maltotriose (Pazur, French and Knapp, 1950; Hopkins and Bird, 1954; Robyt 1962). Porcine pancreatic alpha-amylase yields the same products (Bernfeld, 1951).

The early products obtained by the action of Bacillus subtilis alpha-amylase on amylose and amylopectin are maltotriose, maltopentaose and maltohexaose. The later products are mostly maltose, maltotriose and maltohexaose and smaller quantities of glucose, maltotetraose, maltopentaose and maltoheptaose (Robyt and French, 1963; Hopkins and Bird, 1954). alpha-amylase from Aspergillus oryzae converts amylose to glucose and maltose whereas, glucose is the main product of amylopectin hydrolysis (Hanrahan and Caldwell, 1953).

Alpa-amylase has also been observed in the streptococci. S. bovis produces glucose, maltose and maltotriose and S. equinus produces glucose, maltose, maltotriose and maltotetraose from starch

(Walker, 1965a; 1965b; Boyer and Hartman, 1971). In 1983 Spandau and his coworkers identified an alpha-amylase from an oral strain of Actinomyces viscosus which produced maltotetraose, maltotriose and maltose from starch but never glucose. However, none of these authors investigated the products formed from amylose and amylopectin.

The products which remain after the action of alpha-amylase on amylopectin are called alpha-limit dextrins and contain a mixture of 1,4 and 1,6-bonds. They range from pentasaccharide to larger oligosaccharide molecules which possess two or three alpha-1,6-bonds (Whelan and Roberts, 1952; Roberts and Whelan, 1960). These alpha-limit dextrins are formed because alpha-amylase is unable to split 1,4-bonds in the vicinity of 1,6-branch points (Meyer and Gonon, 1951).

Alpha-amylase from saliva, A. oryzae and the porcine pancreas yield the same tetrasaccharide (Nordin and French, 1958; Hughes, 1959). B. subtilis alpha-amylase yields a pentasaccharide, whereas barley-malt alpha-amylase yields the smallest limit dextrin, the trisaccharide panose (Hughes, 1959; Hughes, Smith and Whelan, 1963; Heller and Schramm, 1964). These different alpha-dextrins are produced because the enzymes show a variation in their specificity towards the 1,4-alpha-D-glucosidic linkages in the vicinity of interchain linkages (Manners, 1989).

4.2.1.1.1 Physical properties

All alpha-amylases that have been investigated are calcium metalloenzymes and require calcium metalloproteins as cofactors. They have at least one calcium ion per molecule and depend upon the metal group for catalytic activity (Vallee et al, 1959; Fischer and Stein, 1960). The strength of binding of the metal depends upon the source of the enzyme and increases in the following order: higher plants < mamalian < bacterial < fungal (Greenwood and Milne, 1968). Calcium may be removed from the amylase molecule by dialysis against ethylene diamine tetra acetate, ammonium salts or electrodyalysis (Stein, Hsui and Fischer, 1964). Calcium free amylases are susceptible to denaturation by heat, acid, urea, trypsin, chymotrypsin, subtilin, pepsin and papain (Yamamoto, 1955; Stein and Fischer, 1958).

Many mammalian alpha-amylases are activated by monovalent anions especially chloride (Myrback, 1926). When chloride ions are removed by dialysis the enzyme loses activity, shows increased solubility and loss of stability in the presence of elevated temperatures and heavy metals (Myrback, 1926; Muus, Brockett and Connelly, 1956). The optimum concentration for activity is 10 mM sodium chloride (Meyer, Fischer and Bernfeld, 1947; Walker and Whelan, 1960).

The physical constants of salivary and [illegible]e bacterial alpha-amylases are given in Table 1.4 (Dunican and Seeley, 1962; Robyt and Whelan, 1968; Boyer and Hartman, 1971).

Table 1.4 Physical constants of several alpha-amylases

| Source | Optimum pH | pH stability range | Optimum temp | Molecular weight |
|---|---|---|---|---|
| Saliva | 6,9 | 4,8 - 11 | 40 | 55,200 |
| A. oryzae | 4,8 - 5,8 | 5,5 - 8,5 | 40 | 51,000 |
| B. subtilis | 6,0 | 4,8 - 8,5 | 40 | 48,700 |
| B. stearothermophilus | 5,0 | ? | 65 | 15,600 |
| S. equinus | 7,2 | ? | 37 | ? |
| S. bovis | 7,3 | ? | 37 | ? |

Alpha-amylase from B. subtilis usually exists as a dimer which dissociates into a monomer with a molecular weight of 48,200 at high dilutions (Isemura, Kakiuchi and Eto, 1960; Isemura and Kakiuchi, 1962; Kakiuchi et al, 1964). Apparently the zinc atom in the molecule is responsible for the monomer-dimer transformation (Stein, 1957; Fisher et al, 1958; Fisher and Stein, 1960).

4.2.1.1.2 Effect of pH

Generally alpha-amylases are denatured by acid but are stable at pH values between 5 and 8,5. The effect of pH is usually ascribed to ionization of side chain groups in the protein. The groups involved include the substrate binding group and the catalytic group. Maximum activity is in the acid region between 4,5 and 7 but this may vary for each individual enzyme (Robyt and Whelan, 1968a).

4.2.1.1.3 Effect of temperature

The alpha-amylases with a full complement of calcium are resistent to heat inactivation and are less heat labile than the beta-amylases (Robyt and Whelan, 1968a). The enzymes of B. subtilis and B. stearothermophilus are particularly stable to heat (Manning and Campbell, 1961). The activity of alpha-amylase increases steadily from 0°C to a maximum of 40°C in the majority of cases. There is a rapid decrease of activity between 40°and 60°C after which it undergoes denaturation (Robyt and Whelan, 1968a).

4.2.1.1.4 Kinetics of action

Three modes of attack have been considered for alpha-amylase activity - the single chain, multi-chain and multiple attack (Figure 1.3). The mechanism which is currently accepted is the multiple attack (Robyt and Whelan, 1968a). Multiple attack may be defined as repeated attacks on a long chain substrate molecule after a single encounter of the enzyme and substrate (Kondo et al, 1978). In the multiple attack mechanism the enzyme forms a complex with the substrate and produces the first cleavage. After the first cleavage the fragment with the new nonreducing end dissociates from the active site while the fragment with the newly formed hemiacetal reducing end remains associated with the active site and repositions itself to give another cleavage and the formation of either maltose or maltotriose. The enzyme remains with one of the fragments of the original substrate and catalyses the hydrolysis of several bonds before it dissociates and forms a new active complex with another substrate molecule (Robyt, 1984).

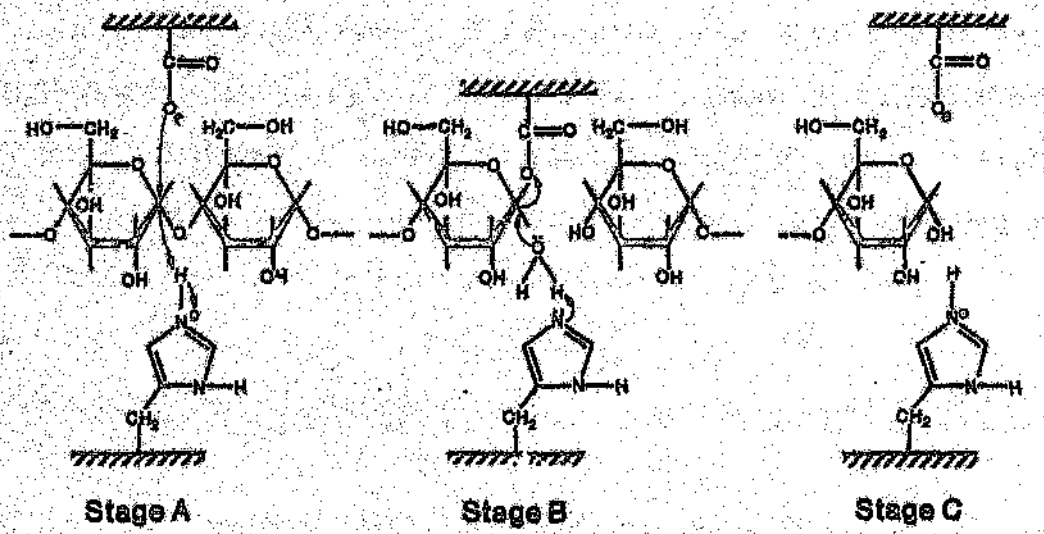



Figure 1.3 Suggested mechanism of the bond-breaking step in the hydrolysis of an alpha-1,4-glucosidic bond by alpha-amylase.

4.2.1.1.5 Measurement of kinetics

The measurement of alpha-amylase kinetics is accomplished by determining either the disappearance of a substrate with time, or the appearance of products usually by measuring the increase in the reducing value of the digest (Bernfeld and Studer-Pecha, 1947a, 1947b; Schwimmer, 1950; Yamamoto, 1955; van Dyk and Caldwell, 1956a, 1956b, 1956c). The latter method is more accurate than measuring the disappearance of substrate by iodine staining because measurements can be related directly to the number of bonds broken. Existing methods of measuring reducing groups are not completely accurate because they do not differentiate between molecules of different degrees of polymerization (Robyt and Whelan, 1968a).

4.2.1.1.6 Mechanisms of action

Meyer and Bernfeld (1941) and Meyer and Gonon (1951) proposed an hypothesis for alpha-amylase action. They considered the alpha-amylase reaction as a random process in which alpha-amylases show equal preference for all alpha-1,4 linkages except those adjacent to the two ends of the substrate chain and in the vicinity of the branch points. Paper chromatography studies have shown maltodextrins specific for particular alpha-amylases are formed in the early and late stages of many amylase digests (Pazur, French and Knapp, 1950; Hopkins and Bird, 1954; Dube and Nordin, 1961; Robyt, 1962; Robyt and French, 1963). French (1957b), Pazur, French and Knapp (1950) and Robyt and French (1963) have developed this hypothesis further to explain the non-random distribution of products.

4.2.1.2 Beta-amylase

Early work indicated "malt amylase" is composed of two starch splitting enzymes. Both enzymes are able to attack starch but their mode of action is different. The first, designated dextrinogenic enzyme produces dextrins and is called alpha-amylase. The second enzyme is saccharogenic because it produces maltose from starch and a residue which stains blue with iodine. This enzyme was later shown to be a beta-amylase (Myrback, 1948).

Beta-amylases liberate maltose in a stepwise fashion from the non-reducing end-group of an alpha-D-1,4-glucan (Myrback and Nycander, 1942; French et al, 1950). The hydrolytic action results in the formation of maltose in the beta form (Marshall, 1974). Chains containing an odd number of residues will yield one molecule of either maltotriose or glucose (Manners, 1989).

Originally beta-amylase was a plant enzyme obtained from cereals. The sweet potato was a rich source of this enzyme (Balls, Walden and Thompson, 1948; Meyer, Fisher and Piguet, 1951; Meyer, Spahr and Fisher, 1953; Nakayama and Amagase, 1963). More recently beta-amylases have been isolated from bacteria, including Bacillus polymyxa and other species (Robyt and French, 1964; Higashihara and Okada, 1974; Shinke, Kunimi and Nishira, 1975a,1975b). Enzymes from different sources appear to act similarly (Greenwood and Milne, 1968).

4.2.1.2.1 Physical properties

Beta-amylase is relatively stable to acid. The pH optimum for most enzymes lies in the range 5 to 6, whereas sweet potato beta-amylase has an optimum between pH 4 and 5 (Balls, Walden and Thompson, 1948; Piguet and Fischer, 1952; Meyer, Spahr and Fischer, 1953). Most beta-amylases are more heat labile than alpha-amylases (Greenwood and Milne, 1968). Sweet potato beta-amylase may be heated to 60°C without loss of activity (Balls et al, 1948). The crystalline enzyme from this source is used at pH 3,6 and 35°C for the hydrolysis of starch (Peat, Pirt and Whelan, 1952).

No co-enzyme requirements are known for beta-amylase. The enzyme however contains sulphydryl groups that can be masked (Balls et al, 1948). Inhibition is complete when the enzyme is treated with reagents that react with the sulfhydryl groups i.e. L-ascorbic acid, p-chloromercuribenzoate, silver, copper, mercury and iodine. This inactivation can be restored with the reducing agents glutathione, hydrogen sulfide and cysteine (England, Sorof and Singer, 1951). The commercial preparation of crystalline sweet-potato beta-amylase loses activity rapidly at pH 4,8 and 35°C but glutathione gives protection (Robyt and Whelan, 1968b).

4.2.1.2.2 Kinetics of action

In contrast to the alpha-amylases the reducing activity generated by beta-amylase is directly proportional to enzyme activity. There appears to be no standard method of measuring reducing power and different substrates have been used (Greenwood and Milne, 1968). When amylose is the substrate the rate of enzyme activity decreases

as the size of the amylose molecule increases (Pfanne Muller, 1967). This barrier to beta-amylolysis has been attributed to the presence of limited branching in the molecule (Banks and Greenwood, 1966).

4.2.1.2.3 Mechanisms of action

The action of beta-amylase has been depicted in the form of a 'mouth and throat' that can admit the non-reducing end of the starch chain. Provided two glucose units can be admitted into the mouth the active centre of the enzyme will be able to sever the penultimate glycosidic bond and release maltose (Figure 1,2). This model explains why the enzyme cannot progress past a branch point in amylopectin, i.e. when a 1,6-bond is encountered (Robyt and Whelan, 1968b).

Beta-amylase was the first enzyme to show a multiple attack mechanism. The enzyme repeatedly cleaves a substrate chain before dissociation. With low molecular weight amylose there are an average of 4 cleavages but with larger molecules the number is much higher (Bailey and French, 1956; French and Youngquist, 1963). The direction of attack is from the non-reducing to the reducing end is opposite to that of alpha-amylase (Robyt, 1984). Susceptibility to hydrolysis decreases when amylose retrogrades but the effect can be modified under normal conditions. The hydrolysis of amylose by beta-amylase has been used extensively to characterize amylose from different sources (Banks, Greenwood and Thomson, 1959b; Banks and Greenwood, 1967).

Perhaps the most important clue to the mechanism of bond splitting is that beta-maltose is released following the splitting of an alpha-glucosidic bond (Robyt and Whelan, 1968b). Studies using $O^{18}$ labelled water have shown the maltose formed by the enzymic degradation of starch contained $^{18}O$ bound to the reducing, anomeric carbon atom. These studies indicate beta-amylase, like alpha-amylase cleaves the C1-O-C4 bond in the alpha-D-1,4 linked D-glucans between C1 and the oxygen atom (Greenwood and Milne, 1968).

4.2.1.3 Amyloglucosidase (EC 3.2.1.33)

Amyloglucosidases have assumed great commercial value because they convert starch directly to glucose (Greenwood and Milne, 1968). Their action is similar to beta-amylase because the enzyme acts only on non-reducing chain ends with the cleavage of alpha-1,4 bonds and the liberation of beta-glucose (Reese, Maguire and Parrish, 1967). They differ from beta-amylase because they do not produce limit dextrins (Robyt, 1984). Terminal alpha-1,6 bonds are cleaved by this enzyme at a slow rate, Maltose is attacked slowly, whereas increasing the chain length up to 5 or 6 glucose units increases the rate of attack (Abdullah et al, 1963).

In principle amyloglucosidase could give complete degradation of amylose or amylopectin. However, when amyloglucosidase is purified and no alpha-amylase is present incomplete hydrolysis occurs because of irregularities in the structure of the starch (Marshall and Whelan, 1970). Amyloglucosidase will trim off the more

accessible glucose units from the non-reducing chain end of branched oligosaccharides produced from amylopectin by alpha-amylase (Abdullah and French, 1970).

Amyloglucosidase is produced by fungi and bacteria and occurs in the mammalian digestive tract (Pazur and Okada, 1967; Pazur, 1970; Kelly and Alpers, 1973; Fleming, 1968). In 1981 Bender isolated an amyloglucosidase from a flavobacterium species which was able to degrade cycloamyloses to glucose.

4.2.1.4 Pullulanase (EC 3.2.1.41)

The enzyme pullulanase was first recognised by Bender and Wallenfels in 1961. It is a 1,6-alpha-glucosidase which specifically attacks the alpha-glucan pullulan. The substrate is hydrolysed at random with the initial production of hexa- and nona-oligosaccharides and thereafter maltotriose. Pullulanase is important in structural studies because it hydrolyses 1,6-alpha-glucosidic linkages in amylopectin provided there are two alpha-1,4-glucosidic links on either side of the alpha-1,6-link. The linear tetrasaccharide 6-alpha-maltosylmaltose is the smallest molecule which meets this requirement (Abdullah and French, 1970; Norman, 1979).

The chains in starch which contain up to 6 to 10 units appear to be susceptible to pullulanase action. It is still not clear why amylopectin and beta-amylase limit dextrins are partially resistant to pullulanase action (Price, 1968). Reduction of the glucose unit

to sorbitol at the reducing end of a branched oligosaccharide may inhibit the hydrolytic activity of this enzyme (Abdullah and French, 1970).

Pullulanase is produced by a number of bacteria including Klesiella aerogenes (Price, 1968; Ohba and Ueda, 1975), Escherichia coli (Palmer, Wober and Whelan, 1973), Escherichia intermedia (Wober, 1976), bacillus species (Morgan, Adams and Priest, 1979) and Clostridium thermohydrosulfuricum (Hyun and Zeikus, 1985). Furthermore, this enzyme has been isolated from Str.mitis a species of viridans streptococci (Walker, 1966). It is specifically induced by maltose, higher maltodextrins and pullulan, but rot glucose (Preiss and Walsh, 1981).

4.2.1.5 Isoamylase (EC 3.2.1.65)

Isoamylase cleaves the 1,6-linkages present in branch points in oligo and polysaccharides provided an A-chain of 3 or more glucose units occurs before the branch point. In contrast pullulanase requires an A-chain of 2 or more units and is able to cleave 1,6-alpha-glucosidic linkages at a branch point and in a linear chain (Marshall, 1975).

A further restriction of this enzyme is that 1,6-alpha-D-glucosidic linkages must be at true branch points i.e. the hydroxyl group of the D-glucose residue must be linked through both O-1 and O-4 (Marshall, 1974). These requirements make the enzyme highly specific. The enzyme, for example debranches glycogen and

amylopectin but is unable to act on pullulan. In addition the beta-limit dextrins of glycogen and amylopectin are not hydrolyzed (Marshall, 1974).

Isoamylase is found in yeasts, Cytophaga, Escherichia, Streptomyces and Pseudomonas (Marshall, 1974; Fogarty and Kelly, 1980). The enzyme from Pseudomonas amyloderamosa has been studied and is extensively used for debranching studies. It is not heat-stable and may be readily inactivated. The pH optimum lies between 3 and 4 and the enzyme is inactivated above pH 6,7. The temperature optimum is 40°C (Harada, Yokobayashi and Misaki, 1968; Yokobayashi, Misaki and Harada, 1970; Harada et al, 1972; Yokobayashi et al, 1973).

4.2.1.6 R-enzyme (EC 3.2.1.41)

R-enzyme was one of the first enzymes shown to debranch starch. It is specific for the hydrolysis of branch points in glucans and removes alpha-1,6 linked branch chains containing 2 or more 1,4 linked alpha-glucose units. The enzyme catalyzes the cleavage of alpha-D-1,6-linkages in amylopectin and releases biose and triose from the beta limit dextrin of amylopectin. After the action of R-enzyme the dextrins are susceptible to further attack by beta-amylase and phosphorylase (French, 1975).

The enzyme has been isolated from potatoes, broad beans, barley and animal tissue (Greenwood and Milne, 1968). According to Greenwood (1970) the R-enzyme activity of broad beans and malted barley may

be fractionated into 2 components, one acts on amylopectin whereas the second attacks small alpha-limit dextrins of amylopectin.

4.2.1.7 Schardinger enzyme (EC 2.4.1.19)

The Schardinger enzyme also known as transglycosylase is an exo-enzyme which produces cyclic dextrins in high yield from starch polysaccharide. It is not hydrolytic and cannot be detected by the release of reducing groups (Bacon, 1979). The cyclic dextrins or Schardinger dextrins were first discovered by Schardinger in 1903 (French, 1957a). These are also known as cycloamyloses and can be recognised because they form crystalline compounds that precipitate out of solution (Myrback 1948; French, 1957a; Bacon, 1979).

The nature of the cyclic dextrins was not clear for many years. Eventually researchers realized the products are rings of mainly six, seven or more alpha-1,4 linked glucose residues. Freudenberg suggested the structure of the polysaccharide makes crystallization possible (Bacon, 1979). If the glucanosyl chain is 6 or 7 glucose units a cyclic molecule is formed and released from the enzyme (French, 1975). Amylose yields more cyclodextrins than amylopectin (Myrback, 1948) which indicates the helical nature of amylose favours the formation of these dextrins.

4.2.1.8 Newer microbial enzymes

4.2.1.8.1 Bacillus polymyxa amylase

Bacillus polymyxa produces an enzyme capable of converting starch primarily into maltose (Robyt and French, 1964). The enzyme system degrades cyclic Schardinger dextrins, presumably by the action of an alpha-amylase and produces 3 maltose units per cyclomaltohexaose. Studies using paper chromatography produced no branched oligosaccharide from amylopectin suggesting the enzyme debranches this starch. The enzyme also acts as a pullulanase (French, 1975).

4.2.1.8.2 Pseudomonas stutzeri amylase

Pseudomonas stutzeri produces an amylase which converts starch into maltotetraose (Robyt and Ackerman, 1971). The enzyme is similar to beta-amylase because it acts from the non-reducing end of the starch chains and produces maltotetraose in the beta-configuration. It acts on amylopectin to give maltotetraose and a limit dextrin which suggests it is unable to bypass the branch points of the substrate (French, 1975).

4.2.1.8.3 G6 enzyme

G6 enzyme is produced from Klebsiella aerogenes and converts amylose to maltohexaose (Kainuma et al, 1972, 1975). Initial studies indicated it was an exoase but it can bypass branch points to incorporate branching into the oligosaccharide. The six-unit basic chain can include a fragment of an A chain, a 1,6-linkage and the reducing end of a B chain (French, 1975).

4.2.2 Enzymes that regulate acid production from starch

As was mentioned previously glycolysis is the major metabolic pathway for the generation of acids by the streptococci, and glucose is the initial substrate for this process (Stanier et al, 1971). Most amylases do not produce large amounts of glucose but hydrolyse starch to a mixture of products including maltose, maltotriose, maltotetraose, maltopentaose and higher oligosaccharides (see Section 4.2.1.1).

Two enzymes secreted by the small intestine and the pancreas can convert these oligosaccharides into glucose. The first is alpha-1,4-glucosidase which converts maltose, maltotriose and maltotetraose into D-glucose by successive action from the non-reducing end. The second is an alpha-1,6-glucosidase which converts branched dextrins to glucose and maltotriose. The latter is subsequently converted to D-glucose by alpha-1,4-glucosidase (Gray, Lally and Conklin, 1978).

There is no evidence that oral streptococci produce alpha-1,4-glucosidase and alpha-1,6-glucosidase. Furthermore, information about the production of glucose and acids from starch by these bacteria is sparse (see Section 1.2.2).

### 4.2.3 Enzymes that produce starch

There are at least 3 enzymes that can synthesize starch. They are phosphorylase, starch synthetase and Q-enzyme. Phosphorylase has been isolated from bacteria whereas starch synthetase and Q-enzyme are found only in starch granules (Greenwood and Milne, 1968).

#### 4.2.3.1 Phosphorylase (EC 2.4.1.1)

The first reports of the biosynthesis of a polysaccharide were made by Cori and Cori in 1939 and Hanes in 1940. These authors were working with phophorylases from plants and muscle. The enzyme is also found in the liver and heart of rabbits, humans, pigeons, dogs and pigs. Phosphorylase from animals is found in two forms, the active a form and the inactive b form. The b form is converted to the a form by phosphorylation (Greenwood and Milne, 1968).

Phosphorylases catalyze the reaction

D-glucosyl phosphate + D-Gn $\leftrightarrow$ phosphate + D-Gn+1.

D-Gn is an alpha-D-1,4 linked chain of D-glucose residues. The degradation of the polymer occurs when high concentrations of inorganic phosphate are present and synthesis occurs if levels of D-glucosyl phosphate are high (Greenwood and Milne, 1968).

In the above equation D-Gn, the primer for synthesis, may be a linear maltodextrin or a branched polysaccharide. The smallest primer for potato phosphorylase activity is the triose but larger

oligosaccharides are more effective (French and Wild, 1953). Phosphorylated maltotetraose and maltohexaose are inefficient primers (Posternak, 1951) which suggests D-glucose residues are added to the non-reducing end of the primer (Greenwood and Milne, 1968). Amylose and amylopectin may also act as primers. Amylopectin was found to be efficient for potato phosphorylase whereas the beta-limit dextrin of this substrate has little action (Hestrin, 1949).

Little is known of the importance of phosphorylase in the metabolism of starch *in vivo*. Studies in man have indicated the enzyme is of prime importance for the degradation of glycogen and provides a high-energy form of glucose from the stored polysaccharide (Robyt, 1984). This enzyme may be used *in vitro* to prepare amylose with a very narrow distribution of molecular weights (Greenwood and Milne, 1968).

4.2.3.2 Starch synthase (EC 2.4.1.21)

Starch synthase was first observed by Leloir and Cardini (1957). It is found in starch granules and has been prepared from sweet corn, potato and spinach leaves (Frydman and Cardini, 1964a, 1964b; Doi, Doi and Nikuni, 1964; Ghosh and Preiss, 1965). This enzyme uses adenosine diphosphate glucose (ADPG) or uridine diphosphate glucose (UDPG) as a high-energy donor (Rocondo and LeLoir, 1961; Frydman 1963).

The reaction proceeds as follows

$$m\ ADPG + Gn \longrightarrow G(n+m) + m\ ADP$$

primer — synthesized polysaccharide chain

ADPG or UDPG may be involved in this reaction. According to Robyt (1984) ADPG is used to synthesize amylopectin and UDPG amylose. Two separate synthase enzymes are involved.

4.2.3.3 Q-enzyme (EC 2.4.1.18)

Q-enzyme appears to be important in the synthesis of starch and is required for the synthesis of alpha-D-1,6 branch linkages. It is probably responsible for the D-glucosyl exchange reaction in which the alpha-D-1,4 linkage is converted into an alpha-D-1,6 linkage. Apparently G40 is the minimum length for rapid action. No branching action occurs on amylose of chain lengths less than 35 to 40 glucose units (Whelan, 1971). The enzyme is capable of introducing additional branches into amylopectin to give glycogen-like polymers (Greenwood and Milne, 1968; Robyt, 1984).

The enzyme catalyses an interchain transfer rather than an intrachain reaction. The mechanism for branching involves inter-turn transfer between the chains of a double helix. This mechanism would explain the requirement for long chains when amylose is the substrate because short chains could not be expected to form a double helix (French, 1975). Random attack of amylose by Q-enzyme would give rise to an amylopectin having an elongated structure containing features of both the laminated and the comb structure as first postuated in 1937 (Hall and Manners, 1980).

Q-enzyme is present in all plants and was first isolated from potato (Haworth, Peat and Bourne, 1944; Barker, Bourne and Peat, 1949). It is heat-stable, stabilized by presence of substrate, activated by inorganic ions and has a molecular weight of 85 000 (Greenwood and Milne, 1968; Preiss and Walsh, 1981).

A diagramatic representation of the action of some of the enzymes that degrade amylose and amylopectin can be found in figures 1.1 and 1.2.

## 5 AIMS OF THE INVESTIGATION

From the foregoing discussion apparently there are many problems associated with research into starch. Thus the variability, insolubility and complex nature of the molecule has made studies of starch difficult. These problems have led to the development of several complimentary methods of analysis. None are entirely satisfactory because after nearly 50 years work researchers still do not agree about the structure of these polymers.

One of the main fields of starch research has been enzymes that modify these polysaccharides. Two groups of enzymes have been discovered, namely, starch degrading enzymes called amylases and starch producing enzymes also known as synthases. Both are identified by the products they form. Research has shown S. mitis produces alpha-amylase, a debranching enzyme called pullulanase and a branching enzyme which converts amylose and amylopectin to glycogen (Walker, 1966, 1968; Walker and Builder, 1971). This

suggests many oral streptococci could produce enzymes that modify starch to acids and polysaccharides. However, the problems associated with the analysis of starch may complicate the detection of these products.

The aims of the present study are

1. To determine some of the products, in particular acids and polysaccharides, produced from amylose and amylopectin by the oral streptococci.

2. To study some of the enzymes responsible for producing these products.

# CHAPTER 2

# MATERIALS AND METHODS

## CONTENTS

1 SELECTION AND CULTURE OF STARCH HYDROLYSING STREPTOCOCCI... 82

1.1 Oral health of subjects..... 82

1.2 Isolation of streptococci..... 85

1.3 Amylose an. amylopectin broth..... 86
1.3.1 Formulation of the broths..... 86
1.3.2 Preparation and sterilization..... 91
1.3.3 Inoculation and incubation..... 92
1.3.4 Sampling of broths..... 93
1.3.5 Measurement of growth in broths..... 94

2 ACID PRODUCTION FROM AMYLOSE AND AMYLOPECTIN..... 95

2.1 Production of glucose and reducing sugars..... 95
2.1.1 Fehling's test for reducing sugars..... 95
2.1.2 The glucose oxidase test..... 96
2.1.3 Ion chromatography.....100

2.2 Production of acids.....102
2.2.1 Change in pH.....102
2.2.2 Identification of acids.....102
2.2.2.1 Gas liquid chromatography.....102
2.2.2.2 Ion exclusion chromatography.....103

3 POLYSACCHARIDE PRODUCTION FROM STARCH.....105

3.1 Production of polysaccharide.....105

3.2 Isolation of extracellular enzymes.....105

3.3 Isolation of polysaccharide.....106

3.4 Chemical characteristics of polysaccharide.....107
3.4.1 Solubility.....107
3.4.2 Iodine staining.....107
3.4.3 Infra-red spectrum.....107
3.4.4 Methylation analysis.....108
3.4.5 Enzymic analysis.....109
3.4.5.1 Dissolving the polysaccharide.....109
3.4.5.2 Debranching with isoamylase.....109
3.4.5.3 Digestion with amylases.....110

4 ENZYMES THAT REGULATE STARCH METABOLISM.....111

4.1 Isolation and extraction of enzymes.....111

4.2 Identification of enzymes.....112
4.2.1 Incubation of enzyme extracts.....112
4.2.2 Identification of the degradation products.....113
4.2.2.1 Thin layer chromatography.....113
4.2.2.2 High performance liquid chromatography.....114

4.3 Molecular weight of enzymes.....114
4.3.1 Gel electrophoresis.....114
4.3.1.1 Sample preparation.....115
4.3.1.2 Electrophoresis.....115

5 STATISTICAL ANALYSIS.....117

LIST OF ILLUSTRATIONS

Table 2.1 Strains of 'viridans' streptococci used..... 87
in the study.

Table 2.2 Growth of Str. mitis on different..... 90
concentrations of tryptone, yeast extract
and amylopectin.

Figure 2.1 The number of decayed, missing and filled..... 83
teeth of the 7 subjects in the study.

Figure 2.2 The dental plaque index of the 7 subjects..... 84
investigated.

Figure 2.3 Standard graph of known concentrations of..... 97
glucose. Glucose was reduced with the
Fehlings reagents

Figure 2.4 Standard graph of known concentrations of..... 99
glucose. Glucose was reacted with the
GOD-PAP(Boehringer Mannheim) reagent.

Figure 2.5 Ion chromatogram of glucose oligomer standards...101

Figure 2.6 Ion chromatogram of standard acids.....104

# 1 SELECTION AND CULTURE OF STARCH HYDROLYSING STREPTOCOCCI

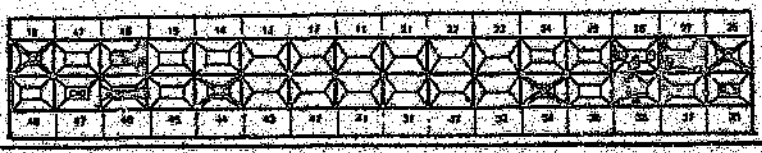
## 1.1 Oral health of subjects

The streptococci studied were isolated from 7 male subjects between the ages of 18 and 35 years. They were divided into 2 groups, the first consisted of 3 subjects with dental caries and the second included 4 caries free subjects. A clinical examination of their teeth was undertaken by a dentist who had experience in examination procedures and was a member of the staff of the Dental Hospital, University of the Witwatersrand, Johannesburg. The criteria of the World Health Organization (1971) were used to determine the number of decayed, missing and filled teeth (DMFT). Bite-wing radiographs were taken of the molar areas to ensure there was no undetected caries in areas inaccesible to normal examination procedures (Figure 2.1).

A plaque index based on the method of Silness and Loe (1964) was used to determine the oral hygiene status of the subjects. All surfaces of the teeth were examined with a number 9 probe. A count of 0 was given if there was no plaque present, 1 if plaque was present on the gingival third of the tooth, 2 if it covered two thirds and 3 if it covered more than two thirds of the tooth. A plaque index was compiled by dividing the sum of these scores by the number of surfaces examined. The indices ranged from 0.51 to 2. The former was obtained in a subject who was caries free and the latter in a patient with dental caries (Figure 2,2).

Subject 1 Age 23

D = 0
M = 4
F = 8
12



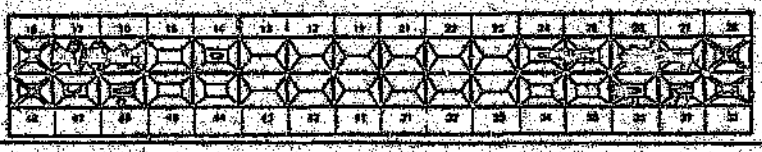
Subject 2 Age 22

D = 0
M = 4
F = 8
12



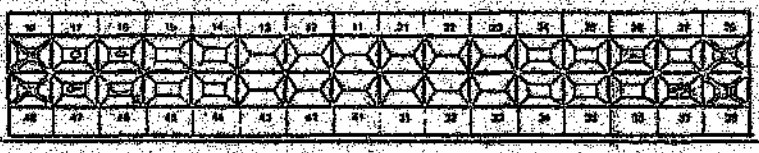
Subject 3 Age 27

D = 0
M = 4
F = 11
15



Subject 4 Age 22

D = 0
M = 4
F = 6
10



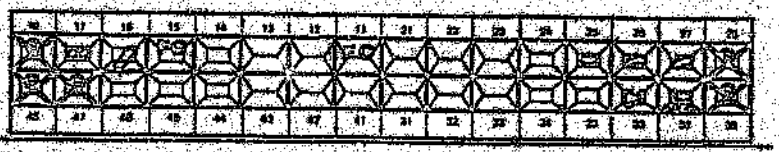
Subject 5 Age 19

D = 5
M = 0
F = 1
6



Subject 6 Age 29

D = 7
M = 5
F = 4
16



Subject 7 Age 22

D = 3
M = 5
F = 4
12

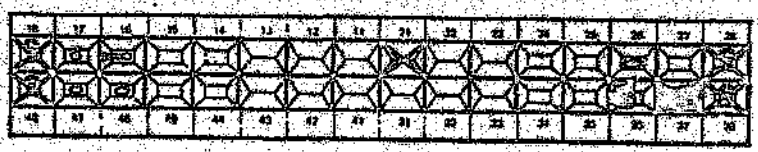


Figure 2.1 The number of decayed, missing and filled teeth of the 7 subjects in the study.

Decayed O , Missing X , Missing due to caries ⊠ ,
Filled ⊡ , Full Crown FC .

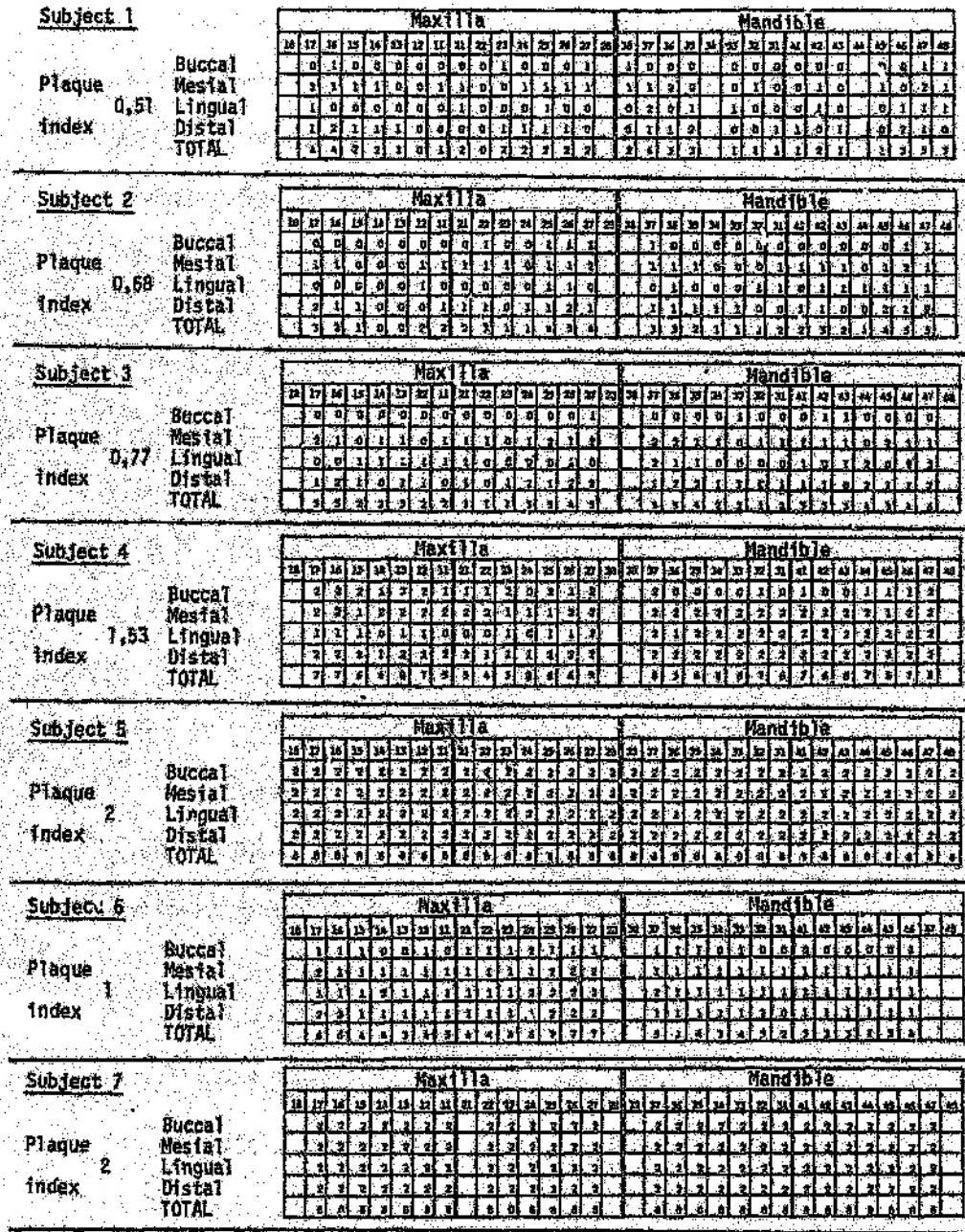



Figure 2.2 The dental plaque index of the 7 subjects investigated.

1.2 Isolation of streptococci

Samples of debris were obtained from either carious lesions or in caries free subjects from dental plaque on the buccal aspect of the smooth surface of the lower incisors (Table 2,1). This site was chosen because experimental animals developed dental caries in this area when challenged by a high starch diet (Grenby, 1965; Frostell and Baer, 1971b; Shaw and Ivimey, 1972; McDonald and Stookey, 1977; Kreitzman, 1978).

The material was placed in 1 ml of sterile phosphate buffered saline pH 7,2 (Cruickshank, 1962) containing 80 mesh glass beads (Merck). The suspensions were agitated with a whirlimixer (Fisons) and diluted in sterile saline by 10 fold dilutions ranging from $10^{-1}$ to $10^{-12}$. A sterile pipette was used to place 0,1 ml of each dilution on a starch agar plate devised by Iverson and Mills in 1974 (see Appendix). The inoculum was spread with a sterile glass rod and the plates were incubated at 37°C for 48 hours in a candle jar to provide carbon dioxide.

The plates were examined and those with separate colonies were chosen for further investigation. Samples of c s with a different macroscopic appearance were stained with Gram's stain (Cruickshank, 1962). Colonies of gram positive cocci that occurred in chains and were catalase negative were selected for further study. Samples of the cells from each colony were subcultured on starch agar and nutrient agar (Oxoid) containing 5 per cent sterile citrated horse blood and incubated at 37°C for 48 hours to obtain pure cultures.

The starch agar plates were flooded with Lugol's iodine (Cruickshank, 1962). Colonies which hydrolysed starch were surrounded by a clear halo in contrast to the remaining agar which turned blue. These plates were discarded because iodine has antibacterial properties. The duplicate cultures on blood agar were inoculated into various media to identify them by the code proposed by Coogan in 1986 which is a modification of the scheme put foreward by Facklam (1977, 1984). Fresh cultures of these starch hydrolysing streptococci were prepared on blood agar plates. The cells were removed from the plates and suspended in horse serum containing 1 per cent glucose and sterile glass beads and stored at -20$^{o}$C until required.

Twenty strains of starch hydrolysing oral streptococci were isolated, 8 from the subjects with dental caries and 12 from caries free subjects. These strains and a stock strain FW 213 were used in the study (Table 2.1).

## 1.3 Amylose and amylopectin broths

### 1.3.1 Formulation of the broths

A culture medium had to be developed which is suitable for studying starch hydrolysis. The medium had to support the growth of streptococci and contain minimal amounts of carbohydrate. The presence of the latter would make it difficult to distinguish between the products of starch hydrolysis and the original carbohydrates present in the medium.

**Table 2.1 Strains of 'viridans' streptococci used in this study**

| Subject | | Tooth | Code | Species |
|---|---|---|---|---|
| Caries free | 1 | 22 | MC 101 | S. mitis |
| | | | MC 102 | S. sanguis I |
| | | | MC 103 | S. sanguis II |
| | 2 | 22 | MC 204 | S. sanguis I |
| | | | MC 205 | S. mitis |
| | | | MC 206 | S. mitis |
| | 3 | 22 | MC 307 | S. anginosus |
| | | | MC 308 | S. mitis |
| | | | MC 309 | S. sanguis II |
| | 4 | 22 | MC 410 | S. mitis |
| | | | MC 411 | S. intermedius |
| | | | MC 412 | S. sanguis II |
| Caries positive | 5 | 47 | MC 513 | S. anginosus |
| | | | MC 514 | S. mitis |
| | 6 | 17 | MC 615 | S. sanguis II |
| | | | MC 616 | S. sanguis I |
| | | | MC 617 | S. intermedius |
| | 7 | 17 | MC 718 | S. sanguis I |
| | | | MC 719 | S. anginosus |
| | | | MC 720 | S. sanguis II |
| Stock stain (Walker) obtained from G Coleman | | | FW 213 | S. sanguis I |

Initially a chemically defined medium devised by Carlson (1970) for the growth of S. sanguis was tested. It consisted of ammonium and magnesium sulphate, vitamins and amino acids in phosphate buffered saline pH 7,5 and potato amylopectin (Sigma No. A8515) at a concentration of 0,5 per cent as a carbon source. The medium was sterilized by filtration through a membrane filter with pore size 0,2µm.

Growth of the starch hydrolysing streptococci on this medium was determined by plating out 0,1 ml of the inoculated broth on blood agar before and after 24 hours incubation. An increase in the number of microorganisms in the broth after 24 hours as indicated by colony counts on the plates showed growth had occurred. Three starch hydrolysing streptococci S. mitis 101, S. sanguis I 102 and S. sanguis II 103 were tested for their ability to grow in this medium. One isolate S. mitis 101 did not grow even after the addition of glucose. For this reason the medium was abandoned.

Studies on S. mutans indicate a complex medium containing proteins and peptones has an advantage over a synthetic medium consisting of vitamins, nucleic and amino acids because it stimulates more enzyme production (Ciardi, 1983). The complex medium described by Boyer and Hartman (1971) for alpha-amylase and transglucosylase production by S. equinus was tested. It consisted of 1 per cent tryptone and 1 per cent yeast extract made up in 0,03 M potassium phosphate buffer adjusted to pH 7,5. Amylopectin was added to this medium at a final concentration of 0,5 per cent. S. mitis 101, S. sanguis I 102 and S. sanguis II 103 all grew well on this medium.

The necessity of adding tryptone and yeast extract was investigated further because a basal medium was needed that contained a minimal amount of constituents which would interfere with starch metabolism and chemical analyses. S. mitis 101 was inoculated into 4 different broths containing either yeast extract, tryptone, yeast extract plus amylopectin or tryptone plus amylopectin. The broths were incubated at 37°C for 24 hours. Each broth was tested in triplicate, the amount of growth was determined turbidimetrically and a mean value was calculated.

Tryptone had little influence on growth whereas 1 per cent yeast extract supported adequate growth (Table 2,2). When amylopectin was added to the yeast extract there was a 17 fold increase in growth (Table 2,2). It was clear the yeast extract provided adequate nutrients for growth whereas tryptone did not appear to enhance growth and could be excluded from the medium.

The starch broths were modified further. According to Myrback (1926), Muus, Brockett and Connelly (1956), Walker and Whelan (1960) and Robyt and Whelan (1968a) chloride ions enhance alpha-amylase activity. These ions must be present at a concentration of at least 0,01M. Chloride ions were added to the broths to achieve this level. The final recipe for the medium used for testing growth, sugar and acid production can be found in the Appendix. It consisted of phosphate buffered saline, yeast extract, sodium chloride and either amylopectin or amylose.

Table 2.2 Growth of *Str. mitis* 101 on tryptone, yeast extract and amylopectin determined by calculating an increase in broth turbidity.

| mg % | Growth in F. T. U. units | | | |
|---|---|---|---|---|
| | tryptone | yeast extract | 0,5% amylopectin | |
| | | | tryptone | yeast extract |
| 2,5 | 4 | 6 | 20 | 62 |
| 1 | 3 | 2,3 | 16 | 51 |
| 0,75 | 2 | 1,8 | 12 | 39 |
| 0,5 | 1 | 1,6 | 9 | 33 |
| 0,25 | 0,5 | 0,4 | 6 | 23 |
| 0,1 | 0,1 | 0,3 | 4 | 16 |

1.3.2 Preparation and sterilization

Amylopectin and amylose are adversely affected by temperatures above 60° C (Greenwood, 1970; Collison, 1968). For this reason the broths in the study were sterilized with bacteriological filters. Three litres of yeast extract broth was pumped under pressure through a sterile Sartorius 25 G membrane filter with a pore size of 0,2 µm and a diameter of 142 mm. A litre was distributed into 4 sterile bottles using aseptic techniques and retained for use as the control medium which did not contain starch.

The amylopectin medium was prepared by adding 5 g of starch to 1 litre of the buffered yeast extract broth to which chloride ions had been added. The broth was heated to 60° C to facilitate dissolution of the carbohydrate and sterilized by filtration. The initial batch of amylopectin dissolved readily at 60° C but a subsequent batch was more insoluble and had to be dissolved by the method of McCready and Hassid (1943). A litre of broth was prepared by moistening 5 g of amylopectin with 20 ml of ethanol. Twenty ml of 10 per cent sodium hydroxide and 100 ml of yeast extract broth was added to the starch and the mixture was stirred until the amylopectin dissolved. The pH of the solution was adjusted to 7,5 with 10 per cent hydrochloric acid and the broth made up to 1 litre with the remaining yeast extract.

Amylose did not dissolve readily. After several unsuccessful attempts the method of McCready and Hassid (1943) described above was adopted. In addition a tissue homogenizer (Ultra turrax) was

used to agitate the amylose suspension for 10 minutes to help the starch dissolve. The medium was sterilized by passing the solution through a series of filters with pore sizes of 8 µm, 0,8 µm, 0,45 µm and 0,2 µm and distributing aseptically into sterile glass bottles.

1.3.3 Inoculation and incubation

The filter sterilized media were incubated overnight at 37°C to ensure no contamination had occurred. In addition the inoculation room was sterilized with formaldehyde gas before use to decrease the chance of contamination.

A fresh culture of streptococci was obtained by inoculating a blood agar plate with a pure culture. The plate was incubated overnight and a loopful of the growth was inoculated into 20 ml of Todd Hewitt broth (see Appendix). The broth was incubated for 18 hours and centrifuged for 10 minutes at 12 000 g. The supernatant was discarded and the cells washed twice in sterile potassium phosphate buffer pH 7,5 (Cruickshank, 1962). The microorganisms were resuspended in 10 ml phosphate buffered saline and 0,5 ml of this suspension was inoculated into 6 bottles, 2 of yeast extract, 2 of yeast extract and amylopectin and 2 of yeast extract and amylose. The bottles were sealed with plastic tape to prevent contamination and incubated at 37°C for 24 hours in a metabolic shaker.

There were 2 reasons for aerating the cultures in a metabolic shaker. Firstly, starch does not penetrate the deeper layers of plaque (Grenby, 1967; Frostell and Bauer, 1971a; Mormann and Muhlemann, 1981; Newbrun, 1982) and would be metabolized on the surface where aerobic conditions are present. Secondly, Evans and coworkers (1979) showed oxygen is necessary for the production of polysaccharides.

Each streptococcal isolate was cultured on yeast extract broth, yeast extract broth plus 0,5 percent amylopectin and yeast extract broth plus 0,5 percent amylose. In addition the isolates from each subject were mixed and cultured on these 3 media. The broths were plated on blood agar plates after incubation to ensure the streptococci had grown and no contamination had occurred.

1.3.4 Sampling of broths

Samples of broth were taken before and during incubation to measure growth and the production of acids and sugars. A 5 ml sample was taken aseptically before incubation and again at 2 hourly intervals for the first 8 hours and after 24 hours. The metabolic activity and growth of microorganisms was stopped by adding 1mg NaF to each ml of broth. The pH and turbidity of these samples were measured and they were stored at -20$^{o}$C for later analyses.

### 1.3.5 Measurement of growth in broths

The opacity of the inoculated broth samples was measured on a Hach turbidometer Model 2100A against Formazine turbidity unit standards (FTU). Growth can be measured by reading changes which have occurred in the opacity of a bacterial suspension. The amount of light scattered by a broth culture is directly proportional to either the dry weight or number of bacteria in suspension (Meynell and Meynell, 1970; Wilson and Miles, 1975). Growth of the bacterial cultures was determined by measuring the increase in turbidity produced in yeast extract, in amylose and in amylopectin broths over a period of 24 hours.

## 2 ACID PRODUCTION FROM AMYLOSE AND AMYLOPECTIN

Two stages in the production of acid from amylose and amylopectin were studied. The first was the degradation of starch to glucose and glucose oligomers and the second the production of acid from these sugars.

### 2.1 Production of glucose and reducing sugars

The samples were tested for the presence of glucose and reducing sugars before inoculation and after 24 hours incubation. All samples were tested in duplicate.

#### 2.1.1 Fehling's test for reducing sugars

One ml samples of the broths were pipetted into 20 ml test tubes. Equal volumes of Reagents A and B (see Appendix) were mixed and 1 ml of the mixture was added to the test solutions. The tubes were placed in a 95°C waterbath and heated for 10 minutes, removed and cooled in water for 1 min. Immediately 3 ml of the phosphomolybdic acid solution (see Appendix) was added, the tubes returned to the waterbath, heated for 1 min, removed and cooled in water. The absorbance of the reduced sugars was read against a reagent blank on a Varian Techtron 635 spectrophotometer set at 420 nm, the wavelength of maximum absorbance.

A standard graph of the absorbance of known concentrations of glucose was drawn up using the Fehlings test (Figure 2,3). The absorbance obtained from the yeast extract and starch broths was compared to this graph to determine the amount of reducing sugars in the broths. Duplicate 50 mg glucose standards were included in every assay to ensure the results were reproducible.

2.1.2 The glucose oxidase test

The glucose enzymatic colorimetric test-GOD-PAP (Boehringer Mannheim) was used to detect glucose. Glucose oxidase catalyses the oxidation of glucose with the formation of gluconic acid and hydrogen peroxide. The hydrogen peroxide formed in this reaction reacts 4-aminophenazone and 2,4-dichlorophenol in the presence of peroxidase to form antipyrylchloroquinone imine. According to Barham and Trinder (1972) the amount of dye formed during the test is proportional to the glucose concentration.

The reagent was made up in double distilled water according to the manufacturer's instructions and stored at 4°C in the dark. Two ml of the reagent were added to 0,1 ml of test solution. The tubes were placed in a waterbath at 37°C and incubated for 30 minutes The absorbance of the dye was read on a Varian Techtron 635 spectrophotometer at 510 nm, the wavelength of maximum absorbance.

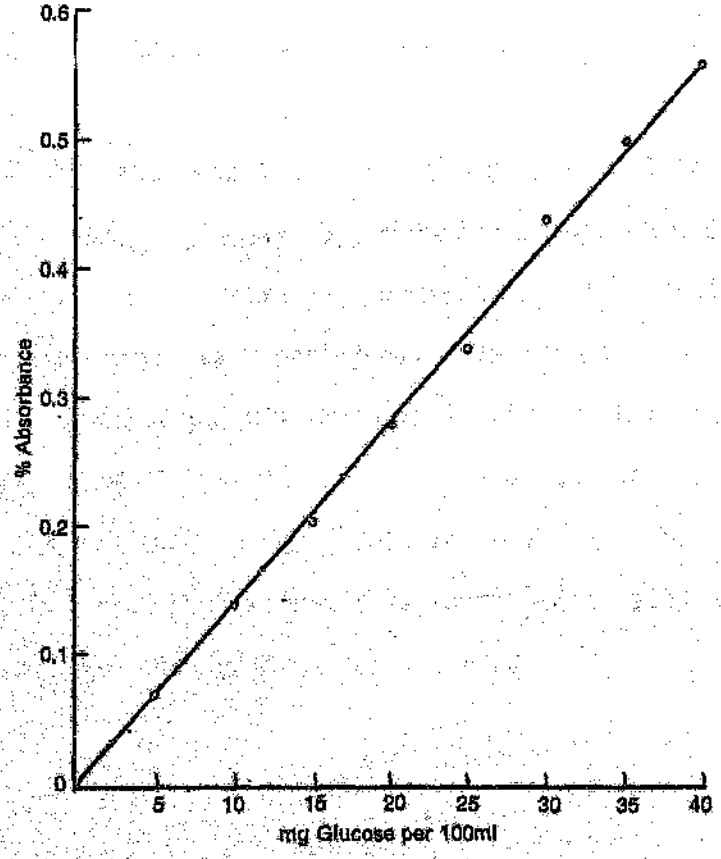



Figure 2.3 Standard graph of known concentrations of glucose Glucose was reduced with the Fehlings reagents and the absorbance was read at 420 nm, the wavelength which gave a maximum reading. The graph was obtained by plotting the concentration of glucose against absorbance.

A standard graph of the absorbance of known concentrations of glucose was drawn up using the glucose oxidase test (Figure 2,4). The amount of glucose in the yeast extract, amylose and amylopectin broths was determined by comparing the absorbance obtained from the broths with the standard graph. Duplicate 50 mg glucose per 100 ml standards were included in every batch to ensure accurate results were obtained.

A comparison of the results from the glucose oxidase and Fehlings tests revealed more glucose than reducing sugars were detected. The problem was solved by removing amylose and amylopectin before the glucose oxidase test was performed. The amylose was retrograded by freezing and thawing and the amylopectin was precipitated by adding 1 ml of ethyl alcohol to 0,1 ml of sample. The suspensions were centrifuged at 12 000 g for 10 minutes and the supernatant was used for the test. The results from this method were satisfactory because the glucose oxidase and Fehlings tests agreed.

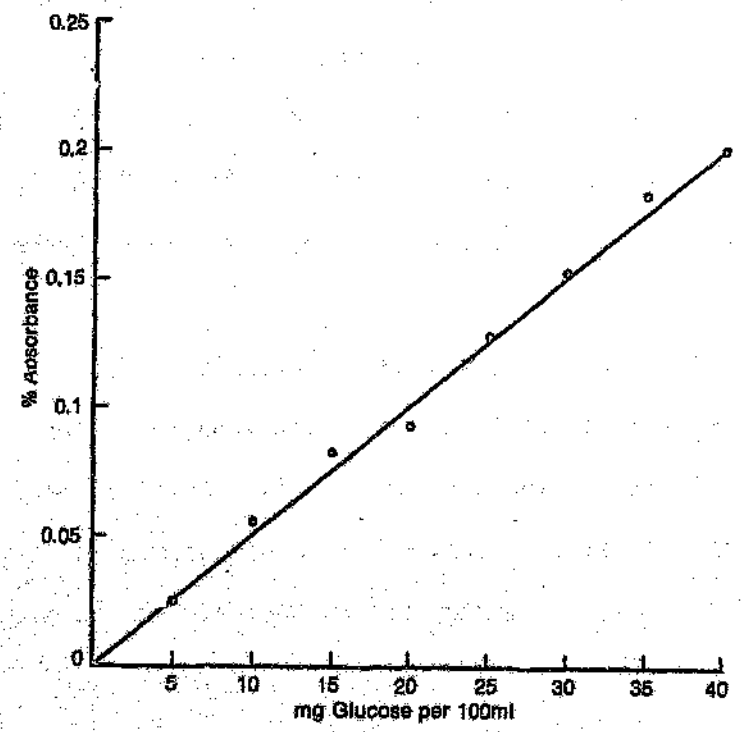



Figure 2.4 Standard graph of known concentrations of glucose. Glucose was reacted with the GOD-PAP(Boehringer Mannheim) reagent. The absorbance of the dye antipyrylchloroquinone which is formed during the reaction was read at 510 nm, the wavelength of maximum absorbance. The graph was obtained by plotting the concentration of glucose against absorbance.

2.1.3 Ion chromatography.

Pure cultures of the oral isolates of streptococci that showed an increase of more than 20 mg reducing sugar per 100 ml of amylose and amylopectin broth after 24 hours incubation were investigated further. The broths were analysed for the presence of glucose and glucose oligomers on a Dionex 2000i ion chromatograph. The species were separated on a HPIC-AS6 separator at 34°C with an eluent of 0,15 M NaOH and 0,15 M NaOAc. The sugars were detected with a Triple-pulse Pulsed Amperometric detector fitted with a gold working electrode.

Standards of glucose, maltotriose, maltotetraose, maltopentaose, maltohexaose were used to calibrate the column (Figure 2.5). The [illegible] ose oligomers was confirmed by comparison of the retent[illegible] in the samples with those of the standard chromatogram.

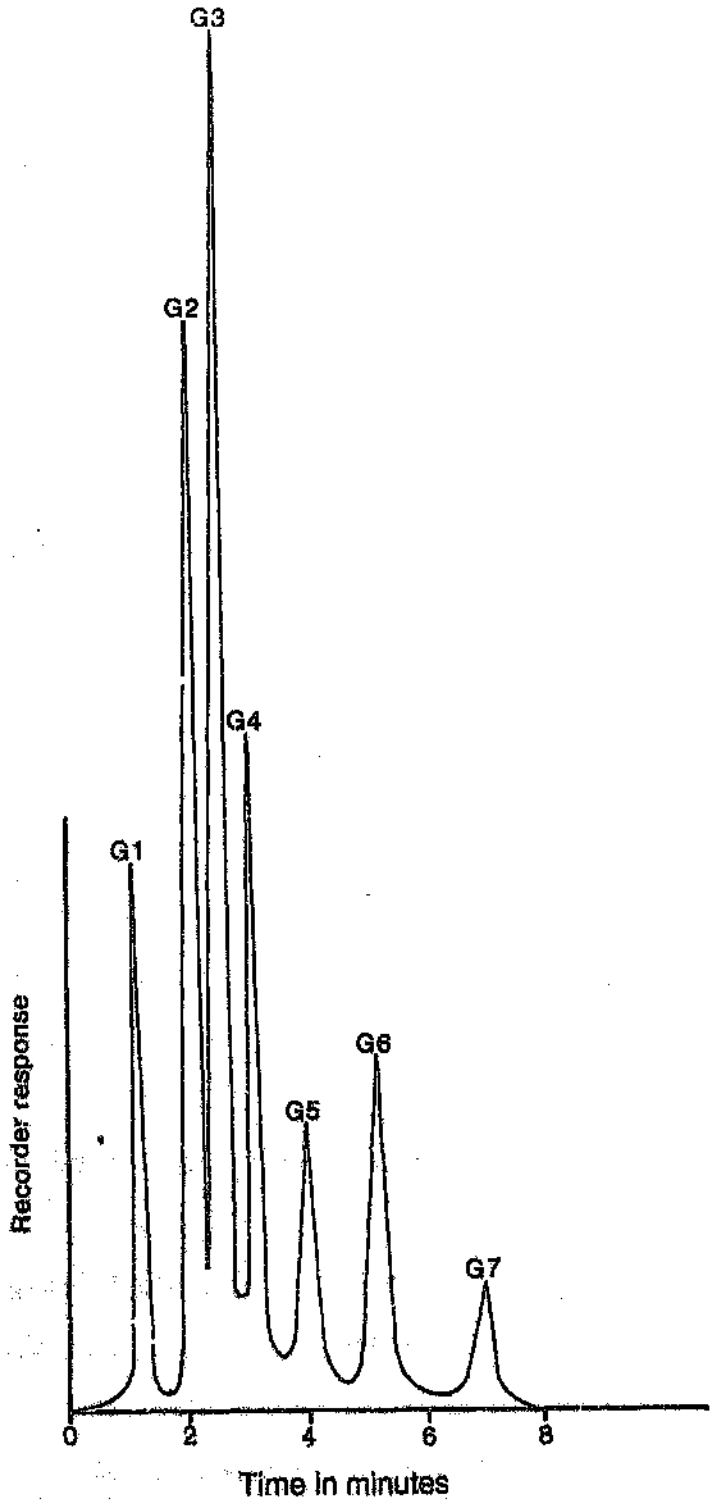



**Figure 2.5** Ion chromatogram of glucose oligomer standards
$G_1$ = glucose (3 mg%), $G_2$ = maltose (7 mg%)
$G_3$ = maltotriose (10 mg%), $G_4$ = maltotetraose (6 mg%)
$G_5$ = maltopentaose (3 mg%), $G_6$ = malthohexoase (6 mg%)
$G_7$ = maltoheptaose (3 mg%)

## 2.2 Production of acids

### 2.2.1 Change in pH

The pH was measured on a Beckman Zeromatic SS 3 pH meter fitted with a combination electrode. The instrument was calibrated with two standard solutions at pH 5 and 7.

### 2.2.2 Identification of acids

The production of acids was determined by testing 1 ml samples of yeast extract, amylose and amylopectin broths before inoculation and after inoculation and 24 hours incubation. The acids were detected by gas chromatography and ion exclusion chromatography. Acid production was calculated by subtracting the amount of acid in the broths before incubation from the amount after 24 hours incubation.

#### 2.2.2.1 Gas liquid chromatography

The method described by Holderman et al, (1977) was used for detecting acids. Volatile acids were extracted directly with ether whereas nonvolatile acids were first methylated. A 1 µl sample was injected into a Hewlett Packard 5710 A gas chromatograph fitted with a SPS 1000 column. The temperature of the detector was set at 300 °C and the injector temperature at 200 °C. The acids were identified by comparing their retention times with those of authentic samples. Their identity was confirmed by mass spectrometry.

2.2.2.2 Ion exclusion chromatography

The acids were also detected by ion exclusion chromatography with a Dionex 2000i ion chromatograph. Samples were diluted 10 fold in deionized water and 50 µl were separated at room temperature on a HPICE-AS1 (9 by 200 mm) Ion Exclusion separator column with 1mM HCl as eluent. Effluent from the separator column was passed into a silver form suppressor column where the chloride was precipitated as the silver salt thus converting the high conductivity HCl eluent to low conductivity water. The organic acids passed through the suppressor column without modification and were measured by conductivity detection. Individual acids were identified and quantified by the addition of measured amounts of known standards. The production of acetic, lactic and succinic acid was confirmed by comparison of the chromatograms with acid standards (Figure 2.6).

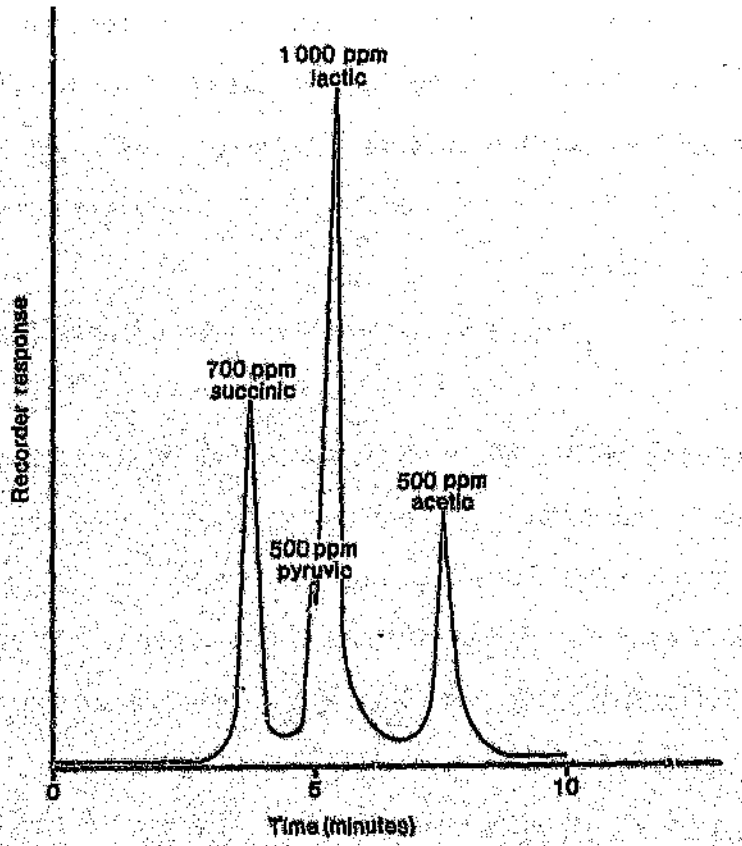



Figure 2.6 Ion chromatogram of standard acids

# 3 POLYSACCHARIDE PRODUCTION FROM STARCH

## 3.1 Production of polysaccharide

The production of polysaccharide was determined by examining the cultures grown in yeast extract broth containing either amylopectin or amylose for production of a gelatinous residue which attached readily to glass.

## 3.2 Isolation of extracellular enzymes

S. sanguis 1 MC 204 produced more polysaccharide from amylopectin than the other strains and for this reason was grown on a special medium to isolate the extracellular polysaccharide producing enzymes. A loopful of bacterial growth obtained from a blood agar plate was inoculated into 250 ml of a chemically defined medium (Janda and Karumitsu, 1976). The broth was incubated at 37°C for 48 hours, centrifuged at 12 000 g and the cells were separated from the supernatant. The cells were inoculated into 100 ml of sterile phosphate buffered saline pH 7,2 containing 0,5 percent amylopectin and 0,01 M sodium chloride. The supernatant which contained extracellular enzymes was passed through a sterile filter with a pore size of 0,2 µm to remove any remaining cells. The suspension was mixed with 250 ml of sterile phosphate buffered saline pH 7,2 containing 1 percent amylopectin and 0,01 M sodium chloride. The cell and enzyme suspensions were incubated in a metabolic shaker at 37°C for 72 hours and examined for polysaccharide production

## 3.3 Isolation of polysaccharide

The insoluble polysaccharide produced from amylopectin by *S. sanguis* 1 MC 204 was prepared from a cell free enzyme suspension of the chemically defined medium of Janda and Karumitsu (1976) containing amylopectin (see Section 3.2). The amylopectin broth was divided into 100 ml amounts, distributed into sterile Boston round bottles and incubated at 37°C for 5 days. After incubation the broth was decanted into sterile bottles and the layer of material which adhered to the glass was removed and the suspension was centrifuged at 12 000 g for 20 minutes. The polysaccharide was washed 4 times in distilled water and dried in a desiccator over phosphorus pentoxide. This procedure was repeated 6 times on the same litre of broth.

A further soluble polysaccharide was obtained from the amylopectin broth. Amylopectin was precipitated by adding 50 ml ethanol to 100 ml of the broth. The broth was centrifuged at 12 000 g for 20 minutes and 20 ml ethanol was added to the supernatant. The suspension was centrifuged at 12 000 g for 20 minutes, the supernatant discarded, then the precipitate washed in several changes of 70 percent alcohol and dried in a desiccator over phosphorus pentoxide.

The insoluble dextrans produced from sucrose by *Streptococcus mutans* ATCC 25175 (mutan) and *S. sanguis* 1 MC 204 (dextran I) were prepared using the method described by Nisizawa *et al* (1976).

## 3.4 Chemical characteristics of polysaccharides

### 3.4.1 Solubility

The solubility of amylopectin, of the insoluble and soluble polysaccharides from amylopectin and of mutan produced from sucrose by S. mutans were tested in 5 percent boric acid, 1 N acetic acid, 1 M potassium hydroxide, ethyl alcoh ', dimethyl sulphoxide, dimethyl formamide, n methyl pyrrolidone and diethyl propylene urea.

### 3.4.2 Iodine staining

The iodine staining characteristics of mutan, the soluble and the insoluble polysaccharides produced from opectin were determined (see appendix) and compared with those produced by amylopectin and amylose using the method of McCready and Hassid (1943).

### 3.4.3 Infra-red spectra

The water soluble and insoluble polysaccharides from amylopectin were subjected to infra-red spectrophotometry. They were compared to the water insoluble dextran produced from sucrose by the same strain of S. sanguis 1 and with the mutan produced by S. mutans ATCC 25175. The samples were dried over phosphorus pentoxide and suspended in a potassium bromide pellet. The spectra were read on a Jasco A-202 Infra-red Spectrophotometer.

### 3.4.4 Methylation analysis

The water soluble and insoluble polysaccharides from S. sanguis 1 204, amylopectin, amylose and maltose were dried in a desiccator for 24 hours in the presence of phosphorus pentoxide. The material was transferred to McCartney bottles, methylated, hydrolysed, reduced and acetylated using the method described by Harris et al, (1984). The insoluble polysaccharide was ground into a fine powder by means of a pestle and mortar in an effort to improve the solubility of this sample. In addition the method for the methylation of insoluble samples was used (Harris et al, 1984). The preliminary methylation step was repeated 3 times and the sample was heated to 60°C for 60 minutes before proceeding with the methylation. Myo-inositol was added to each sample after methylation but before acetylation and served as an internal standard.

The resultant alditol acetates were analysed by gas chromatography-mass spectroscopy. A Carlo Erba Fractovap Series 4200 gas chromatograph fitted with a SE 30 column was used. The carrier gas was hydrogen. The oven temperature was held at 40°C for 3 minutes, raised by 4°C per minute to 100°C and then raised by 8°C to 250°C. The identity of each peak was confirmed by mass spectrometry-gas chromatography on a Micromas 16F mass spectrometer. The ion source was set at 220°C and the electron energy level at 70 electron volts. The spectra were identified by comparison with the mass spectra in the NBS Library Search. The molar percentages of the glucitol acetates were determined by comparing the areas of the peaks from the gas chromatographs.

### 3.4.5 Enzymic analysis

#### 3.4.5.1 Dissolving the polysaccharides

Amylopectin and the soluble polysaccharide produced from amylopectin by S. sanguis I 204 were prepared for enzyme analysis by moistening 25 mg of the sample with 0,3 ml ethyl alcohol, adding 1 ml 10 percent potassium hydroxide and stirring with a magnetic stirrer. Previous unpublished studies by the author showed the amyloglucan from S. sanguis I 204 did not dissolve readily. In an effort to overcome this problem the polysaccharide was ground to a fine powder in a pestle and mortar, suspended in the alcohol alkali mixture, heated to 60°C for 1 hour and stirred for 1 week. This method was not entirely satisfactory because some of the amyloglucan did not dissolve. No solution to this problem could be found.

#### 3.4.5.2 Debranching with isoamylase

Isoamylase was used to determine the lengths of A and B chains and the ratio of A to B chains. The pH of the dissolved starches was adjusted to 3,6 with 18 M acetic acid and the solution was made up to 5 ml with 100 mM acetate buffer pH 3,6. The starches were debranched with 1,5 units isoamylase from Pseudomonas amyloderamosa ATCC 21262 (Hayashibara Biochemical Laboratories) for 18 hours at 37° C. The debranched polysaccharides were separated by chromatography on a Sephadex G-50 column 100 cm by 1,5 cm with degassed distilled water as the eluent. Fractions of the

eluent were collected in a fraction collector and analysed for reducing sugars using phenol-sulphuric acid (Dubois et al, 1956).

3.4.5.3 Digestion with amylases

The digestion of amylopectin, amyloglucan and soluble polysaccharide by alpha-amylase and beta-amylase was tested. Ten mg samples of the polysaccharides were prepared for each enzyme as described in Section 3.4.5.1. The pH of the susupensions for testing alpha-amylase activity was adjusted to 7,4 with 10 M hydrochloric acid and made up to 3 ml with 0,03 M potassium phosphate buffer pH 7,4 containing 5 mM calcium chloride. The solutions for determining the beta-amylolysis limit were adjusted to pH 4,8 with 18 M acetic acid and made up to 3 ml with 50 mM acetate buffer pH 4,8.

One mg of alpha-amylase obtained from Bacillus subtilis (Boehringer Mannheim GmbH), 60 unit of beta-amylase from Ipomoea batatas the sweet potato (Boehringer Mannheim GmbH) was added to the suspensions. The tubes were incubated at 37°C for 18 hours and the enzyme action stopped by heating in boiling water for 1 minute. The undigested suspensions and the digests were tested for reducing sugars with the Fehlings reagent (see Appendix). The undigasted suspensions were also tested for total carbohydrate by the phenol-sulphuric acid method (Dubois et al, 1956).

# 4 ENZYMES THAT REGULATE STARCH METABOLISM

## 4.1 ISOLATION AND EXTRACTION

Crude enzyme extracts were prepared from each of the 21 strains of streptococci using a modification of the method described by Walker and Builder (1971). A loopful of growth from a fresh blood agar plate was inoculated into 250 ml of the chemically defined medium of Janda and Karumitsu (1976) which contained 0,5 percent glucose and 0,005 M calcium chloride. The cultures were incubated at 37°C for 72 hours in order to obtain a heavy suspension of enzyme producing cells. Strains which produced minimal amounts of enzyme were cultured on the same broth with 0,5 percent amylopectin as the sole carbon source and incubated at 37°C for 5 days. According to Foster (1965) these conditions increase amylolytic enzyme production.

The cells were removed by centrifugation at 12 000 g for 30 minutes. Ammonium sulphate was added to the supernatant while stirring to give 45 percent saturation (Wood, 1976). The suspension was allowed to stand for one hour and centrifuged at 27 000 g for 10 minutes. The precipitate was discarded and additional ammonium sulphate added to the supernatant to give 72 percent saturation. The suspension was allowed to stand for 18 hours at 4° C and centrifuged at 27 000 g for 10 minutes. The precipitate was dissolved in 5 ml 0,03 M phosphate buffer pH 7,2 containing 0,005 M calcium chloride and dialysed against the same buffer for 24 hours to remove the ammonium sulphate.

## 4.2 IDENTIFICATION OF ENZYMES

### 4.2.1 Incubation of enzyme extracts

Conditions that would accommodate most starch degrading enzymes were selected. The choice of buffer was critical because alpha-amylase is inhibited by citrate and phosphate buffers and pululianase by citrate, phosphate and Tris-buffer (Robyt and Whelan, 1968a; Marshall, 1974). These buffers are inhibitory because they bind $Ca^{2+}$ required for enzyme activity. In addition, alpha-amylase needs $Cl^-$ for activity (Robyt and Whelan, 1968a). To meet these requirements 0,03 M potassium phosphate buffer containing 0,005 M $CaCl_2$ was used.

The optimal temperature and pH for starch hydrolysing enzyme activity varies for each enzyme (Dunican and Seeley, 1962; Boyer and Hartman, 1971; Robyt and Whelan, 1968a; Kainuma et al, 1972; Norman, 1979; Stavn and Granum, 1979; Fogarty and Kelly, 1980). The optimal pH lies between 5 and 8,5 and the optimal temperature between 20° and 60° C. A temperature of 37°C and a pH of 7,2 was chosen because these are optimal for the growth of streptococci.

A 0,5 percent solution of amylose and amylopectin was prepared in 0,03 M potassium phosphate buffer pH 7,2 containing 0,005 M calcium chloride according to the methods described by McCready and Hassid (1943). Five ml of the crude enzyme extract were added to 25 ml of these starch solutions. The enzymes were allowed to act for 10 minutes at 20°C. Ten ml of the solution were removed, inactivated by plunging into boiling water for one minute and stored at -20°C.

The remaining solution was incubated at 37°C for 24 hours, similarly inactivated and frozen. The suspensions were heated to room temperature and centrifuged at 27 000 g to remove the enzymes and retrograded starches. The supernatant which contained the oligosaccharides was freeze dried.

4.2.2 Identification of the degradation products

4.2.2.1 Thin layer chromatography

The thin layer chromatographic method of Hansen (1975) was used for the identification of oligosaccharides formed from starch. Silica gel 60 F254 HPTLC plates measuring 10 cm by 10 cm were activated at 105°C for one hour, removed and stored in a desiccator until required. The silica gel surfaces of the plates were marked with a pencil 0,5 cm from one edge to identify the origin. One µl samples of the amylose and amylopectin suspensions were applied to the marks on the plate. The plates were developed in isopropanol : acetone : 1 M lactic acid (2:2:1) until the solvent front reached the upper edge of the plates. The plates were removed, dried in a stream of hot air and visualised with aniline diphenylamine reagent (Sigma).

For comparative purposes standards of glucose, maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose and maltoheptaose (Sigma) were included in each plate.

#### 4.2.2.2 High performance liquid chromatography

High performance liquid chromatography was used to determine the amount of oligosaccharides produced by the crude enzyme extract. The freeze dried samples were dissolved in 1 ml distilled water and filtered through a Millipore filter with a pore size of 0,45 µm. The oligosaccharides were separated on a Waters Sugar-pak column using degassed reagent quality (RQ) water as the mobile phase. The sugars were detected with a Waters 401 refractometer. Standards of glucose, maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose and maltoheptaose were used to calibrate the column.

## 4.3 MOLECULAR WEIGHT OF ENZYMES

### 4.3.1 Gel electrophoresis

The molecular weight of the enzyme were studied using a crude extract. The extracts were separated by sodium dodecyl sulphate polyacrylyamide gel electrophoresis (SDS-PAGE). Molecular weight markers were included in the gel to determine the size of the enzyme. Further information was gained by incubating the gel in a buffer containing sucrose and studying the products formed. After incubation the gel was stained with the periodic acid Schiff stain (PAS). Soluble glucans with predominantly alpha-1,6-linkages stain purple, water insoluble alpha-1,3 glucans are insensitive to the stain whereas fructans stain pink (Mukasa, 1986). Fructan-synthesizing enzymes were distinguished from glucan-producing enzymes by their ability to utilize raffinose and produce bands in the presence of this sugar (Russell, 1979).

4.3.1.1 Sample preparation

Gel electrophoresis was carried out on the enzymes which produced the most oligosaccharides from starch. S. mitis 101 and S. sanguis 1 204 were grown on the chemically defined medium of Janda and Kuramitsu (1976) which contained glucose as the sole carbon source. A fresh crude enzyme extract was prepared from the broth as described in Section 4.1.

4.3.1.2 Electrophoresis

SDS-PAGE was performed using the system described by Laemmli (1970) and modified by Russel (1976, 1979). All the solutions were prepared immediately before use because enzyme activity was inhibited by solutions that were allowed to stand for more than a week. A 5 percent stacking gel and a separating gel containing 8 percent acrylamide and 0,5 percent amylopectin were used (see Appendix).

The amount of protein in the enzyme extract was determined by the method of Bradford (1976). Samples were suspended in distilled water to give a protein concentration of approximately 700 µg per ml. Forty µl of these suspensions were mixed with 40 µl of sample buffer (see Appendix) and heated to 45°C for 90 minutes. These conditions were chosen because the activities of the enzymes were destroyed above 45°C and heating for 90 minutes resulted in compact protein bands. After heating 40µl of the samples were applied to the gels.

Molecular weight markers for the determination of polypeptides in the range 20 000 to 340 000 Da (Combithek, Boehringer Mannheim) were prepared according to the manufacturers instructions. They included alpha-2-macroglobulin (Mr 170 000, reduced), phophorylase b (Mr 97 400), glutamate dehydrogenase (Mr 55 400), lactate dehydrogenase (Mr 36 500) and trypsin inhibitor (Mr 20 100). A further standard containing Bacillus subtilis alpha-amylase (Mr 48 700) was included to indicate amylopectin hydrolysis. It was prepared at a concentration of 0,005 µg per ml because higher concentrations of the enzyme hydrolysed amylopectin extensively.

Electrophoresis was carried out at a constant current of 36 mA until the bromophenol blue ran off the gel. After electrophoresis the gel was washed by shaking in several changes of 0,05 M phosphate buffer pH 6.5 and incubated at 37°C for two days in buffer containing either 5 percent sucrose, 0,5 per cent amylopectin or 5 per cent raffinose (see Appendix). After incubation the gel was stained with periodic acid Schiff stain (PAS). Thereafter, the stained gel was fixed in 30 percent ethanol overnight and stained with silver stain to visualize the protein markers (see Appendix).

# 5 STATISTICAL ANALYSIS

Growth, change in pH and the production of acids were compared in cultures from subjects with healthy teeth and carious lesions. An analysis of variance was undertaken using an IBM 370/158 computer and the Statistical Analysis System (SAS Institute, 1982). A General Linear Models procedure for a nested analysis of variance was used because the streptococci were treated as randomly chosen subgroups, while the source of the isolates, healthy or carious teeth, were considered as main groups. When significant differences between the main groups were detected, they were considered significant above any differences that may have existed in the streptococci.

# CHAPTER 3

# RESULTS

## CONTENTS

1 GROWTH ON AMYLOSE AND AMYLOPECTIN.....123

2 ACID PRODUCTION FROM STARCH.....132

2.1 PRODUCTION OF GLUCOSE AND REDUCING SUGARS.....132

2.1.1 Changes in yeast extract.....132
2.1.2 Changes in amylopectin and amylose.....132

2.2 PRODUCTION OF ACIDS.....137

2.2.1 Change in pH.....137
2.2.2 Identification of acids.....141

3 POLYSACCHARIDES FROM STARCH.....147

3.1 PRODUCTION OF GLUCANS.....147

3.2 CHEMICAL CHARACTERISTICS OF THE GLUCANS.....149

3.2.1 Adherence and solubility.....149
3.2.2 Iodine staining.....151
3.2.3 Infra-red spectrum.....151
3.2.4 Methylation analysis.....153
3.2.5 Enzymic analysis.....162
3.2.5.1 Debranching with isoamylase.....162
3.2.5.2 Digestion by amylases.....164
3.2.6 Conclusion.....165

4 ENZYMES THAT REGULATE STARCH METABOLISM.....166

4.1 IDENTIFICATION OF ENZYMES.....166

4.1.1 Products of amylopectin degradation.....166
4.1.2 Products of amylose degradation.....168
4.1.3 Molecular weight of enzyme.....170
4.1.4 Conclusion.....171

LIST OF ILLUSTRATIONS

TABLES

Table 3.1 Mean growth values determined by calculating.....127
an increase in broth turbidity. Streptococci were
grown in the broths at 37 C for 24 hours. Turbidity
readings were compared using a general linear models
procedure for a nested analysis of variance.

Table 3.2 The effect of amylopectin and amylose on the.....130
growth of streptococci. Growth was either inhibited (-)
stimulated (+) or unaffected (0).

Table 3.3 Changes in the amount of glucose and reducing.....131
sugars detected in broths inoculated with streptococci
and incubated at 37 C for 24 hours.

Table 3.4 Glucose and glucose oligomers detected in.....134
broths inoculated with streptococci and incubated at
37 C for 24 hours.

Table 3.5 Mean change in mg in the amount of glucose.....135
and reducing sugars detected in broths inoculated
with streptococci and incubated at 37 C for 24 hours.
The amount of sugars either decreased (-) or increased (+).
Changes were compared using a general linear models
procedure for a nested analysis of variance.

Table 3.6 Mean change in mg in the amount of glucose.....136
and reducing sugars detected in broths inoculated with
streptococci which grew either vigorously or poorly.
The amount of sugars either decreased (-) or increased (+).
Changes were compared using a general linear models
procedure for a nested analysis of variance.

Table 3.7 Mean changes in pH of broth inoculated with.....142
streptococci and incubated at 37 C for 24 hours. The
pH was compared using a general linear models procedure
for a nested analysis of variance.

Table 3.8 Acids detected in broths inoculated with.....143
streptococci and incubated at 37 C for 24 hours.

Table 3.9 Mean mmol acids detected in broths inoculated.....145
with streptococci and incubated at 37 C for 24 hours.
Acid production was compared using a general linear
models procedure for a nested analysis of variance.

Table 3.10 The presence of insoluble polysaccharide.....148
and dextran in broths inoculated with starch
hydrolysing streptococci.

Table 3.11 Adherence to glass, solubility and iodine.....150
staining characteristics of amylose, amylopectin,
insoluble and soluble polysaccharide produced from
amylopectin by S. sanguis 1 204 and insoluble mutan
produced from sucrose by S. mutans ATCC 25175.

Table 3.12 Molar percentages of partially methylated.....159
glucitol acetates obtained during the methylation analysis
of amylopectin, amyloglucan and the soluble glucan

Table 3.13 Comparison of the chemical properties of.....161
amylose, amylopectin, amyloglucan and the soluble glucan
produced from amylopectin by S. sanguis 1 204.

Table 3.14 Glucose and glucose oligomers produced.....167
from amylopectin by streptococcal enzymes.

Table 3.15 Glucose and glucose oligomers produced.....169
from amylose by streptococcal enzymes.

FIGURES

Figure 3.1 Changes in the turbidity of uninoculated.....124
broths incubated at 37 C for 24 hours. Turbidity was
measured in formazine units.

Figure 3.2 The growth of S. sanguis 1 W 213 in.....124
broths incubated at 37 C for 24 hours. Growth was
determined by measuring an increase in turbidity of
the broths in formazine turbidity units.

Figure 3.3 Growth of streptococci from healthy teeth.....125
in broths incubated at 37 C for 24 hours. Growth was
determined by measuring an increase in turbidity of the
broths in formazine turbidity units.

Figure 3.4 Growth of streptococci from carious lesions.....126
in broths incubated at 37 C for 24 hours. Growth was
determined by measuring an increase in turbidity of the
broths in formazine turbidity units.

Figure 3.5 Changes in the pH of broths inoculated with.....138
S. sanguis 1 FW 213 and incubated at 37 C for 24 hours.

Figure 3.6 Changes in the pH of broths inoculated with.....139
pure and mixed cultures of streptococci from healthy teeth
and incubated at 37 C for 24 hours.

Figure 3.7 Changes in the pH of broths inoculated with.....140
streptococci from carious lesions and incubated
at 37 C for 24 hours.

Figure 3.8 Infra-red absorption spectrum of amylopectin.....152
amyloglucan, a soluble polysaccharide produced from
amylopectin by S. sanguis 1 204, the dextran produced
from sucrose by S. sanguis 1 204 and the mutan produced
from sucrose S. mutans ATCC 25175.

Figure 3.9 Gas chromatographs of the alditol acetates.....154
formed by the methylation of maltose and glycogen.

Figure 3.10 Gas chromatographs of the alditol acetates.....155
formed by the methylation of amylopectin and amylose.

Figure 3.11 Gas chromatographs of the alditol acetates.....156
formed by the methylation of amyloglucan and the soluble
polysaccharide produced from amylopectin.

Figure 3.12 Mass spectra of the alditol acetates formed.....157
by the methylation of maltose, glycogen, amylopectin,
amylose, amyloglucan and the soluble polysaccharide
produced from amylopectin.

Figure 3.13 Fractionation products of debranched.....163
amylopectin, amyloglucan and the soluble polysaccharide
produced from amylopectin by S. sanguis 1 204. The
polysaccharides were debranched with isoamylase and
the fractions were separated on a Sephadex G-50 column.

Figure 3.14 Photograph of SDS gel electrophoresis of a.....172
crude enzyme extract os S. sanguis 1 204.

Figure 3.15 High magnification of SDS gel stained with.....172
periodic acid Schiff stain.

1 GROWTH ON AMYLOSE AND AMYLOPECTIN

The streptococci were grown on amylose and amylopectin to test their ability to produce enzymes that enabled them to utilize these starches as a source of nutrients. Growth was measured by determining an increase in turbidity of the broths after incubation. A comparison of the turbidity readings of the uninoculated broths with the broths containing streptococci showed all the cultures grew in yeast extract broth and broth containing amylopectin and amylose after 24 hours incubation (Figures 3.1, 3.2, 3.3 and 3.4).

A statistical comparison using a nested analysis of variance confirmed there was no difference between the growth of pure cultures and mixtures of these isolates. Furthermore the analysis showed the growth of the mixed cultures did not depend upon the growth of the individual isolates making up these mixtures (Table 3.1). Therefore mixed cultures were treated as individual isolates for the remainder of the analysis.

An examination of the growth curves after 24 hours incubation showed 5 different patterns. Firstly, S. intermedius 411 did not grow well in amylopectin broth and showed a decrease in turbidity after 24 hours incubation. Secondly, a third of the cultures showed a gradual increase in growth in yeast extract, amylopectin and amylose broth with a maximum turbidity of 50 FTU. This pattern was produced by S. anginusus 307, S. mitis 514 and 206, S. sanguis II 309, the mixtures 3 and 5 and all the cultures from subject 6 (Figure 3.3 and 3.4).

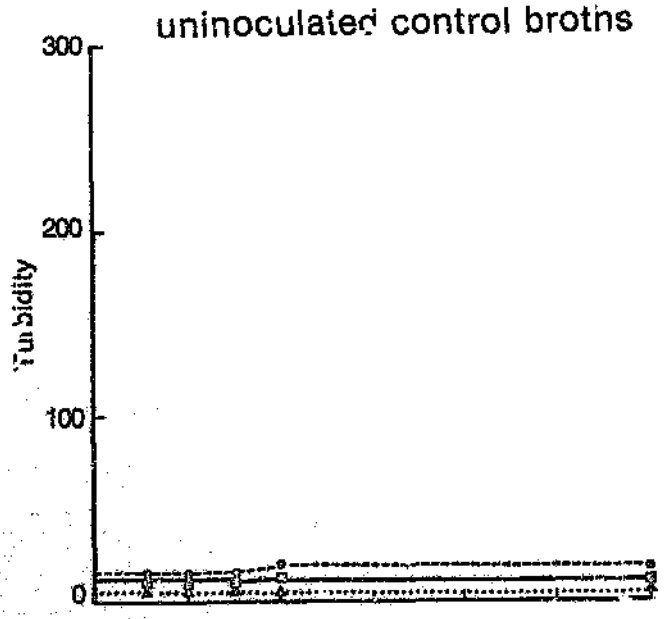



Figure 3.1 Changes in the turbidity of uninoculated yeast extract broth (△·····△), yeast extract broth containing 0,5 percent amylopectin (□——□) and yeast extract broth containing 0,5 per cent amylose (○----○). The broths were incubated at 37 C for 24 hours. Turbidity was measured in formazine units.

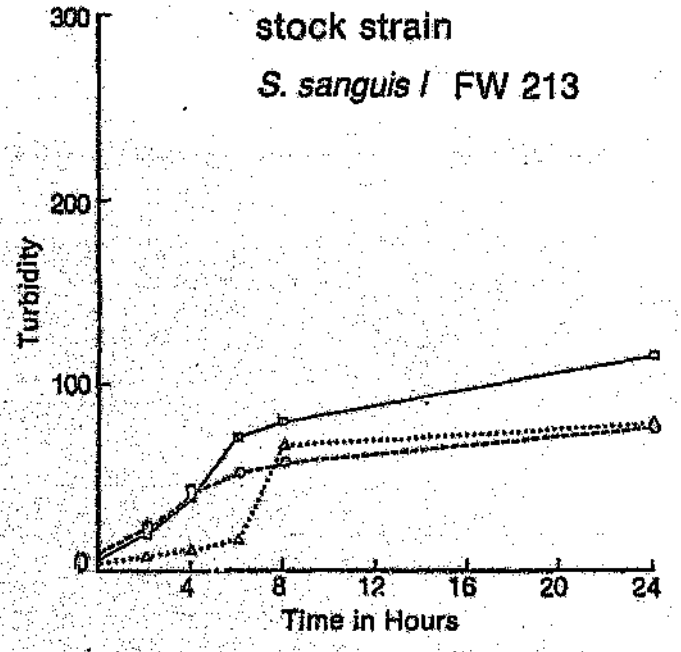



Figure 3.2 The growth of S. sanguis I FW 213 in yeast extract broth (△····△), yeast extract broth containing 0,5 per cent amylopectin (□——□) and yeast extract broth containing 0,5 per cent amylose (○----○). The broths were incubated at 37 C for 24 hours. Growth was determined by measuring an increase in turbidity of the broths in formazine turbidity units.

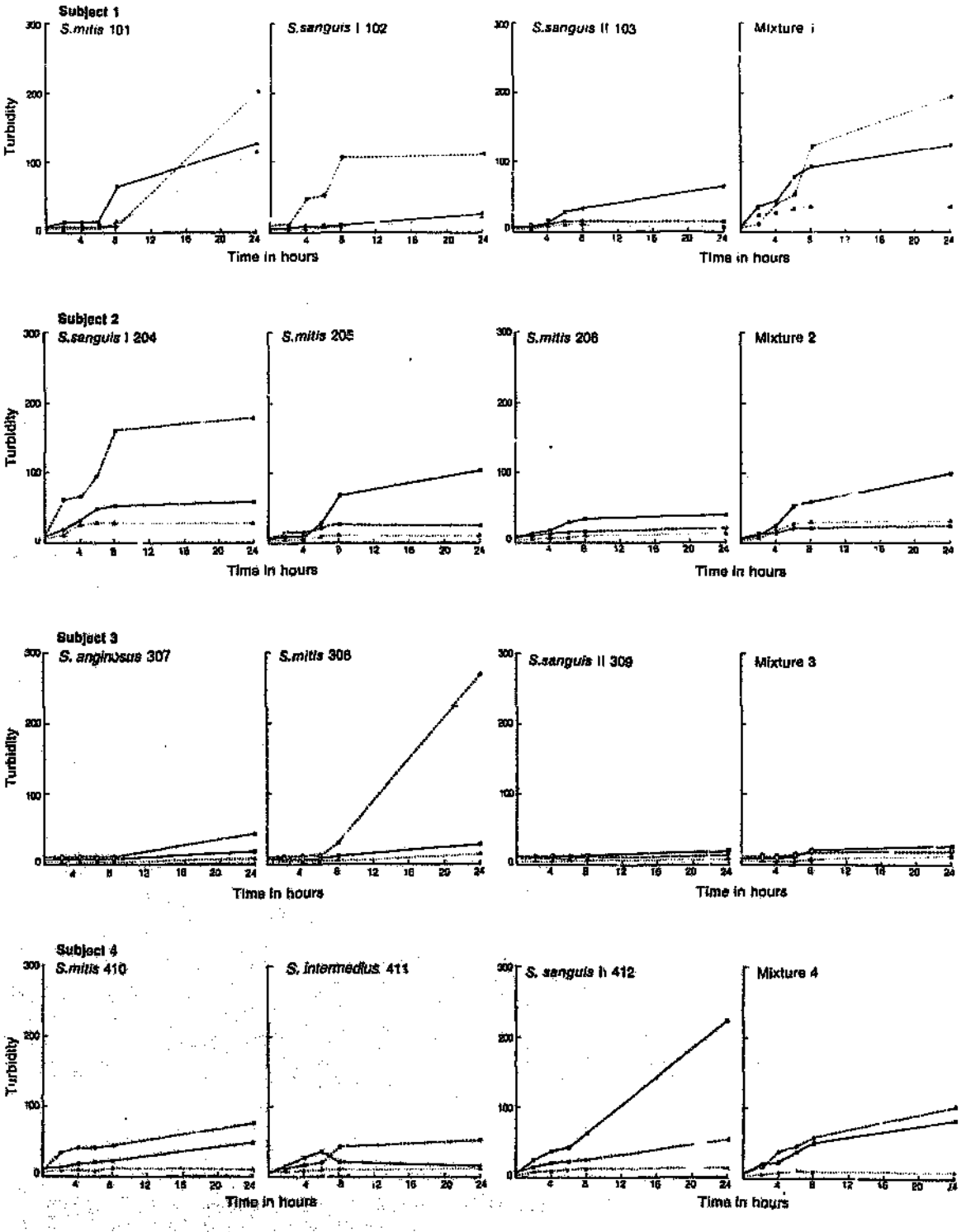



Figure 3.3 The growth of pure and mixed cultures of streptococci from healthy teeth in yeast extract broth (△···△), yeast extract broth containing 0,5 per cent amylopectin (□——□) and yeast extract broth containing 0,5 per cent amylose (○---○). The broths were incubated at 37 C for 24 hours. Growth was determined by measuring an increase in turbidity of the broths in formazine turbidity units.

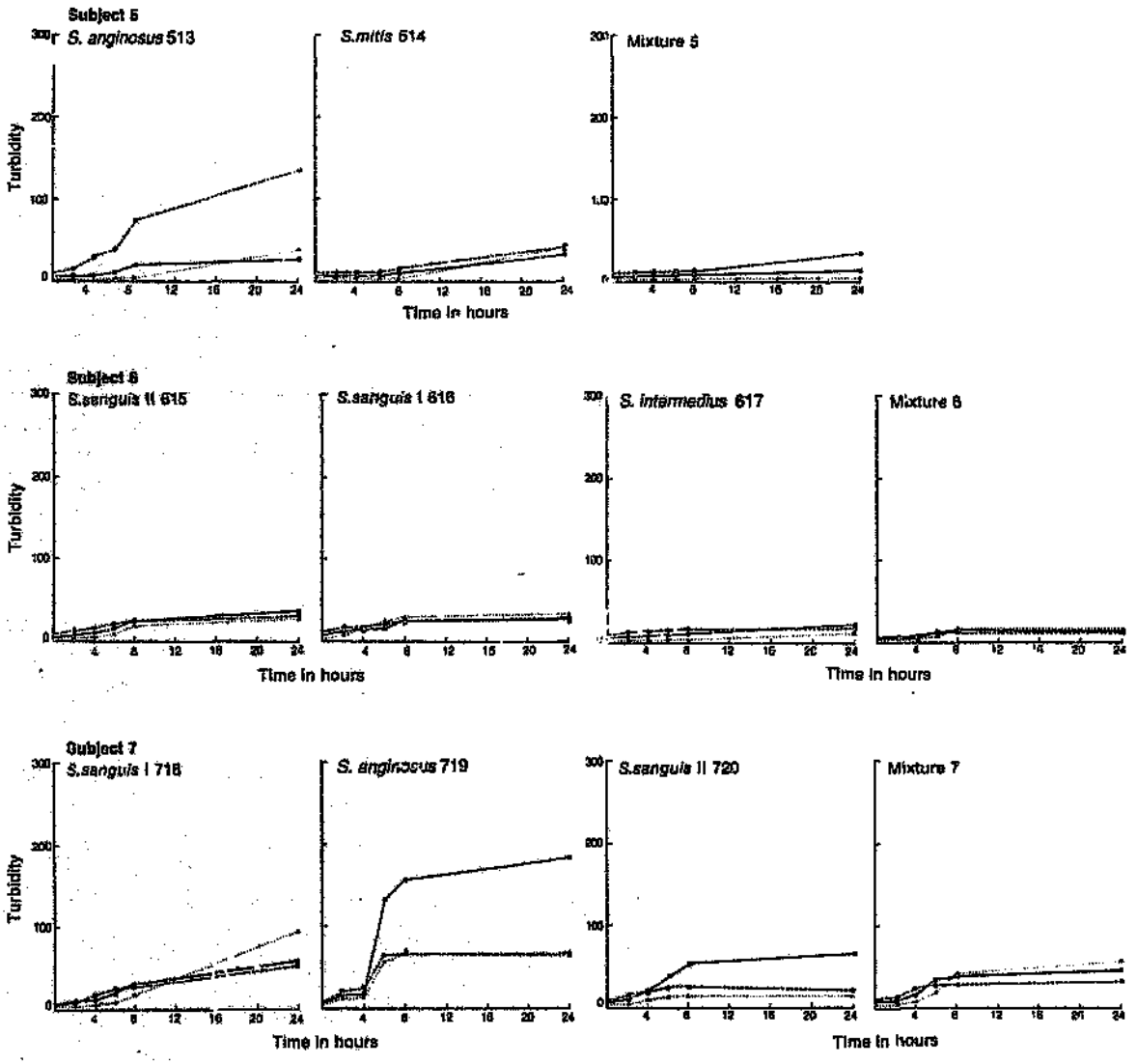



Figure 3.4 The growth of pure and mixed cultures of streptococci from carious lesions in yeast extract broth (△ ··· △), yeast extract broth containing 0,5 per cent amylopectin (□——□) and yeast extract broth containing 0,5 per cent amylose (○---○). The broths were incubated at 37 C for 24 hours. Growth was determined by measuring an increase in turbidity of the broths in formazine turbidity units.

Table 3.1 Mean growth values determined by calculating an increase in broth turbidity. *Streptococci* were grown in the broths at 37°C for 24 hours. Turbidity readings were compared using a general linear models procedure for a nested analysis of variance.

| Source of strains | Mean turbidity of Culture broth | | |
|---|---|---|---|
| | Yeast extract broth $\bar{X}$ (n) | Yeast extract broth plus 0,5% amylopectin $\bar{X}$ (n) | Yeast extract broth plus 0,5% amylose $\bar{X}$ (n) |
| Stock strain FW213 | 79 (2) | 114 (2) | 78 (2) |
| Healthy teeth | ++21 (32) | * 66 (32) | †87 (32) |
| Carious lesion | 34 (22) | 43 (22) | 47 (22) |
| Total | * 30 (56) | 61 (56) | *72 (56) |

Standard Error 17,88
†* p = 0,02
 * P = 0,01

A third pattern was characterized by a long lag phase of 6 to 8 hours followed by vigorous growth. S. mitis 101 showed this pattern in all the test media whereas S. mitis 308 adapted to growth in amylose (Figure 3.3). The fourth pattern which is known as diaxic growth was seen with 6 cultures. The isolates grew vigorously initially followed by a lag phase of minimal change and thereafter accelerated growth. S. sanguis 11 412 showed diaxic growth in amylopectin, S. sanguis 1 102, S. sanguis 1 204, S. anginosus 513 in amylose and mixtures 1 and 4 in amylopectin and amylose (Figures 3.3 and 3.4).

With the fifth pattern there was a gradual increase of growth in all the broths for the first 4 to 6 hours and thereafter a marked increase for the remaining 18 to 24 hours. Six cultures produced this type of growth in amylopectin i.e. the mixture 2, S. mitis 205, S. sanguis 11 103 and 720 and S. anginosus 719. S. mitis 101, 308 and 410 grew vigorously in amylose and S.sanguis 1 718 in yeast extract broth (Figures 3.3 and 3.4). In the fifth pattern, the strains grew well and did not require time to adapt. S. sanguis 1 FW 213 and mixture 4 adapted readily to growth in both starches whereas S. sanguis 11 412 grew rapidly in amylose (Figures 3.2 and 3.3).

A comparison of the growth of streptococcal strains in yeast extract broth after 24 hours showed only 5 cultures were well adapted and reached a turbidity value of more than 50 FTU. They included S. anginosus 719, S. mitis 101, S. sanguis 1 718 and FW 213 and the mixture from subject 7. The readings for the remainder ranged from 6 to 40 FTU (Figures 3.2, 3.3 and 3.4).

The addition of amylopectin and amylose to the yeast extract broth either inhibited, had no effect or stimulated growth. At the one extreme these starches inhibited the growth of S. sanguis 1 718 but had no influence on a further 4 cultures. On the other hand amylose and amylopectin stimulated the growth of six isolates. Some streptococci grew well in the presence of amylose and not amylopectin and others were stimulated by amylopectin and not amylose (Table 3.2).

Statistical analysis of the growth after 24 hrs showed streptococci grew more vigorously ($p=0,01$) in amylose (mean = 72 FTU) than in yeast extract (mean = 30). There was no significant difference between growth in amylose and amylopectin, and yeast extract and amylopectin. Isolates from healthy teeth grew more vigorously in amylopectin (mean = 66 FTU) and amylose (mean = 87 FTU) than in yeast extract (mean = 21 FTU). These differences were less significant ($p=0,02$) (Table 3.1).

When isolates from caries free subjects and carious lesions were compared 9 of the 12 cultures that grew vigorously (FTU > 50) in amylose and amylopectin were from caries free subjects i.e. S. mitis 101, 205 and 308, S. sanguis 1 102 and 204, S. sanguis II 412 and the mixtures 1, 2 and 4 whereas only 3 isolates, S. anginosus 513 and 719 and S. sanguis II 720 were from carious lesions (Figures 3.2 and 3.3). Despite the apparent differences between individual cultures there was no statistically significant difference between the growth of microorganisms isolated from carious lesions and healthy teeth after 24 hrs incubation (Table 3.1).

Table 3.2 The effect of amylopectin and amylose on the growth of *Streptococci*. Growth was either inhibited (-), stimulated (+) or unaffected (o).

| Streptococcal strain | Code | Growth on amylose | Growth on amylo-pectin |
|---|---|---|---|
| S. sanguis I | 718 | - | - |
| S. intermedius | 617 | o | o |
| S. mitis | 514 | o | o |
| S. sanguis I | 616 | o | o |
| S. sanguis II | 309 | o | o |
| | 615 | o | o |
| S. anginosus | 513 | + | - |
| S. intermedius | 411 | + | o |
| S. mitis | 101 | + | o |
| S. sanguis I | 102 | + | o |
| S. anginosus | 719 | o | + |
| S. mitis | 206 | o | + |
| S. sanguis I | FW213 | o | + |
| S. sanguis II | 103 | o | + |
| | 720 | o | + |
| S. anginosus | 307 | + | + |
| S. mitis | 205 | + | + |
| | 308 | + | + |
| | 410 | + | + |
| S. sanguis I | 204 | + | + |
| S. sanguis II | 412 | + | + |

## 2 ACID PRODUCTION FROM STARCH

Two stages of acid production were studied. Firstly, the release of glucose and glucose oligomers from amylose and amylopectin and secondly, the production of acids from these carbohydrates.

### 2.1 Production of glucose and glucose oligomers

Glucose was measured by the glucose oxidase test whereas glucose oligomers were detected as reducing sugars by the Fehlings test. Before incubation approximately 4 mg glucose were present in 100 ml of the yeast extract broth and 27 mg in the broths containing amylopectin and amylose.

#### 2.1.1 Changes in yeast extract

After 24 hours incubation the concentration of glucose and reducing sugars decreased in almost half the samples. In contrast *S. sanguis* I 616 and 718, *S. anginosus* 719 and mixture 2 released between 5 and 32 mg glucose and reducing sugars, whereas glucose increased in the broths inoculated with *S. sanguis* I 102, *S. sanguis* II 412 and *S. anginosus* 513 (Table 3.3).

#### 2.1.2 Changes in amylopectin and amylose

The majority of cultures grown in amylopectin and amylose produced a slight change in the glucose content of the medium. *S. sanguis* I 616 and the mixture of strains isolated from subject 4 increased the glucose content of amylopectin and amylose broth by between 8

Table 3.3 Changes in the amount of glucose (A) and reducing sugars (B) detected in broths inoculated with *streptococci* and incubated at 37°C for 24 hours. The amount of sugars either decreased (-) or increased (+).

| Source | Streptococcal code | mg sugar in 100 ml broth | | | | | |
|---|---|---|---|---|---|---|---|
| | | Yeast Extract broth | | Yeast Extract broth + amylo-pectin | | Yeast Extract broth + amy-lose | |
| | | A | B | A | B | A | B |
| Healthy tooth | 101 | + 1 | + 3 | 0 | - 7 | + 7 | +21 |
| | 102 | +15 | + 1 | - 2 | +10 | 0 | +25 |
| | 103 | - 2 | - 7 | - 3 | -20 | - 3 | -10 |
| | mixture 1 | + 8 | - 5 | + 7 | +15 | + 9 | +20 |
| Healthy tooth | 204 | + 1 | - 6 | + 6 | + 4 | 0 | + 4 |
| | 205 | + 3 | 0 | + 4 | +11 | + 4 | - 6 |
| | 206 | - 4 | - 4 | + 1 | - 3 | + 7 | - 2 |
| | mixture 2 | +10 | -16 | - 5 | +73 | - 2 | 0 |
| Healthy tooth | 307 | 0 | + 1 | + 7 | +29 | - 3 | 0 |
| | 308 | + 1 | + 2 | +13 | +33 | - 3 | + 9 |
| | 309 | + 1 | - 3 | - 8 | -27 | + 1 | +17 |
| | mixture 3 | - 1 | -10 | - 2 | - 6 | + 1 | - 1 |
| Healthy tooth | 410 | - 3 | - 7 | + 1 | +30 | - 5 | + 6 |
| | 411 | + 1 | -26 | 0 | - 4 | - 6 | -27 |
| | 412 | +12 | -22 | + 4 | +24 | + 2 | -20 |
| | mixture 4 | - 3 | + 4 | +16 | +74 | +10 | + 8 |
| Carious lesions | 513 | + 8 | + 1 | + 8 | + 5 | + 3 | + 8 |
| | 514 | - 1 | + 3 | - 2 | + 8 | 0 | + 4 |
| | mixture 5 | - 2 | - 2 | - 2 | +10 | - 2 | - 8 |
| Carious lesions | 615 | - 4 | + 6 | 0 | +54 | + 2 | + 4 |
| | 616 | + 18 | + 5 | +13 | +35 | +10 | + 2 |
| | 617 | - 1 | + 6 | - 1 | +35 | - 1 | 0 |
| | mixture 6 | + 6 | -12 | +12 | + 5 | + 6 | +27 |
| Carious lesions | 718 | + 8 | +32 | 0 | +24 | +10 | + 1 |
| | 719 | +12 | +12 | - 3 | +26 | +16 | + 1 |
| | 720 | - 1 | -10 | - 1 | +23 | + 1 | - 9 |
| | mixture 7 | + 5 | - 2 | 0 | + 5 | + 7 | - 2 |
| Stock | FW 213 | - 5 | + 4 | 0 | +24 | + 2 | +60 |

and 16 mg per 100 ml. A similar increase was seen when the mixture from subject 1, S. sanguis 1 718 and S. anginosus 719 were grown in amylose and mixture 6, S. mitis 308 and S anginosus 513 cultured in amylopectin (Table 3.3).

A greater change was seen in the reducing sugars. Almost half the cultures grown in amylopectin produced more than 22 mg reducing sugars in 24 hours (Table 3.3). These changes were caused by the release of glucose oligomers into the broths. S. sanguis 1 718, for example produced maltohexaose, S. sanguis 11 412 and 720 maltose and maltohexaose whereas S. mitis 308 and S. anginosus 718 released glucose, maltose, maltotriose, maltohexaose and maltooctaose. Five isolates produced detectable oligosaccharides from amylose. S. mitis 308 and S. sanguis 1 102 produced maltose, S. mitis 101 maltotriose, S. sanguis 1 FW 213 glucose, maltose and maltotriose whereas S. sanguis 11 309 produced maltose, maltotriose, maltononaose and a dextrin with 12 glucose units (Table 3.4).

Statistical analysis showed cultures produced significantly more reducing sugars from amylopectin and amylose than from yeast extract broth ($p=0,05$). However, there was no significant difference between amylose and amylopectin. Isolates from healthy teeth did not produced more sugars than those from carious lesions nor did strains which grew vigorously produce more sugars than less vigorous cultures (Tables 3.5 and 3.6).

Table 3.4 Glucose and glucose oligomers detected in broths inoculated with *Streptococci* and incubated at 37°C for 24 hours

| Broth | Species | Code | Glucose and glucose oligomers | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | $G_1$ | $G_2$ | $G_3$ | $G_5$ | $G_6$ | $G_7$ | $G_8$ | $G_9$ | $G_{12}$ |
| Yeast extract broth plus 0,5% amylose | S. mitis | 308 | | + | | | | | | | |
| | S. sanguis I | 102 | + | + | | | | | | | |
| | | FW213 | + | + | + | | | | | | |
| | S. mitis | 101 | + | | + | | | | | | + |
| | S. sanguis II | 309 | | + | + | | | | | + | + |
| yeast extract broth plus 0,5% amylopectin | S. sanguis I | 718 | | | | | + | | | | |
| | S. sanguis II | 412 | | + | | | + | | | | |
| | | 720 | | + | | | + | | | | |
| | | 615 | + | | + | + | | | | | |
| | S. sanguis I | FW213 | + | | + | + | + | | | | |
| | S. intermedius | 617 | + | | | | + | + | | | |
| | S. anginosus | 307 | + | | + | | + | | | | + |
| | S. sanguis I | 616 | + | | | | + | | + | + | + |
| | S. mitis | 410 | + | + | + | | + | | | | |
| | | 308 | + | + | + | | + | | + | | |
| | S. anginosus | 719 | + | + | + | | + | | + | | |

Table 3.5 Mean change in mg in the amount of glucose (A) and reducing sugars (B) detected in broths inoculated with streptococci and incubated at 37°C for 24 hours. The amount of sugars either decreased (-) or increased (+). Changes were compared using a general linear models procedure for a nested analysis of variance.

| Streptococcal isolates | mean change in mg sugar per 100 mℓ broth | | | | | | Standard error | |
|---|---|---|---|---|---|---|---|---|
| | yeast extract broth $\bar{X}$ (n) | | yeast extract broth plus amylopectin $\bar{X}$ (n) | | yeast extract broth plus amylose $\bar{X}$ (n) | | | |
| | A | B | A | B | A | B | A | B |
| Stock strain FW 213 | -4,50 (2) | +4,00 (2) | 0,0 (2) | +24,00 (2) | +2,00 (2) | +59,50 (2) | 6,60 | 15,95 |
| Strains from healthy teeth | +2,75 (32) | -4,34 (32) | +2,28 (32) | +15,22 (32) | +1,19 (32) | +2,94 (32) | 1,65 | 3,99 |
| Strains from carious lesions | +5,14 (22) | +3,36 (22) | +2,27 (22) | +20,59 (22) | +4,68 (22) | +2,41 (22) | 1,99 | 4,81 |
| Total | +3,43 (56) | *o -1,018 (56) | +2,20 (56) | * +17,64 (56) | +2,59 (56) | o +4,75 (56) | 1,25 | 3,02 |

- = mean decrease
+ = mean increase
* o P = 0,05

Table 3.6 Mean change in mg in the amount of glucose (A) and reducing sugars (B) detected in broths inoculated with streptococci which grew either vigorously or poorly. The amount of sugars either decreased (-) or increased (+). Changes were compared using a general linear models procedure for a nested analysis of variance.

| Streptococcal isolates | | mean change in mg sugar per 100 mℓ broth | | | | | |
|---|---|---|---|---|---|---|---|
| | | Yeast extract broth | | Yeast extract broth plus amylopectin | | Yeast extract broth plus amylose | |
| | | A | B | A | B | A | B |
| Vigorous strains FTU > 50 | $\bar{X}$ (n) | +3,90 (10) | +8,80 (10) | +1,70 (20) | +23,85 (20) | +2,70 (20) | +7,27 (30) |
| | Standard error | 2,95 | 7,13 | 2, | 5,05 | 1,71 | 4,12 |
| Less vigorous strains FTU < 50 | $\bar{X}$ (n) | +3,33 (46) | -3,15 (46) | +2,47 (36) | -* (36) | +2,46 (26) | +1,85 (26) |
| | Standard error | 1,38 | 3,33 | 1,56 | 3,76 | 1,83 | 4,42 |

## 2.2 Production of acids

The production of acids was observed as a decrease in pH of the broths. The acids that caused this decrease were identified by gas liquid and ion chromatography, and their identity confirmed by mass spectroscopy.

### 2.2.1 Change in pH

During incubation the pH of the uninoculated yeast extract broth and the broths containing amylopectin and amylose remained at 7,3. Most cultures grown in yeast extract decreased the pH to below 7 after 24 hours incubation with the exception of S. anginosus 307, S. intermedius 411 and 617, S. mitis 101, 205, 206, 300 and 410, S. sanguis I 204, S. sanguis II 103, 309 and 720 and mixture 4 (Figures 3.5, 3.6 and 3.7).

Over 80 per cent of the cultures grown in amylose and amylopectin decreased the pH to between 6,5 and 6,9. However, in half these cultures there was only a slight difference between the pH of amylose and amylopectin and yeast extract broth. They included S. anginosus 513, S. intermedius 411 and 617, S. mitis 206 and 514, S. sanguis I 102 and 616, S. sanguis II 309 and 615 and mixtures 3, 5, 6 and 7 (Figures 3.6 and 3.7).

The addition of amylopectin produced a greater decrease in pH than amylose in the broths inoculated with S. anginosus 719, S. mitis 205, S. sanguis I 718, S. sanguis II 103 and 720 and the mixtures 1, 2 and 4 whereas S. anginosus 307, S. mitis 101 and

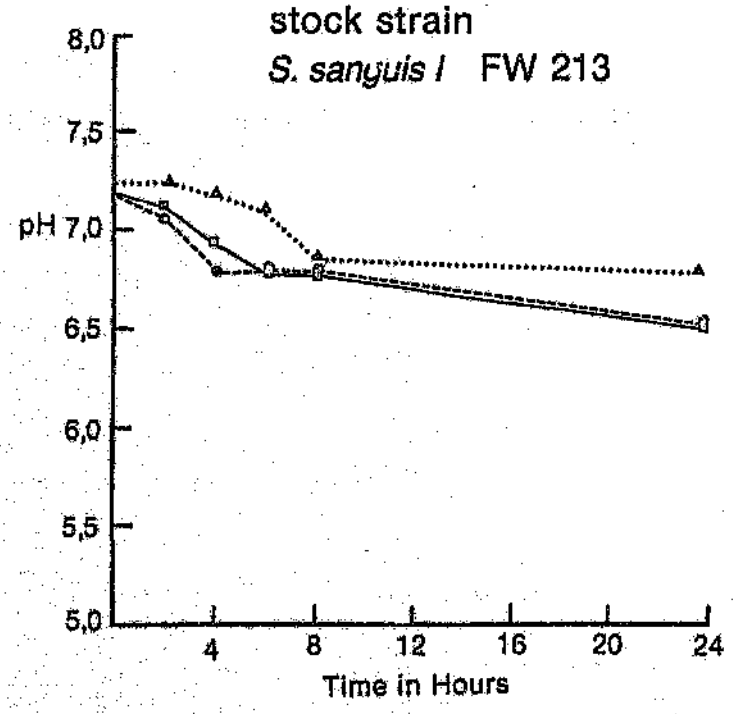



Figure 3.5 Changes in the pH of yeast extract broth (△····△), yeast extract broth containing 0,5 percent amylopectin (□——□) and yeast extract containing 0,5 per cent amylose (○---○). The broths were inoculated with S. sanguis 1 FW 213 and incubated at 37 C for 24 hours.

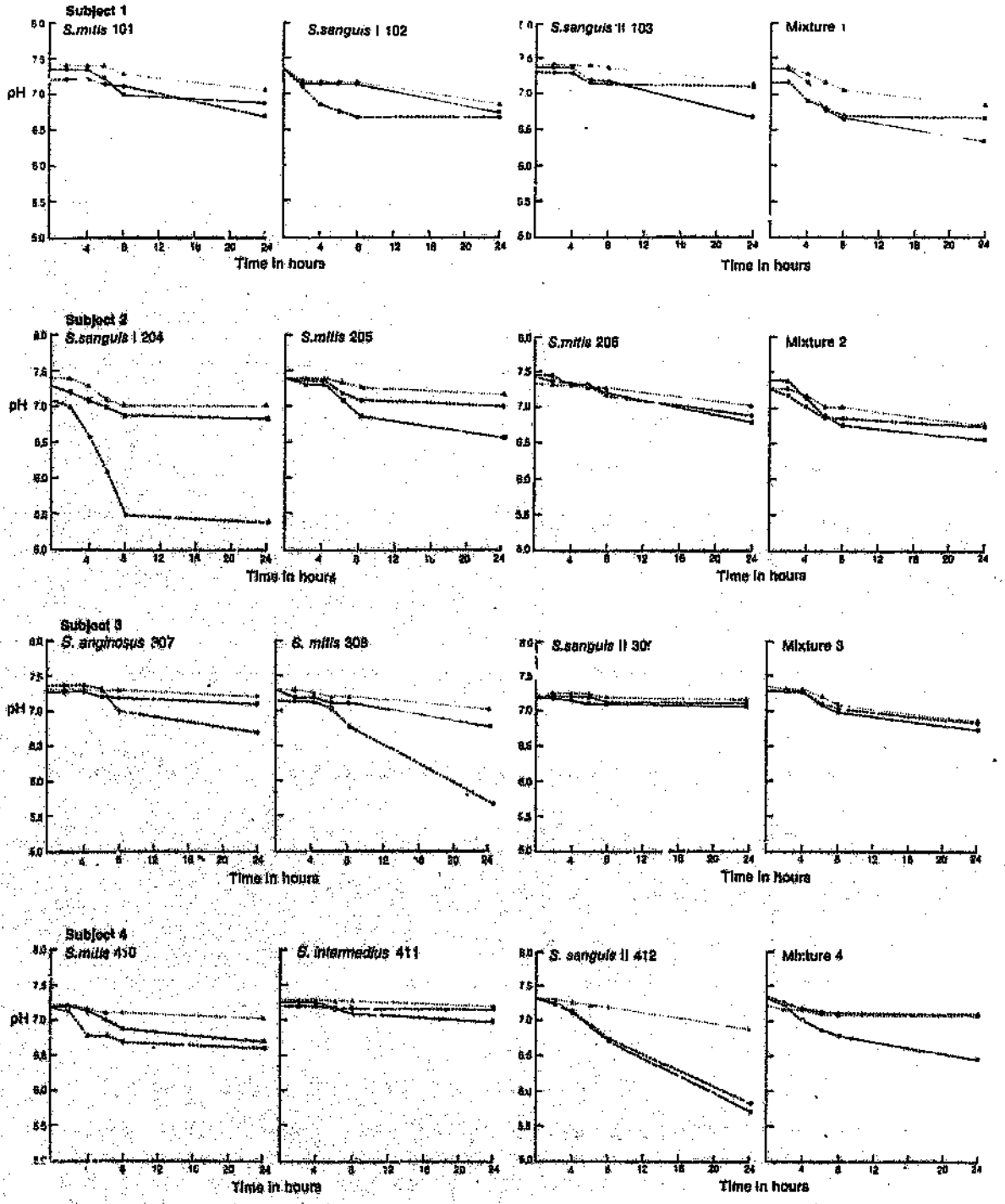



Figure 3.6 Changes in the pH of yeast extract broth (Δ···Δ), yeast extract broth containing 0,5 percent amylopectin (■—■) and yeast extract broth containing 0,5 per cent amylose (●---●). The broths were inoculated with pure and mixed cultures of streptococci from healthy teeth and incubated at 37 C for 24 hours.

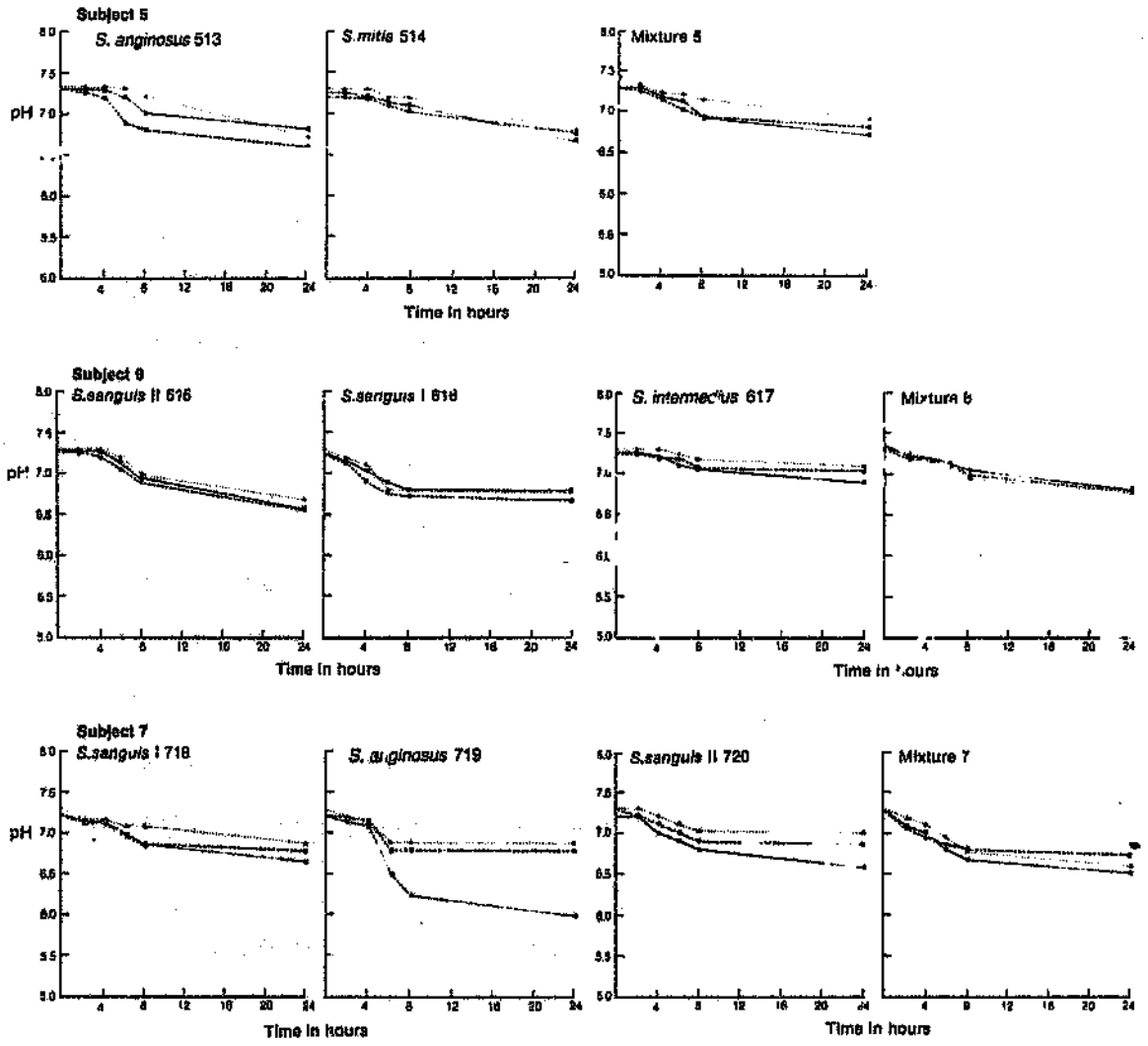



Figure 3.7 Changes in the pH of yeast extract broth (△····△), yeast extract broth containing 0,5 per cent amylopectin (▣——▣) and yeast extract broth containing 0,5 per cent amylose (◎---◎). The broths were inoculated with pure and mixed cultures of streptococci from carious lesions and incubated at 37 C for 24 hours.

308 and S. sanguis 1 204 produced more change in pH of the amylose than the amylopectin broth. Three isolates grown on amylose lowered the pH to below 6. S. sanguis I 204 decreased the pH to 5,4, S. mitis 308 to 5,7 and S. sanguis II 412 to 5,8 whereas the latter also fermented amylopectin to 5,7 (Figures 3.6 and 3.7).

The isolates which exhibited diaxic growth, S. sanguis I 204, S. arginosus 513 and the mixtures of organisms from subjects 1 and 4 did not decrease the pH of amylose and amylopectin broth more slowly than the remaining cultures (Figures 3.5 and 3.6).

There was a significant difference ($p = 0,01$) between yeast extract (mean = 6,95), amylose (mean = 6,73) and amylopectin (mean = 6,67) when an analysis of variance was used to compare the mean decrease in pH after 24 hours incubation. There was a similar difference when isolates that grew vigorously were compared ($p = 0.01$). However, there was no significant difference between isolates from healthy teeth and carious lesions (Table 3.7).

2.2.2 Identification of acids

Lactic acid was produced by all the cultures and predominated in 3 of the 4 isolates which decreased the pH to below 6. These isolates were S. sanguis II 412 grown in amylopectin and S. mitis 308 and S. sanguis I 204 grown in amylose. Lactic acid also predominated in 60 percent of the cultures grown in amylose (Table 3.8).

Table 3.7 Mean changes in pH of broth inoculated with *Streptococci* and incubated at 37°C for 24 hours. The pH was compared using a general linear models procedure for a nested analysis of variance.

| Source of Strains | Mean pH of culture broths | | |
|---|---|---|---|
| | yeast extract broth $\bar{X}$ (n) | yeast extract broth plus 0,5% amylopectin $\bar{X}$ (n) | yeast extract broth plus 0,5% amylose $\bar{X}$ (n) |
| Stock strain FW213 | 6,80 (2) | 6,50 (2) | 6,55 (2) |
| Healthy teeth | 7,02 (32) | 6,70 (32) | 6,70 (32) |
| Carious lesions | 6,85 (22) | 6,64 (22) | 6,79 (22) |
| Vigorous growth FTU>50 | ǂ*6,89 (8) | ǂ6,40 (20) | *6,60 (28) |
| Less vigorous growth FTU<50 | 6,97 (46) | 6,80 (34) | 6,90 (26) |
| Total | †+6,95 (56) | †6,67 (56) | †6,73 (56) |

Standard error 0,06
ǂ, *, †, + p = 0,01

Table 3.8. Acids detected in broths inoculated with *Streptococci* and incubated at 37°C for 24 hours

| Subject | Strain | | mmol acid per litre of yeast extract broth | | | mmol acid per litre of yeast extract broth plus 0,5% amylopectin | | | mmol acid per litre of yeast extract broth plus 0,5% amylose | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | lac-tic | ace-tic | suc-cinic | lac-tic | ace-tic | suc-cinic | lac-tic | ace-tic | suc-cinic |
| 1 | S. mitis | 101 | 3,63 | 3,30 | 1,26 | 4,96 | 4,58 | 1,43 | 3,90 | 3,90 | 1,66 |
| healthy | S. sanguis I | 102 | 1,04 | 1,10 | 1,04 | 3,57 | 2,64 | 1,50 | 4,18 | 1,0 | 0 |
| teeth | S. sanguis II | 103 | 1,45 | 4,30 | 1,45 | 1,21 | 7,0 | 0,30 | 1,30 | 2,0 | 1,70 |
| | mixture 1 | | 3,22 | 5,10 | 3,22 | 8,53 | 6,5 | 1,30 | 3,80 | 5,20 | 0,80 |
| 2 | S. sanguis I | 204 | 3,03 | 3,57 | 1,0 | 5,97 | 5,71 | 1,0 | 8,33 | 7,49 | 1,10 |
| healthy | S. mitis | 205 | 3,53 | 3,80 | 1,06 | 5,71 | 4,54 | 0,70 | 4,54 | 2,72 | 0,90 |
| teeth | S. mitis | 206 | 0,10 | 3,94 | 1,70 | 2,13 | 3,30 | 2,30 | 1,60 | 1,90 | 2,0 |
| | mixture 2 | | 2,40 | 3,60 | 1,30 | 6,36 | 4,60 | 1,10 | 3,40 | 2,45 | 0,90 |
| 3 | S. anginosus | 307 | 4,30 | 1,60 | 0,64 | 1,80 | 0,15 | 2,40 | 1,70 | 1,50 | 1,90 |
| healthy | S. mitis | 308 | 2,18 | 3,0 | 1,80 | 6,12 | 5,17 | 2,70 | 3,97 | 3,68 | 1,53 |
| teeth | S. sanguis II | 309 | 2,90 | 3,70 | 2,20 | 1,0 | 0,80 | 2,20 | 1,05 | 0,40 | 1,40 |
| | mixture 3 | | 0,72 | 2,16 | 1,05 | 0,88 | 1,70 | 3,90 | 1,20 | 1,32 | 1,20 |
| 4 | S. mitis | 410 | 2,84 | 2,35 | 1,26 | 2,90 | 0 | 2,60 | 2,84 | 0 | 2,10 |
| healthy | S. intermedius | 411 | 1,22 | 1,37 | 0,65 | 2,61 | 5,09 | 1,15 | 2,90 | 1,37 | 1,40 |
| teeth | S. sanguis II | 412 | 1,42 | 0,39 | 1,05 | 1,64 | 1,76 | 0,90 | 2,20 | 1,57 | 1,45 |
| | mixture 4 | | 1,76 | 1,64 | 0,90 | 3,20 | 3,14 | 1,28 | 3,20 | 1,60 | 1,85 |
| 5 | S. anginosus | 513 | 2,93 | 2,83 | 0,54 | 1,91 | 2,08 | 0,58 | 5,06 | 2,30 | 0,63 |
| carious | S. mitis | 514 | 1,84 | 1,92 | 0,60 | 6,19 | 3,35 | 1,30 | 2,02 | 5,83 | 0,80 |
| lesion | mixture 5 | | 0,79 | 6,44 | 1,30 | 2,50 | 2,82 | 0 | 4,80 | 3,32 | 0 |
| 6 | S. sanguis II | 615 | 3,59 | 5,01 | 2,10 | 4,09 | 5,86 | 0,70 | 4,16 | 3,96 | 1,30 |
| carious | S. sanguis I | 616 | 3,08 | 5,49 | 1,30 | 2,43 | 1,50 | 1,40 | 6,80 | 4,30 | 1,0 |
| lesion | S. intermedius | 617 | 3,03 | 3,57 | 0,90 | 2,43 | 3,20 | 0,80 | 3,50 | 3,02 | 1,30 |
| | mixture 6 | | 0,80 | 2,80 | 0,60 | 1,40 | 2,26 | 0,91 | 3,01 | 2,07 | 0,90 |
| 7 | S. sanguis I | 718 | 2,52 | 3,59 | 0,80 | 2,14 | 2,15 | 0,70 | 0,78 | 1,56 | 0 |
| carious | S. anginosus | 719 | 1,45 | 3,09 | 0 | 4,46 | 8,58 | 0,90 | 1,05 | 3,23 | 0 |
| lesion | S. sanguis II | 720 | 1,04 | 3,02 | 0 | 3,80 | 5,40 | 0 | 4,0 | 1,91 | 0 |
| | mixture 7 | | 2,10 | 3,10 | 1,60 | 4,08 | 3,07 | 2,40 | 2,78 | 4,63 | 1,90 |
| | S. sanguis I | FW213 | 2,29 | 5,49 | 1,30 | 3,06 | 10,96 | 1,20 | 1,73 | 2,71 | 0 |

Acetic was the major acid in three quarters of the cultures grown in yeast extract, 40 per cent of those incubated in amylose and in most cultures from carious lesions grown in amylopectin. Acetic acid was absent in amylopectin and amylose inoculated with S. mitis 410. S. sanguis II 412, an isolate that decreased the pH below 6, produced more acetic than lactic acid from amylose (Table 3.8). The stock strain S. sanguis I FW 213 was the only culture that produced significantly more acetic acid from amylopectin than from yeast extract and amylose ($p<0,001$) (Table 3.9).

Succinic acid was found in small amounts but was the major acid in 6 cultures from healthy teeth, 5 from subject 3. It was produced by S. anginosus 307 cultured in yeast extract and amylose, S. sanguis II 309 grown in amylose and amylopectin and the mixture of microorganisms from this patient incubated in amylopectin. Succinic acid also predominated in yeast extract inoculated with S. sanguis I 102. It was not produced by S. sanguis I 102 grown in amylose and by 3 isolates from patient 7 cultured in yeast extract, amylopectin and amylose (Table 3.8).

A comparison of the mean m mol acid per litre showed that acetic acid was the most abundant acid (mean = 3,35 mM) followed by lactic (mean = 2,92 mM) and succinic (mean = 1,2 mM). Acetic acid was the predominant acid produced from yeast extract (mean = 3,26 mM) followed by lactic acid (mean = 2,18 mM). Amylopectin yielded the most acid i.e. 4,03 mM acetic and 3,4 mM lactic acid and amylose less acid, 3,17 mM lactic and 2,75 mM acetic. Small amounts of succinic acid were produced from yeast extract, amylopectin and amylose i.e. 1.2 mM, 1.34 mM and 1.06 mM respectively. None of these differences were statistically significant (Table 3.9).

Table 3.9 Mean mmol acids detected in broths inoculated with *Streptococci* and incubated at 37°C for 24 hours. Acid production was compared using a general linear models procedure for a nested analysis of variance.

| Streptococcal isolates | Acid | mean mmol acid per litre of broth | | | Standard error |
|---|---|---|---|---|---|
| | | Yeast extract broth $\bar{X}(n)$ | Yeast extract broth plus 0,5% amylopectin $\bar{X}$ (n) | Yeast extract broth plus 0,5% amylose $\bar{X}$ (n) | |
| Stock strain FW213 | lactic | 2,29(2) | 3,06(2) | 1,73(2) | 0,39 |
| | acetic | ‡5,49(2) | ‡†10,96(2) | † 2,71(2) | 0,43 |
| | succinic | 1,30(2) | 1,20(2) | 0 (2) | 0,17 |
| Strains from healthy teeth | lactic | 2,23(32) | 3,55(32) | 3,13(32) | 0,39 |
| | acetic | 2,80(32) | 3,97(32) | 2,81(32) | 0,43 |
| | succinic | +1,64(32) | †† 1,67(32) | ‡‡1,36(32) | 0,17 |
| Strains from carious lesions | lactic | 2,11(22) | 3,22(22) | 3,36(22) | 0,39 |
| | acetic | 3,20(22) | 3,78(22) | 3,28(22) | 0,43 |
| | succinic | +0,88(22) | †† 0,88(22) | ‡‡0,71(22) | 0,17 |
| Vigorous strains FTU>50 | lactic | 2,44(10) | * 4,28(22) | 3,63(30) | 0,39 |
| | acetic | 3,27(10) | 4,41(22) | 2,79(30) | 0,43 |
| | succinic | 0,91(10) | 1,09(22) | 1,06(30) | 0,17 |
| less vigorous strains FTU<50 | lactic | 2,14(46) | 2,91(34) | 2,95(26) | 0,39 |
| | acetic | 3,26(46) | 3,40(34) | 2,66(26) | 0,43 |
| | succinic | 1,23(46) | 1,50(34) | 1,14(26) | 0,17 |
| Total | lactic | 2,18(56) | 3,40(56) | 3,17(56) | 0,39 |
| | acetic | 3,26(56) | 4,03(56) | 2,75(56) | 0,43 |
| | succinic | 1,20(56) | 1,34(56) | 1,06(56) | 0,17 |

+, ††, ‡‡, * $p = 0,02$ ‡, † $p < 0,001$

Streptococci from healthy teeth produced significantly more succinic acid ($p \leq 0,02$) from yeast extract, amylose and amylopectin than isolates from carious lesions. Furthermore, these isolated produced more lactic acid from yeast extract and amylopectin than isolates from carious lesions. However, none of these differences were statistically significant (Table 3.9).

# 3 POLYSACCHARIDES FROM STARCH

Most strains of streptococci produced polysaccharides from amylopectin. They were designated glucans because they contained chains with alpha-1,4-linkages. Their structure was analysed using methods for amylopectin because they were derived from this polymer. Iodine staining indicated the presence of helices and branching in the molecules. Infra-red spectroscopy confirmed the anomeric configuration of the sugars. Methylation analysis determined the length of the chains as well as the sugar linkages in the molecules, whereas the enzymic analyses yielded information about the A and B chains and the exterior and interior chain lengths.

## 3.1 PRODUCTION OF GLUCANS

All the species of streptococci investigated contained strains that produced an insoluble polysaccharide from amylopectin and not amylose. They included S. sanguis 1 FW 213, 8 of the 12 strains from healthy teeth and 5 from carious lesions (Table 3.10). S. sanguis 1 MC 204 produced the most polysaccharide which was designated amyloglucan because it was derived from amylopectin and contained mainly alpha-1,4-linkages. Both the cells and the cell free enzyme extract of S. sanguis 1 MC 204 produced amyloglucan which accumulated on the surface of the glass bottles.

Further investigation of S. sanguis 1 204 showed it also produced a soluble polysaccharide from amylopectin. This polymer was called a soluble glucan because it was soluble and contained chains with alpha-1,4-linkages (Table 3.13).

Table 3.10 Presence of insoluble polysaccharide and dextran in broths inoculated with starch hydrolysing streptocci

| Species | Code | Insoluble polysaccharide in amylopectin broth | Dextran in sucrose broth |
|---|---|---|---|
| S. anginosus | 307 | + | 0 |
| | 719 | + | + |
| | 513 | 0 | 0 |
| S. intermedius | 617 | + | 0 |
| | 411 | 0 | + |
| S. mitis | 514 | + | 0 |
| | 101 | + | + |
| | 308 | + | + |
| | 205 | 0 | 0 |
| | 206 | 0 | 0 |
| | 410 | 0 | 0 |
| S. sanguis I | FW213 | + | + |
| | 204 | + | + |
| | 718 | + | + |
| | 102 | + | 0 |
| | 616 | 0 | + |
| S. sanguis II | 720 | + | + |
| | 103 | + | 0 |
| | 309 | + | 0 |
| | 412 | + | 0 |
| | 615 | + | 0 |

Eight of the 13 strains that produced an insoluble polysaccharide from amylopectin did not produce a detectable dextran from sucrose. Furthermore, 25 per cent of the dextran producing strains did not produce polysaccharide from amylopectin (Table 3.10).

## 3.2 CHEMICAL CHARACTERISTICS OF THE GLUCANS

### 3.2.1 Adherence and solubility

Amyloglucan and the mutan produced from sucrose by S. mutans were the only polymers that adhered readily to glass. This indicates they will adhere to teeth.

The soluble glucan produced from amylopectin by S. sanguis 1 MC 204 dissolved readily in most of the solvents tested with the exception of ethyl alcohol. In contrast the amyloglucan was similar to amylose because it was insoluble in water, boric and acetic acid. Unlike amylose it was only slightly soluble in dimethyl sulphoxide and ethyl alcohol (Table 3.11) and insoluble in dimethyl formamide, N-methylpyrrolidone and diethylpropylene urea. Furthermore, even though it was exposed to 10 percent potassium hydroxide for 2 weeks it was only slightly soluble in this solvent.

A comparison of the polysaccharides derived from amylopectin and the insoluble mutan produced from sucrose by S. mutans showed the mutan was similar to amyloglucan because it did not dissolve in water and boric acid and was slightly soluble in acetic acid. However, the mutan was not as insoluble as amyloglucan and dissolved in the remaining solvents (Table 3.11).

Table 3.11 Adherence to glass, solubility and iodine staining characteristics of amylose, amylopectin, amyloglucan and the soluble glucan produce from amylopectin by *S. sanguis I 204* an insoluble mutan produced from sucrose by *S mutans* ATCC 25175.

| Property | amylose | amylopectin | amyloglucan | soluble glucan | mutan |
|---|---|---|---|---|---|
| Adherence to glass | 0 | 0 | + | 0 | + |
| Solubility | | | | | |
| Water | 0 | + | 0 | + | 0 |
| 1 N Acetic acid | 0 | + | 0 | + | slightly |
| 5% Boric acid | 0 | + | 0 | + | 0 |
| 1 M Potassium hydroxide | + | + | slightly | + | + |
| Dimethyl sulphoxide | + | + | slightly | + | + |
| 95% Alcohol | slightly | + | slightly | slightly | + |
| Iodine colouration | blue | purple | purple | blue | colourless |
| $\lambda$ max (nm) | 620 | 540 | 560 | 570 | 0 |
| $E_{1\%}$ | 99,7 | 14,5 | 1 | 17,8 | 0 |

### 3.2.2 Iodine staining

The amyloglucan-iodine complex gave a purple stain with an E1% of 1 (i.e. the theoretical absorbance of a 1 percent solution of amyloglucan) and a λmax of 550 nm. These figures indicate amyloglucan is similar to amylopectin but binds iodine poorly. In contrast the soluble glucan stained blue with iodine (λmax 570 nm and E1% 17,8). In this respect it had a value between amylopectin (λmax 540 nm and E1% 14,5) and amylose (λmax 620 nm and E1% 96,7). This is evidence that the soluble glucan is slightly less branched than amylopectin and contains longer chains. The mutar did not stain with iodine (Table 3.11).

### 3.2.3 Infra-red spectrum

Infra-red analysis showed there was a similarity between the spectra for amylopectin, amyloglucan and the soluble glucan especially in the fingerprint region for sugars which lies between 1000 and 650 cm-' (Nisizawa et al. 1976). There was a peak in the spectra for amyloglucan and the soluble glucan at 844 and not 891 cm-' which indicated the glucose units in these polysaccharides were alpha and not beta-linked. The absorbance peak for 1,4-linkages at 930 cm-' was similer for amylopectin and the soluble glucan, but slightly larger for amyloglucan. The second peak for 1,4-linkages at 758 cm-' was more marked in the soluble glucan than in amyloglucan and amylopectin. The absorbance peaks for 1,6-linkages which occurs at 917 and 768 cm-' were present in amyloglucan and the soluble glucan, but were difficult to interpret because they were close to the peaks for 1,4-linkages. Furthermore there were no marked peaks at 793 cm-', the absorbance region for 1,3-linkages (Figure 3.8).

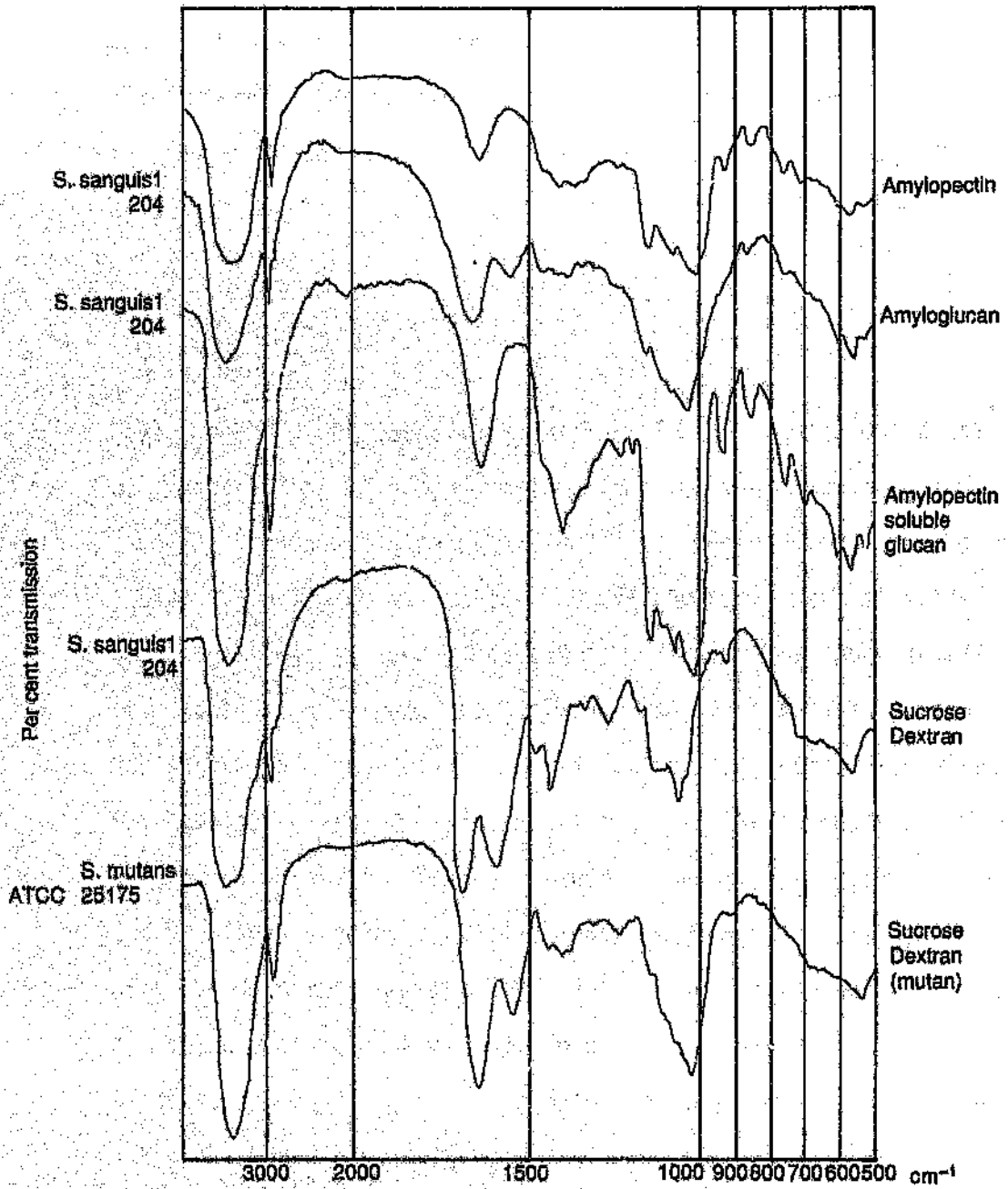



**Figure 3.8** Infra-red absorption spectrum amylopectin, amyloglucan, a soluble glucan produced from amylopectin by *S. sanguis 1* 204, the dextrin produced from sucrose by *S. sanguis 1* 204 and the mutal produced from sucrose *S. mutans* ATCC 25175.

### 3.2.2 Iodine staining

The amyloglucan-iodine complex gave a purple stain with an E1% of 1 (i.e. the theoretical absorbance of a 1 percent solution of amyloglucan) and a λmax of 550 nm. These figures indicate amyloglucan is similar to amylopectin but binds iodine poorly. In contrast the soluble glucan stained blue with iodine (λmax 570 nm and E1% 17,8). In this respect it had a value between amylopectin (λmax 540 nm and E1% 14,5) and amylose (λmax 620 nm and E1% 96,7). This is evidence that the soluble glucan is slightly less branched than amylopectin and contains longer chains. The mutar did not stain with iodine (Table 3.11).

### 3.2.3 Infra-red spectrum

Infra-red analysis showed there was a similarity between the spectra for amylopectin, amyloglucan and the soluble glucan especially in the fingerprint region for sugars which lies between 1000 and 650 cm-' (Nisizawa et al, 1976). There was a peak in the spectra for amyloglucan and the soluble glucan at 844 and not 891 cm-' which indicated the glucose units in these polysaccharides were alpha and not beta-linked. The absorbance peak for 1,4-linkages at 930 cm-' was similar for amylopectin and the soluble glucan, but slightly larger for amyloglucan. The second peak for 1,4-linkages at 758 cm-' was more marked in the soluble glucan than in amyloglucan and amylopectin. The absorbance peaks for 1,6-linkages which occurs at 917 and 768 cm-' were present in amyloglucan and the soluble glucan, but were difficult to interpret because they were close to the peaks for 1,4-linkages. Furthermore there were no marked peaks at 793 cm-', the absorbance region for 1,3-linkages (Figure 3.8).

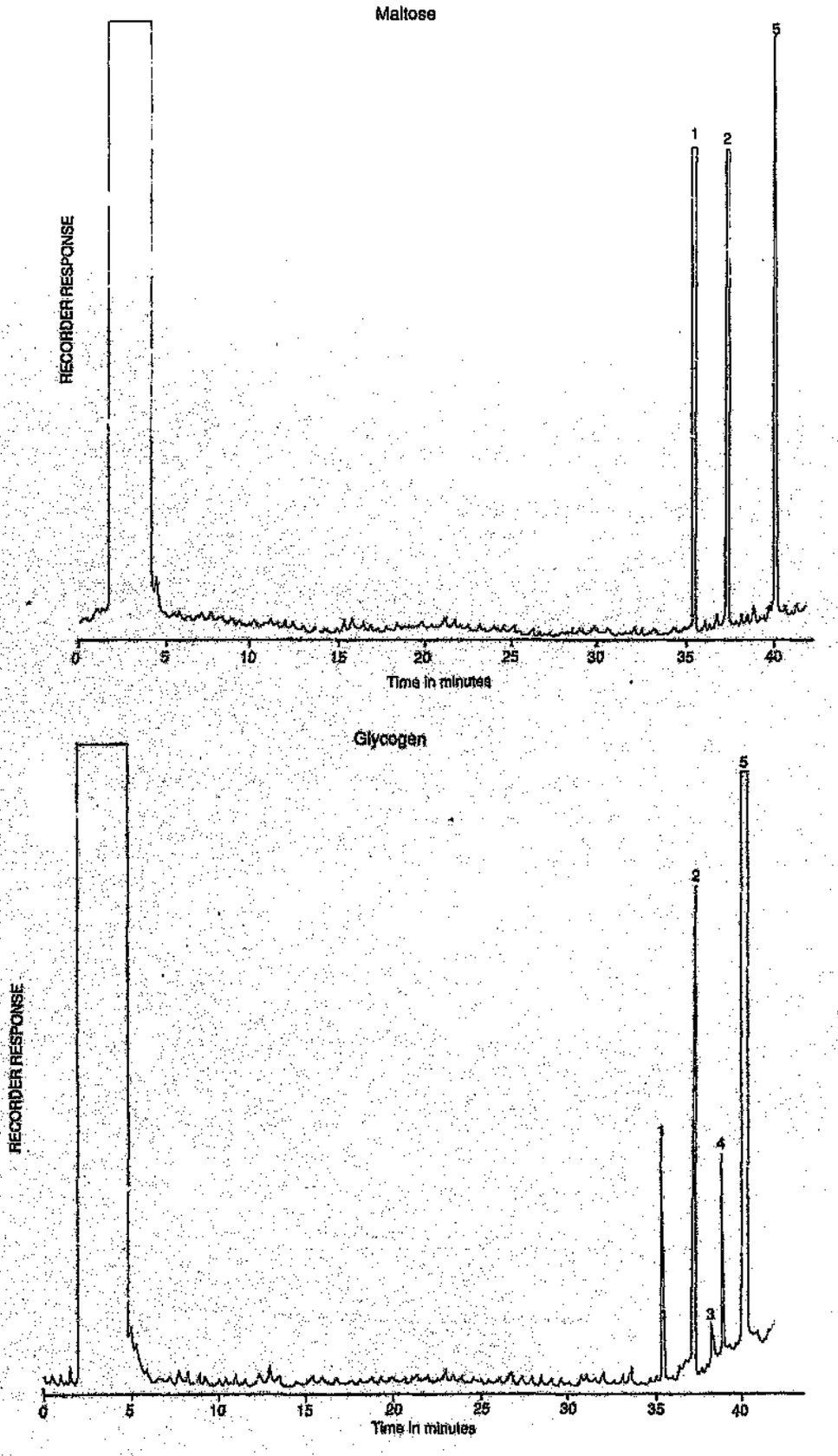



**Figure 3.9** Gas chromatographs of the alditol acetates formed by the methylation of maltose and glycogen.

1. 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol
2. 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl-D-glucitol.
3. 1,3,4,5-tetra-O-acetyl-2,6-di-O-methyl-D-glucitol.
4. 1,4,5,6-tetra-O-acetyl-2,3,-di-O-methyl-D-glucitol.
5. myo-inositol hexa-acetate (internal standard).

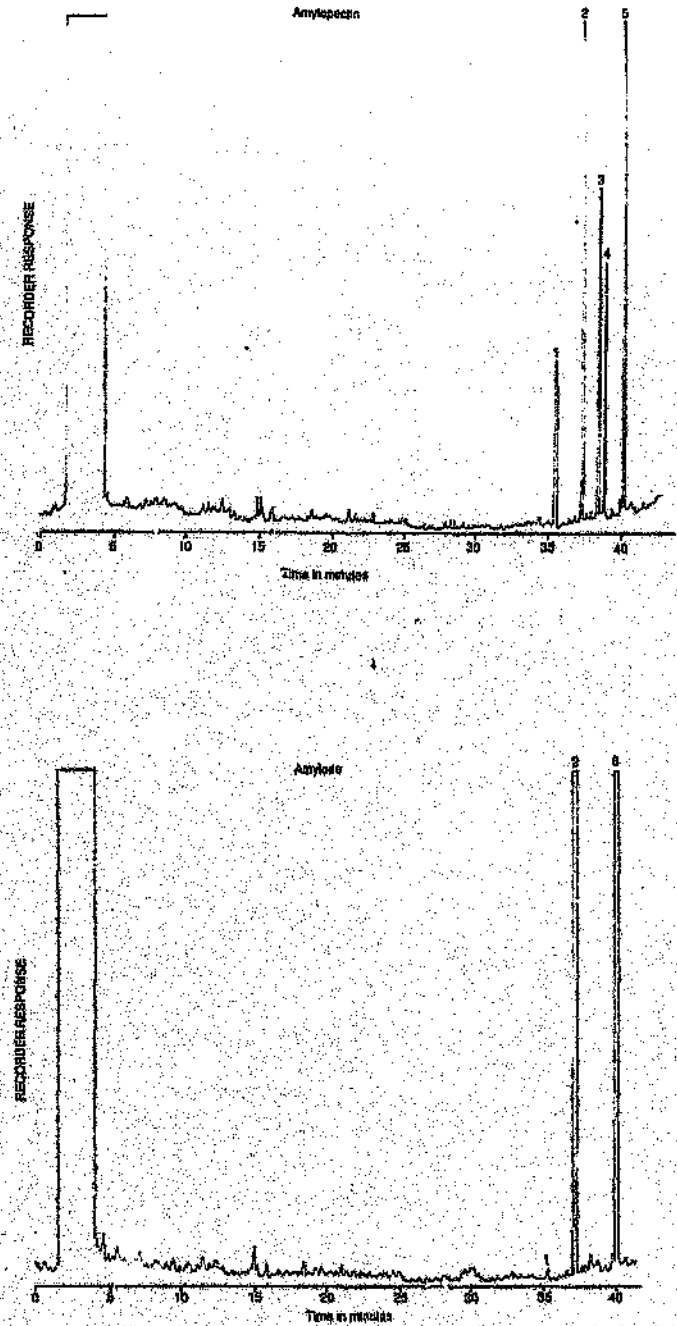



Figure 3.10 Gas liquid chromatograms of the alditol acetates formed by the methylation of amylopectin and amylose.

1. 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol
2. 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl-D-glucitol
3. 1,3,4,5-tetra-O-acetyl-2,6-di-O-methyl-D-glucitol
4. 1,4,5,6-tetra-O-acetyl-2,3-di-O-methyl-D-glucitol
5. myo-inositol hexaacetate (internal standard).

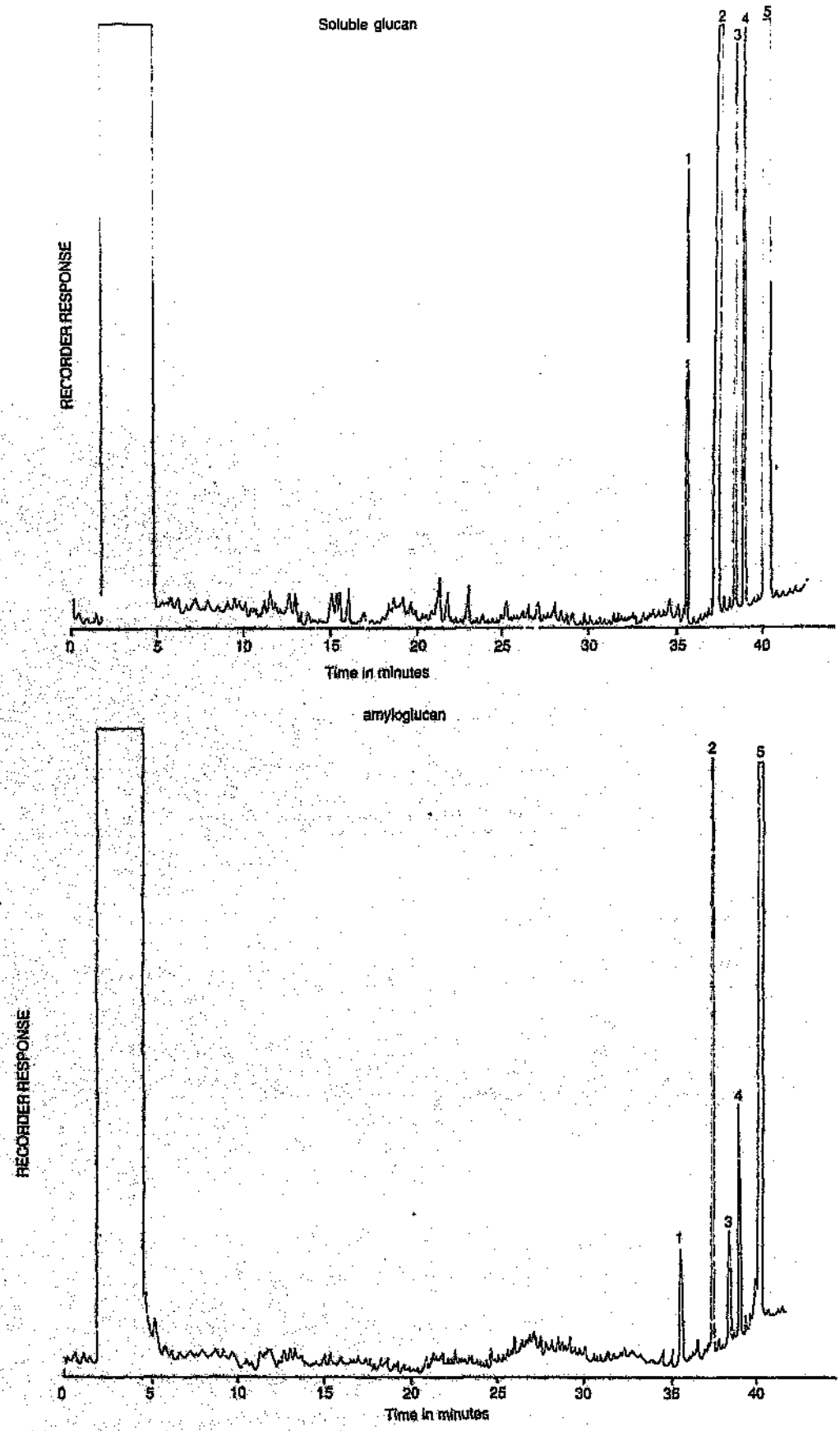



**Figure 3.11** Gas chromatographs of the alditol acetates formed by the methylation of amyloglucan and the soluble polysaccharide produced from amylopectin.

1. 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol
2. 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl-D-glucitol.
3. 1,3,4,5-tetra-O-acetyl-2,6-di-O-methyl-D-glucitol.
4. 1,4,5,6-tetra-O-acetyl-2,3,-di-O-methyl-D-glucitol.
5. myo-Inositol hexa-acetate (internal standard).

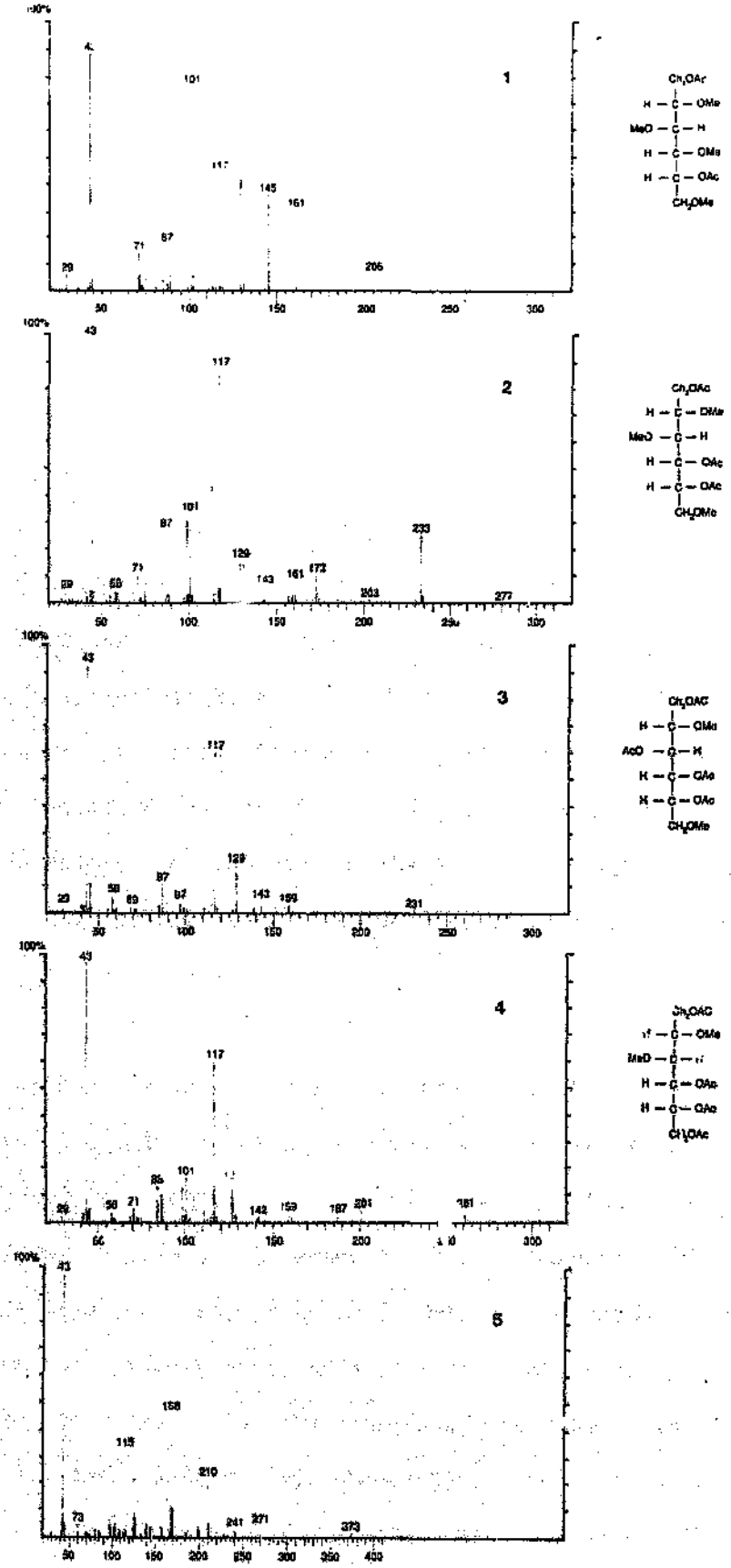



**Figure 3.12** Mass spectra of alditol acetates formed by the methylation of maltose, glycogen, amylopectin, amylose, amyloglucan and soluble glucan produced from amylopectin.

1. 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol
2. 1,45-tri-O-acetyl-2,3,6-tri-O-methyl-D-glucitol.
3. 1,3,4,6-tetra-O-acetyl-2,6-di-O-methyl-D-glucitol.
4. 1,4,5,6-tetra-O-acetyl-2,3,-di-O-methyl-D-glucitol.
5. myo-inositol hexa-acetate (internal standard).

Ac acetate ($CH_3CO$–) GROUP
Me methyl ($CH_3$–) GROUP

amylopectin. An unexpected finding was the presence of 1,3,4,5-tetra-O-acetyl-2,6-di-methyl-D-glucitol. Amyloglucan and soluble glucan contained smaller amounts of this glucitol acetate than amylopectin (Table 3.12).

In the methylation analysis of amylopectin undertaken by Kvernheim in 1987 no 1,3,4,5-tetra-O-acetyl-2,6-di-methyl-D-glucitols were found. Furthermore, infra-red analysis did not show the presence of 1,3-linkages which occur at 793 $cm^{-1}$ (Figure 3.8). These observations suggest the 1,3,4,5-tetra-O-acetyl-2,6-di-methyl-D-glucitols either were produced from contaminants in the amylopectin or were artifacts formed during the methylation analysis. They were obtained during the analysis of glycogen as well as of amylopectin, amyloglucan and the soluble glucan which suggests they were not contaminants from amylopectin but were formed during the methylation analysis.

A further reason to suspect the 1,3,4,5-tetra-O-acetyl-2,6-di-methyl-D-glucitols were artifacts was the short chain length of 21 obtained for amylopectin. This is less than the 24 obtained in previous studies (Greenwood, 1970; Manners, 1985a). If the percentage of 1,3,4,5-tetra-O-acetyl-2,6-di-methyl-D-glucitols was added to 1,4,5-tri-O-acetyl-2,3,6-tri-methyl-D-glucitols the figure would be closer to the published results. This suggests the 1,3,4,5-tetra-O-acetyl-2,6-di-methyl-D-glucitols were formed from alpha-1,4-D-glucose units because there was undermethylation. This proposal is supported by Manners (1989) who states other isomers may be formed under these conditions. The same glucitols were present amyloglucan and the soluble glucan which indicates similar

Table 3.12 Molar percentages of partially methylated glucitol acetates (Glc) obtained during methylation analysis of amylopectin, amyloglucan and the soluble glucan. Retention (R) times are relative to myo-inositol hexaacetate which was used as an internal standard

| Glucan | 2,3,4,6-Glc | 2,3,6-Glc | 2,6-Glc | 2,3-Glc |
|---|---|---|---|---|
| amylopectin | 3,9 | 82,6 | 7,7 | 5,8 |
| amyloglucan | 2,8 | 89,7 | 2,6 | 4,9 |
| soluble glucan | 2,9 | 84,6 | 4,8 | 7,7 |
| Relative R time | 0,887 | 0,932 | 0,957 | 0,969 |

adjustments should be made for these polymers. Therefore, the chain length for amyloglucar was adjusted from 32 to 33 and the soluble glucan from 30 to 31 (Table 3.13).

Apparently from the above information the chains in amyloglucan and the soluble glucan consist of glucose units joined by 1,4-linkages. In addition there are 1,4,6-linkages present in these polymers.

Table 3.13 Comparison of the chemical properties of amylose, amylpectin, amyloglucan and the soluble glucan produced from amylopectin by *S. sanguis* I 204. Information on amylose and amylopectin was obtained from Bathgate and Manners (1966), Bender, Siebert and Stadler-Szoke (1982) and Manners (1985a). The figure for amylopectin in brackets were the results obtained from the present study.

| Property | Amylose | Amylopectin | Amyloglucan | Soluble glucan |
|---|---|---|---|---|
| General structure | essentially unbranched | branched | less branched | less branched |
| Average chain length | approximately $10^3$ | 24 (23) | 33 | 31 |
| Exterior chain length | | 15 (15) | 19 | 21 |
| Interior chain length | | 9 ( 7) | 13 | 9 |
| % Conversion to maltose | | | | |
| with β-amylase | approximately 70 | approximately 55 (56) | 62 | 59 |
| with α-amylase | approximately 110 | approximately 90 (92) | 89 | 86 |
| Bonds chains | 1, 4 | 1, 4 | 1, 4 | 1, 4 |
| branches | 1, 4, 6 (few) | 1, 4, 6 | 1, 4, 6 | 1, 4, 6 |

3.2.5 Enzymic analysis

Debranching of amyloglucan and soluble glucan by isoamylase indicated the length of A and B-chains whereas digestion by beta-amylase determined the exterior and interior chain lengths.

3.2.5.1 Debranching with isoamylase

Examination of the chain profile by debranching with isoamylase and fractionation of the debranched polysaccharides by gel permeation chromatography showed a bimodal distribution of chains. The two major peaks corresponded to chain lengths of approximately 20 and 40. Chains with lengths of 20 glucose units had a partition coefficient (Ka) of 0,75 and consisted of A-chains and short B-chains whereas the longer chains had a Ka of approximately 0,3 and represented long B-chains (Figure 3.13). The partition coefficient was determined from the following formula

$$Ka = \frac{Ve-Vo}{Vt-Vo}$$

Where Vo = void volume

Ve = elution volume

Vt = total volume

The chain profiles for amylopectin, amyloglucan and the soluble glucan were similar but the second peak was larger in samples of amyloglucan and the soluble glucan than in amylopectin. This confirmed the glucans contained more of the long B-chains than their parent polymer.

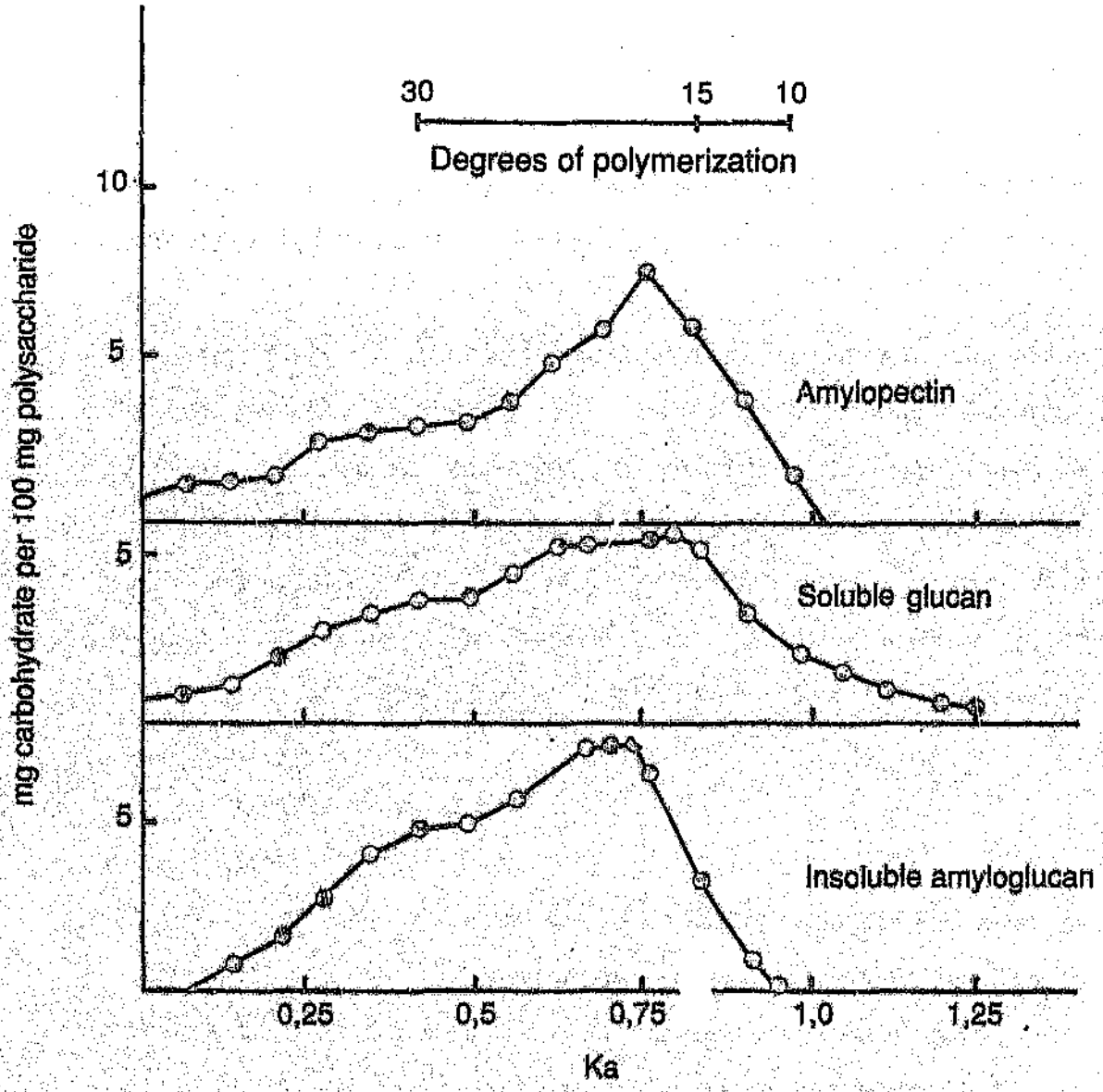



**Figure 3.13** Fractionation products of debranced amylopectin, amyloglucan and the soluble glucan produced from amylopectin by *S. sanguis 1* 204. The polysaccharides were debranced with isoamylase and the fractions were separated on a Sephadex G-50 column.

3.2.5.2 Digestion with amylases

Both amyloglucan and the soluble glucan were slightly less susceptible to hydrolysis by alpha-amylase than amylopectin. Thus 89 percent of the amyloglucan and 86 percent of the soluble glucan were converted to maltose (Table 3.13).

The digestion of amylopectin with beta-amylase gave a value of 56 percent which is slightly larger than published figures (Manners, 1985a). The enzyme converted 52 percent of the amyloglucan to maltose. This was slightly smaller than the 56 percent observed with amylopectin. On the other hand 59 percent of the soluble glucan was converted to sugars (Table 3.13). The exterior and interior chain lengths were calculated from these values and determined by the formula

$$ECL=(CL\times\beta\text{-limit})+2$$

$$ICL=CL-ECL-1.$$

The exterior and interior chains in amyloglucan and the exterior chains in the soluble glucan were about a third longer than in amylopectin. In contrast the interior chains in the soluble glucan did not change and were similar to those in amylopectin (Table 3.13).

## 3.3 Conclusion

The above results show amyloglucan is a highly insoluble polysaccharide that adheres readily to glass. Furthermore it contains longer exterior and interior chains than amylopectin. In contrast the soluble glucan is non-adherent. The lengths of the interior chains are similar to amylopectin and the exterior chains to amyloglucan. The glucose units in both polysaccharides are alpha-1,4-linked with branch points involving alpha-1,4,6-linkages.

# 4 ENZYMES THAT REGULATE STARCH METABOLISM

In the fourth part of this study the enzymes responsible for the degradation of amylose and amylopectin and the production of polysaccharides were studied.

## 4.1 IDENTIFICATION OF ENZYMES

The identity of the enzymes was determined initially by isolating the products of enzymic degradation. Further information about their molecular weight was obtained by separating some of the enzymes by gel eletrophoresis. The production of dextrans from sucrose by these enzymes confirmed they were glucosyltransferases.

### 4. Products of amylopectin degradation

The enzymes from S. mitis MC 101 and S. sanguis 1 MC 204 were the only extracts that degraded amylopectin to glucose and glucose oligomers detectable by high performance liquid chromatography. After 10 minutes incubation S. mitis MC 101 produced glucose, maltose, maltotriose, maltotetraose, maltopentaose and maltohexaose. Further incubation increased the concentration of these sugars, with the exception of maltopentaose which was absent. S. sanguis 1 MC 204 produced glucose, maltose and maltotriose after 10 minutes but no sugars after 24 hours incubation. Ten percent of the available amylopectin was digested by the enzyme extract from S. mitis MC 101 and 3 percent by S. sanguis 1 MC 204 (Table 3.14).

Table 3.14 Glucose and glucose oligomers produced from amylopectin by streptococcal enzymes.
(+ = present, 0 = not detected)

| Streptococcal species | Code | mg glucose and glucose oligomers produced from 500 mg amylopectin | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | G1 | G2 | G3 | G4 | G5 | G6 | G7 | G8 | G9 |
| S. anginosus | 719 | 0 | + | + | + | 0 | 0 | 0 | 0 | 0 |
| | 513 | 0 | 0 | + | + | 0 | + | 0 | 0 | 0 |
| | 307 | 0 | 0 | 0 | + | 0 | 0 | 0 | 0 | 0 |
| S. intermedius | 617 | 0 | 0 | + | + | 0 | + | 0 | 0 | 0 |
| | 411 | 0 | 0 | 0 | + | 0 | 0 | 0 | 0 | 0 |
| S. mitis | 101 | 10 min 8,3 | 8 | 3,4 | 2,1 | 1 | 1,1 | 0 | 0 | 0 |
| | | 24 h 14 | 15,3 | 8,1 | 5,9 | 0 | 5,7 | 0 | 0 | 0 |
| | 206 | 0 | + | + | + | + | + | 0 | 0 | 0 |
| | 410 | 0 | + | + | + | + | + | 0 | 0 | 0 |
| | 308 | 0 | + | 0 | + | 0 | + | + | 0 | 0 |
| | 514 | 0 | 0 | + | + | 0 | + | 0 | 0 | 0 |
| | 205 | 0 | 0 | + | + | 0 | 0 | 0 | 0 | 0 |
| S. sanguis I | FW213 | 0 | 0 | + | + | + | + | 0 | 0 | 0 |
| | 204 | 10 min 5,4 | 5,2 | 2,2 | 1 | 0 | 0 | 0 | 0 | 0 |
| | | 24 h 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 616 | 0 | 0 | + | + | 0 | + | + | 0 | 0 |
| | 102 | 0 | 0 | + | + | 0 | + | 0 | 0 | 0 |
| | 718 | 0 | 0 | 0 | + | 0 | 0 | 0 | 0 | 0 |
| S. sanguis II | 103 | 0 | + | + | + | + | + | 0 | 0 | 0 |
| | 412 | 0 | 0 | + | + | 0 | + | 0 | 0 | 0 |
| | 615 | 0 | 0 | + | + | 0 | + | 0 | 0 | 0 |
| | 720 | 0 | + | + | 0 | 0 | 0 | 0 | 0 | 0 |
| | 309 | 0 | 0 | 0 | + | 0 | 0 | 0 | 0 | 0 |

High performance liquid chromatography of the remaining digests did not show the presence of glucose oligomers even though they were concentrated tenfold by freeze drying. However thin layer chromatography showed they produced a variety of oligomers from amylopectin. They ranged from maltose to maltoheptaose but were present at a concentration of less than 0,1 mg percent which is below the limit of detection by high performance liquid chromatography (Table 3.14).

A large peak identified as soluble dextrins consisting of 12 to 15 glucose units was present in some of the samples analysed by high performance liquid chromatography. The amount varied from 9 mg for S. anginosus MC 307, S. mitis MC 308 and S. sanguis 1 MC 102 to approximately 200 mg for S. sanguis 1 MC 204 and MC 610.

4.1.2 Products of amylose degradation

Amylose digests were tested for glucose and glucose oligomers after 10 minutes and 24 hours. Sugars were produced but their concentration was less than 0,1 mg per 100 ml amylose broth. This was below the limit for the detection by high performance liquid chromatography. Thin layer chromatography however, showed the extracts from S. sanguis 1 MC 204 produced glucose, S. mitis MC 101 maltose and maltononaose, S. mitis MC 205 and S. sanguis 1 MC 718 maltotetraose and S. mitis MC 410 maltooctaose. The remaining enzymes produced a variety of glucose oligomers but not glucose (Table 3.15).

Table 3.15 Glucose and glucose oligomers produced from amylose by streptococcal enzymes. (+ = present, o = not detected)

| Streptococcal species | Code | Glucose and glucose oligomers produced from amylose | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | G1 | G2 | G3 | G4 | G5 | G6 | G7 | G8 | G9 |
| S. anginosus | 513 | 0 | + | + | + | 0 | + | 0 | 0 | 0 |
| | 719 | 0 | + | + | + | 0 | 0 | 0 | 0 | 0 |
| | 307 | 0 | 0 | 0 | 0 | + | 0 | 0 | 0 | 0 |
| S. intermedius | 617 | 0 | 0 | + | + | + | 0 | 0 | 0 | 0 |
| | 411 | 0 | 0 | 0 | + | 0 | 0 | 0 | 0 | 0 |
| S. mitis | 206 | 0 | + | + | + | + | + | 0 | 0 | 0 |
| | 308 | 0 | 0 | 0 | + | 0 | + | 0 | 0 | 0 |
| | 514 | 0 | 0 | 0 | + | 0 | + | 0 | 0 | 0 |
| | 101 | 0 | + | 0 | 0 | 0 | 0 | 0 | 0 | + |
| | 105 | 0 | 0 | 0 | + | 0 | 0 | 0 | 0 | 0 |
| | 410 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | + | 0 |
| S. sanguis I | FW213 | 0 | + | + | + | + | + | + | 0 | 0 |
| | 102 | 0 | 0 | + | + | + | + | + | + | + |
| | 616 | 0 | + | + | + | + | + | + | 0 | 0 |
| | 204 | + | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 718 | 0 | 0 | 0 | + | 0 | 0 | 0 | 0 | 0 |
| S. sanguis II | 103 | 0 | + | + | + | + | 0 | 0 | 0 | 0 |
| | 412 | 0 | + | + | + | 0 | + | 0 | 0 | 0 |
| | 720 | 0 | + | + | + | 0 | + | 0 | 0 | 0 |
| | 615 | 0 | 0 | + | + | + | + | 0 | 0 | 0 |
| | 309 | 0 | 0 | 0 | + | 0 | + | + | 0 | 0 |

The products obtained from the digestion of amylopectin and amylose suggest the enzyme extracts contained alpha-amylases that were not as active as similar enzymes from other sources.

4.1.3 Molecular weight of the enzyme

As was mentioned previously the molecular weight of the amylopectin hydrolysing enzyme was determined by separating the enzymes on SDS polyacrlyamide gel containing amylopectin and comparing their migration distance with molecular weight markers. In addition the production of dextran from sucrose was studied by incubating these gels in a buffer with sucrose.

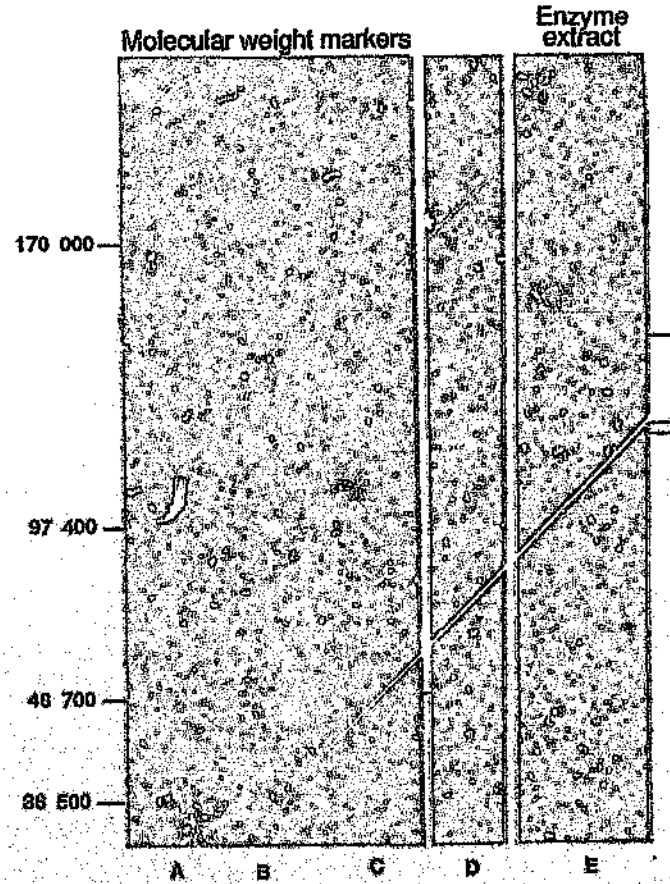
The crude enzyme extracts from S. sanguis 1 MC 204 and S. mitis MC 101 both contained starch hydrolysing enzymes. The extract from S. sanguis 1 MC 204 was more active than from S. mitis MC 101. An amylopectin gel containing these enzymes stained pink after several hours treatment with periodic acid Schiff stain. Hydrolysis of amylopectin was observed as clearing of the gel in the region of 120 000 Da. Possible dextran production appeared as blue bands in the centre of this clearing (Figures 3.14 and 3.15). A single starch hydrolysing enzyme was present in the extracts because only one area of clearing was observed.

The production of dextrans by glucosyltransferases appeared as one major band of 156 000 Da and several minor bands. Initially this area did not stain with periodic acid Schiff stain but developed a dark blue colour overnight. Dextran production by the major band

was prolific so that it projected above the surface of the gel after staining (Figures 3.14 and 3.15). The dextran producing enzymes in the gel did not produce polysaccharides when they were incubated in the presence of raffinose which proves they were glucosyltransferases and not fructosyltransferases (Russell, 1979). Likewise, the amylopectin hydrolysing enzymes in the gel did not produce visible glucans when they were incubated in buffer containing amylopectin. Furthermore, the presence of dextran T 10 in the buffer did not influence glucan production.

### 4.1.4 Conclusion

The above results indicate S. sanguis 1 204 and S. mitis MC 204 both possess alpha-amylases that metabolise starch to produce glucose and oligosaccharides and glucosyltransferases that produce dextran from sucrose. The glucosyltransferases may also produce amyloglucan and soluble polysaccharide from amylopectin.



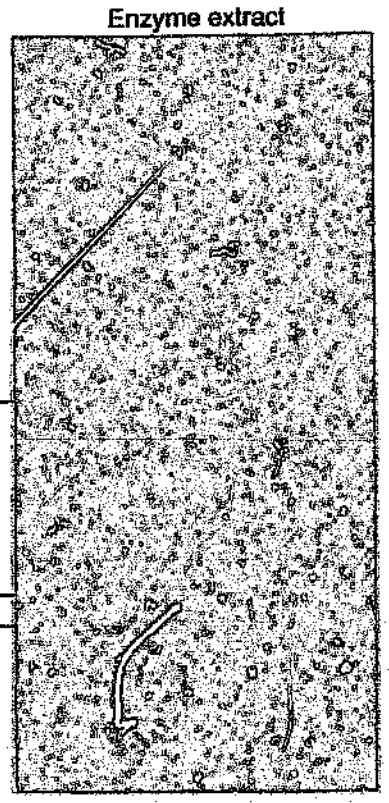




**Figure 3.15**
High magnification of SDS gel stained with periodic acid Schiff stain. Hydrolysis of amylopectin shows as a clear area whereas dextrans appear as blue bands.

Figure 3.14 Photograph of SDS gel electrophoresis of a crude enzyme extract of S. sanguis 1 204 separated on 7,5 percent acrylamide containing 0,5 percent amylopectin. After electrophoresis the gel was incubated in 5 percent sucrose for 72 hours and stained with the periodic acid Schiff stain. Amylopectin stained pink, hydrolysis of amylopectin appeared as clear areas in the gel and dextran production as blue bands. Molecular weight markers were stained with a silver stain. Lane A shows lactate dehydrogenase (Mr 36 500), lane B phophorylase b (Mr 97 400), lane C alpha-2-macroglobulin (Mr 170 000), lane D clearing of amylopectin by alpha-amylase (Mr 48 000) and lane E clearing of amylopectin by the enzyme extract with minor dextran producing bands of Mr 123 000 and 120 000 within the clear area. The major dextran producing band stained darkly with an Mr of 156 000.

# CHAPTER 4

# DISCUSSION

## CONTENTS

1 GROWTH OF BACTERIA.....174

1.1 Growth on amylose and amylopectin.....174
1.2 Stimulation and inhibition.....176
1.3 Conclusion.....176

2 ACID PRODUCTION FROM STARCH.....177

2.1 Production of glucose and reducing sugars.....177
2.2 Acid production.....178
2.2.1 Change in pH.....178
2.2.2 Lactic acid production.....178
2.2.3 Acetic acid production.....179
2.2.4 Succinic acid production.....180
2.3 Acids and dental caries.....182

3 GLUCAN PRODUCTION FROM STARCH.....183

3.1 Production and adherence of amyloglucan.....183
3.2 Solubility of amyloglucan.....183
3.3 Synthesis.....184
3.4 Changes in chain length.....185
3.6 Conclusion.....186

4 ENZYMES THAT REGULATE STARCH METABOLISM.....187

4.1 Enzyme production.....187
4.2 Identification of the enzymes.....188

5 CONCLUSION.....191

LIST OF ILLUSTRATIONS

Figure 4.1 The production of lactic, acetic and succinic acid from starch.....181

# 1 GROWTH OF BACTERIA

Growth of bacteria can be measured by observing an increase in bacterial numbers. If the logarithm of the number of bacteria in a culture is plotted against time a growth curve will be obtained. The curve can be divided into 4 phases, the lag phase, the logarithmic phase, the phase of stationary growth and the phase of decline (Wilson & Miles, 1975).

## 1.1 Growth on amylose and amylopectin

The streptococci produced 4 different growth curves on amylose and amylopectin. They either showed poor adaptation, required time to adapt but grew vigorously, exhibited diauxic growth or adapted well and grew vigorously (Chapter 3, Figures 3.2, 3.3 and 3.4).

Strains may have adapted poorly because there was a low concentration of nucleotides in the broths and streptococci have a specific requirement for these substances (Doelle, 1969). Another reason may be the glucose concentration, because strains grown in Todd Hewitt broth which contains 0,2 percent glucose were transferred to starch broths with less than 0,01 percent glucose (Chapter 3, Section 2.1). Cells from Todd Hewitt would have low levels of glucose-phosphoenolpyruvate phosphotransferase and glyceraldehyde-3-phosphate dehydrogenase and low concentrations of metabolites produced by these enzymes. When they were transfered to the starch broths they would require time to increase these intracellular metabolites.

Another reaction of isolates was a long lag phase followed by a period of growth. This may be caused by many factors including the need to produce energy to activate sugar transport systems. A glucose sugar transport system involving a phosphoenolpyruvate phosphotransferase system has been described in S. mutans and S. salivarius (Kanapka and Hamilton, 1971; Schachtele and Mayo, 1973; Schachtele, 1975; Schachtele and Leung, 1975). A similar system probably operates in the starch hydrolysing streptococci.

A further reaction of some strains was diauxic growth. These isolates probably possessed constitutive enzymes that enabled them to utilize glucose and other simple constituents in the medium. Once this source was exhausted, growth slowed down until they synthesize new inducible enzymes for the utilization of amylose and amylopectin. All strains that exhibited diauxic growth grew vigorously after the induction of starch hydrolysing enzymes which shows enzyme induction was highly successful.

The remaining 40 percent of isolates grew vigorously. Vigorous isolates were obtained from all subjects with the exception of patient 6. The rapid growth may be the result of 'downhill flow' as distinct from active transport which requires the expenditure of energy. Under these conditions the system becomes uncoupled from the energy supply and functions as a passive transport system which enables sugars to diffuse into the cell (Davis et al, 1980).

## 1.2 Stimulation and inhibition

Some isolates were stimulated by amylose and others by amylopectin (Chapter 3, Table 3.2). This reaction suggests the streptococci possessed different enzymes for the utilization of starches. If amylose stimulated growth they possessed enzymes for the degradation of long chains of glucose units. On the other hand if amylopectin stimulated growth, they have enzymes that act in the vicinity of either branches or end groups because amylopectin is highly branched with 4 percent of the glucose units available as end groups and another 4 percent as branch points (Williams, 1968).

Some isolates were inhibited by the presence of starch (Chapter 3, Table 3.2). This inhibition may be caused by many factors including amylose and amylopectin competing for enzymes in the sugar transport system or catabolite repression by cyclic adenosine monophosphate (Stent and Calender, 1971; Adams et al. 1981).

## 1.3 Conclusion

The present study has shown some streptococci adapted more readily than others to growth on starch. These differences could either be caused by factors present in the growth medium, by transport systems in the cells or by enzymes produced by these isolates. Almost half the isolates possessed constitutive enzymes that degraded both amylose and amylopectin. Others exhibited dauxic growth which indicated their starch degrading enzymes were inducible. However, the growth patterns in these starches were not similar which suggests different enzymes are involved in the hydrolysis of amylose and amylopectin.

## 2 ACID PRODUCTION FROM STARCH

The production of acids from starch was preceeded by the degradation of amylose and amylopectin to glucose and reducing sugars.

### 2.1 Production of glucose and reducing sugars

Cultures grown in yeast extract, amylose and amylopectin produced a slight change in the glucose content of the medium (Chapter 3, Table 3.3). However, vigorous strains either reduced the level of glucose or released small amounts of this sugar into the broths. Furthermore, strains that grew poorly allowed glucose to accumulate. These changes suggest that glucose was either utilized immediately for growth and acid production or it accumulated and growth was poor because the streptococci were unable to metabolize this sugar.

More reducing sugars were released from amylopectin than amylose (Chapter 3, Table 3.3). This difference may have been caused by the availability of end groups in the starch molecule. There are approximately 7000 ends in an amylopectin molecule whereas amylose contains only 8 end groups (Greenwood, 1970: Takeda et al , 1984). Streptococcal enzymes probably attacked these ends and released glucose and oligosaccharides. Many of these sugars accumulated (Chapter 3, 3.4) which suggests that several isolates were unable to utilize oligosaccharides. This finding was not unexpected because Glor, Miller and Spandau (1988) showed almost half the oral streptococci they studied were unable to utilize all the oligosaccharides they tested.

## 2.2 Acid production

The production of acids was studied by measuring a change in pH and identifying acids present in the broths.

### 2.2.1 Change in pH

Only three isolates lowered the pH of starch broth to between 5,4 and 5.7 (Chapter 3, Figures 3.5, 3.6 and 3.7) which is within the limits for the decalcification of teeth (van Houte, 1980). However, these strains were grown in pure culture whereas all communities in the mouth are mixed. A more accurate reflection of their caries potential could be gained from the mixtures containing these isolates. They lowered the pH to between 6,5 and 7,1 (Chapter 3, Figure 3.6). None of these values are within the critical pH which suggests *in vivo* demineralization of enamel by these streptococci would be slow.

### 2.2.2 Lactic acid production

Lactic acid was produced by all the cultures and predominated in almost half the cultures and in most isolates which decreased the pH to below 6 (Chapter 3, Table 3.8). Lactate may have predominated because the cultures grew vigorously and an ATP-energized system generating a proton motive force would have functioned during the transport of sugars (Ellwood and Hamilton, 1982; Hamilton and St. Martin, 1982).

2.2.3 Acetic acid production

Most isolates produced a mixture of lactic, acetic and succinic acids whereas acetic was the major acid in almost half these cultures (Chapter 3, Table 3.8). This change to acetic acid production may have been caused by the incubation atmosphere and glucose concentration. The cultures were aerated and an aerobic atmosphere changes the products of fermentation from mainly lactic to acetic acid (Gottschalk, 1979; Stamer and Stoyla, 1967) by inhibiting lactate dehydrogenase (Mickelson 1969,1972; Thomas, Ellwood and Longyear, 1979). Some isolates did not produce much acetic acid which suggests they possessed lactate dehydrogenase that was insensitive to oxygen.

The activity of lactate dehydrogenase may also be influenced by the glucose content of the broths. With glucose limitation a mixture of acids is produced whereas under conditions of excess lactate predominates (Ellwood, Hunter and Longyear, 1974; Carlsson and Griffiths, 1974; Hamilton, Phipps and Ellwood, 1979; Kemp et al, 1983). Four cultures probably used starch to produce high levels glucose and changed their acid production from mainly acetic to lactic acid when they were grown in starch as opposed to yeast extract (Chapter 3, Table 3.8).

Some strains may have produced acetic acid because they formed hydrogen peroxide. The streptococci contain peroxidases which catalyse the oxidation of organic compounds or NADH with hydrogen peroxide. When NADH is oxidized with oxygen, pyruvate can be converted to acetyl-coenzyme A and additional ATP can be formed by the acetate kinase reaction (Carlson, Iwami and Yamada, 1983). A

further source of acetate may be via a phophoclastic reaction. According to Doelle (1975) Acetyl-Coenzyme A (CoA) is formed in the presence of acetyl transferase which transfers the C2 unit from the pyruvate formate exchange system to the acceptor. A phosphate acetyltransferase and acetate kinase converts acetyl-CoA via acetyl phosphate to acetate with a concomitant synthesis of ATP (Figure 4.1).

### 2.2.4 Succinic acid production

Succinic acid was found in small amounts in most cultures but was the major acid produced by 3 strains from subject 3 (Chapter 3, Table 3.8). This metabolic diversion from pyruvate which has not been described in the streptococci could be caused by the functioning of pyruvate carboxylase which converts pyruvate to oxalacetate (Doelle, 1975).

Lactate is oxidized to pyruvate in bacteria in a reaction requiring a flavoprotein as hydrogen acceptor. Oxalacetate is formed from pyruvate in a transcarboxylation reaction with (S)-methylmalonyl-coenzyme A as carbon dioxide donor and biotin as carbon dioxide carrier of the enzyme pyruvate carboxylase. The subsequent action on oxaloacetate of malate dehydrogenase and fumarase yields fumarate which is reduced to succinate by fumarate reductase (Figure 4.1). Reduction is coupled to ATP formation by electron transport phosphorylation (Gottshalk, 1979). The mechanism would be similar to the production of succinate in the Propionibacteriae via enzymes of the tricarboxylic acid cycle working in reverse (Doelle, 1975).

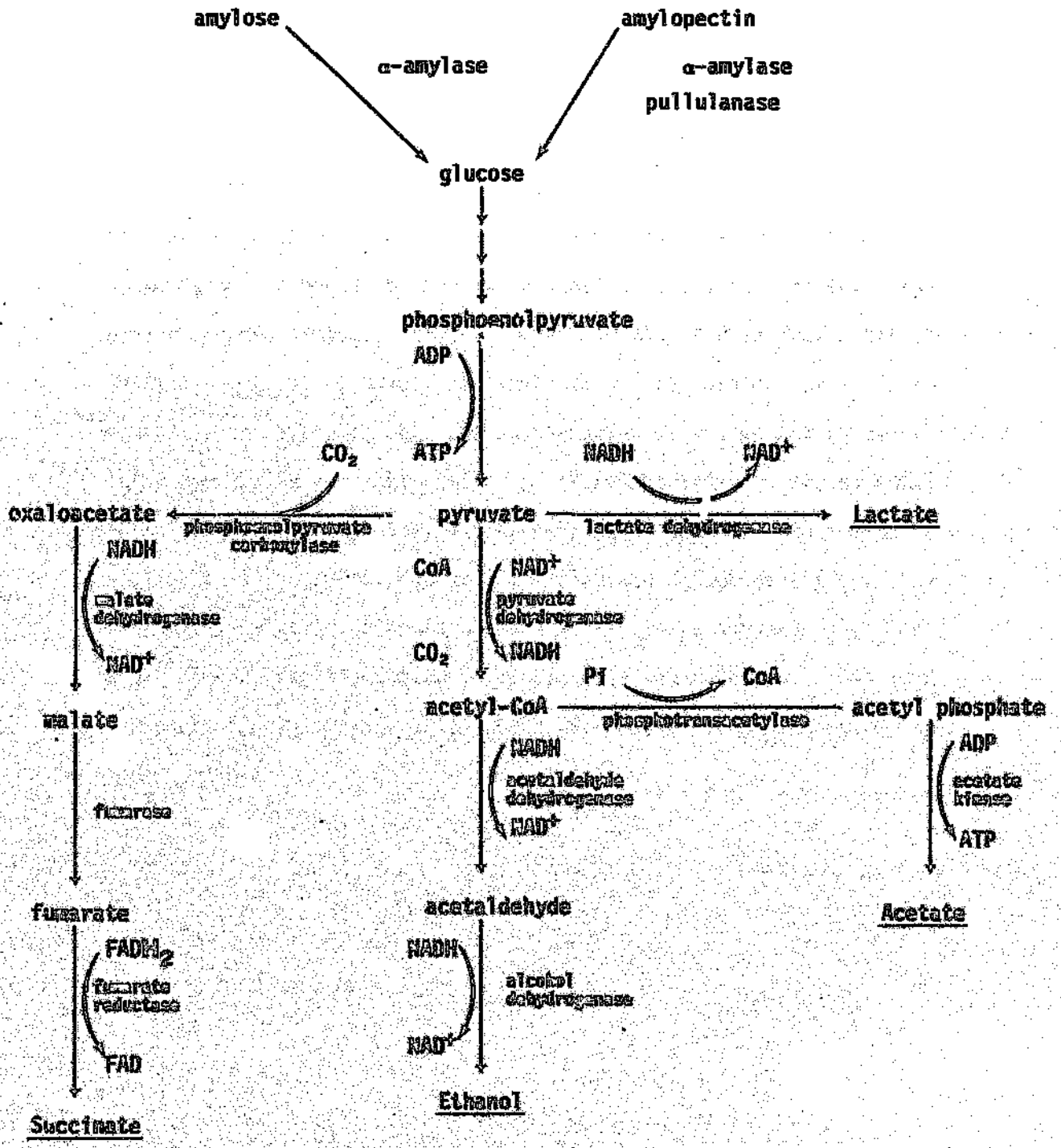



Figure 4.1. The production of lactic, acetic and succinic acid from starch

## 2.3 Acids and dental caries

In the present study cultures from carious lesions did not produce more lactic or acetic acid than isolates from healthy teeth (Part II, Chapter 3, Table 3.5). This implies there is no difference between organisms from carious lesions and healthy teeth, an observation supported by Drucker and Melville (1968). Nevertheless caries prone subjects produce more acid than caries free patients (Vratnos et al , 1979). An increase in acid production could be caused, for example, by the consumption of a high starch diet. This may stimulate the growth of starch hydrolysing streptococci that produce amyloglucan, encourage plaque formation and create an anaerobic environment that activates lactate dehydrogenase. If the starch hydrolysing streptococci release large amounts of glucose from starch the production of lactic acid would follow. This acid is particularly harmful to teeth (Geddes, 1972; Featherstone and Rogers, 1981).

## 3 GLUCANS PRODUCED FROM STARCH

Two glucans were produced from amylopectin by starch hydrolysing streptococci. The first, a soluble polymer did not adhere to glass and the second amyloglucan was insoluble and adherent.

### 3.1 Production and adherence of amyloglucan

The present study has shown most starch hydrolysing strains produced an insoluble amyloglucan (Chapter 3, Table 13.10) that adhered to glass (Chapter 2, Section 3.1). The ability to attach to glass is proof that a polysaccharide will attach to teeth and accumulate in plaque (Wood and Critchley, 1966; Guggenheim and Schroeder, 1967). Several workers have reported the presence of polysaccharide with a high glucose content in plaque and pellicle (Guggenheim and Schroeder, 1967; Newbrun, Lacey and Christie, 1971; Nalbandian et al, 1974; Sonju et al, 1975; Johnson et al, 1977). One of these products could be amyloglucan because it attaches to glass and consists of glucose units.

### 3.2 Solubility of amyloglucan

Amyloglucan did not dissolve in any of the solvents tested (Chapter 2, Table 3.11) which suggests it will accumulate on teeth. The polymer was also resistant to attack by alpha-amylase (Chapter 3, Table 3.13) therefore, it would not be degraded by this enzyme in saliva and plaque (Fisher and Stein, 1960, 1961; Ruby and Gerencser, 1974). In this respect it may be similar to mutan which

accumulates in plaque and enables S. mutans to attach to teeth (Rolla et al, 1983). Furthermore, amyloglucan is less soluble than mutan (Chapter 3, Table 3.11) which suggests it would accumulate more readily.

Amyloglucan may be insoluble for the same reasons that amylose and mutan do not dissolve readily. For example, amylose is a linear molecule with a uniform structure that forms strong intermolecular bonding, has extended ribbon-like structures and contains few branches (Whistler, 1973). Mutan has similar characteristics, it is a linear molecule consisting of alpha-1,3-linkages that form ribbon-like molecules that are nearly fully extended. The chains pack readily into dense, completely water-insoluble microfilms with extensive intra- and intersheet hydrogen bonding (Marchessault and Deslandes, 1981). Elongation of the chains in amyloglucan may have enabled them to form ribbon-like structures with strong intermolecular bonding.

## 3.3 Synthesis

Amyloglucan and the soluble glucan could be synthesized either from the degradation products of amylopectin or by modification of this molecule. There are several reasons to support the idea they are modified amylopectin molecules. Firstly, the infra-red spectrum for amyloglucan and the soluble glucan produced by S. sanguis 1 204 was similar to amylopectin and not the dextran produced from sucrose and the mutan produced by S. mutans. Secondly, all three polymers contained alpha-1,4-linked glucose units (Chapter 3,

Figure 3.8). Thirdly, the methylation analysis showed the glucose units in the three polymers were alpha-1,4-linked with alpha-1,4,6-branches (Chapter 3, Table 3.13). Fourthly, digestion of amylopectin, amyloglucan and the soluble glucan with isoamylase yielded similar chain profiles (Chapter 3, Figure 3.13).

## 3.4 Changes in chain length

An important finding in this study was the increase in chain lengths. The first indication was obtained from debranching with isoamylase. The peak for the longer B-chains consisting of 40 glucose units was larger for amyloglucan and the soluble glucan than for amylopectin (Chapter 3, Figure 3.13). These results were confirmed by the methylation analysis which showed there was an overall increase in the chain length in both amyloglucan and the soluble glucan (Chapter 3, Table 3.13). Furthermore, there was a slight increase in the iodine binding capacity of the soluble glucan when compared with amylopectin (Chapter 3, Table 3.11).

A more detailed study using enzymes showed the exterior chain lengths increased in the soluble glucan whereas both the exterior and interior chain lengths were longer in amyloglucan (Chapter 3, Table 3.13). These changes could be caused by the removal of side chains from the amylopectin molecule to form amyloglucan. The formation of soluble glucan could be the first stage in this process whereby side chains in the exterior part of the molecule are removed. This would increase the length of the remaining exterior chains. Thereafter, further side chains could be removed

from the interior part of the molecule. This would result in an increase in the length of interior and exterior chains and lead to the formation of amyloglucan. Some of the released side chains could be added to the ends of the remaining chains to increase their length even further.

## 3.5 Conclusion

The present study has shown amylopectin can be modified by the oral streptococci to form a highly adherent insoluble amyloglucan and a nonadherent soluble glucan. They are both produced by modification of the amylopectin molecule and contain glucose units that are alpha-1,4-linked with branch points involving alpha-1,4,6-linkages. The soluble glucan with long exterior chains binds iodine readily. In contrast, amyloglucan has elongated interior and exterior chains but has lost the ability to bind iodine. This decrease in iodine binding capacity may not be associated with changes in chain length but could be caused by an increase in the complexity of the amyloglucan molecule.

# 4 ENZYMES THAT REGULATE STARCH METABOLISM

The identity of the starch hydrolysing enzymes produced by the oral streptococci was determined by studying the products of enzyme activity. Further properties of these enzymes were examined by gel electrophoresis.

## 4.1 Enzyme production

Both cells and the cell free extract produced amyloglucan (Chapter 3, Section 3.1) which is proof the enzymes responsible for amyloglucan production are extracellular. If they were produced in the mouth some would attach to plaque bacteria and others would be released and occur in saliva.

Amyloglucan accumulated on the surface of the bottles (Chapter 3, Section 3.1) probably because the polymer producing enzymes were attached to the glass. This suggests they may absorb to enamel and produce insoluble adherent amyloglucan on the tooth surface. In this respect they may be similar to the glucosyltransferase from *S. mutans* which are found in saliva and adsorb to the teeth where they produce an insoluble alpha-1,3-glucan that accumulates and contributes to the formation of dental plaque (Rolla *et al*, 1983).

The dextran producing enzymes in the gel did not produce polysaccharides when they were incubated in the presence of raffinose which proves they were glucosyltransferases and not fructosyltransferases (Russell, 1979). However, the amylopectin

hydrolysing enzymes did not produce visible amyloglucans when they were incubated in buffer with amylopectin solution. Furthermore, the presence of dextran T 10 in the buffer did not influence amyloglucan production. This does not necessarily mean amyloglucan production did not occur in the gel. There may be two reasons for the non-detection. First, the production of this polymer is not as prolific as dextran production and second, amyloglucan does not stain with the periodic acid Schiff stain

## 4.2 Identification of the enzymes

The enzyme extracts produced glucose and glucose oligomers from both amylopectin and amylose (Tables 3.14 and 3.15). These products are similar to the oligomers produced by the alpha-amylases of S. bovis and S. equinus (Walker, 1965a, 1965b; Boyer and Hartman, 1971). However, the digest also contained a soluble glucan and an insoluble amyloglucan (Chapter 3 Section 3.1). This action suggests the starch metabolising oral streptococci produce an unusual alpha-amylase that releases a greater variety of products than has previously been ascribed to these enzymes.

A reason for believing these enzymes are not previously identified alpha-amylases is their molecular weight. Salivary and bacterial alpha-amylases have weights that vary from 15 000 to 55 000 Da. In contrast the enzymes from S. mitis 101 and S. sanguis 1 204 consisted of 2 bands with a molecular weight of 123 000 and 120 000 Da (Chapter 3, Figures 3.14 and 3.15).

The enzymes in the present study are not glycogen synthase-branching enzymes for two reasons. Firstly, they are extracellular in contrast to the intracellular glycogen synthase-branching enzyme of S. mitis (Builder and Walker, 1970; Hamilton, 1976; Tanzer et al, 1976; Dewar, 1977; Birkhed and Tanzer, 1979). Secondly, they produced amyloglucan which is less branched than amylopectin and their action would have decreased rather than increased the number of alpha-1,6-linkages in the molecule.

The most likely possiblity is that these enzymes are glucosyltransferases with hydrolytic function because the amylopectin did not stain initially where dextrans were produced in the 156 000 Da region (Chapter 3, Section 4.2). The basic mechanism for glucosyltransferase activity is disproportionation in which fructose is released from sucrose and glucose is formed into a polymer consisting of alpha-1,6-linkages (Ditson and Mayer, 1984). Glucosyltransferases acts on the alpha-1,6-linkages in glucans (Chassy, 1883) which suggests they will hydrolyse these linkages in amylopectin.

The methylation studies have shown amyloglucan has longer chains than amylopectin (Chapter 3, Table 3.13) and fewer alpha-1,4,6-linkages. The decrease in the number of 1,4,6-linkages may be caused by the removal of A-chains and the ends of B-chains by the hydrolysis of 1,6-linkages by the 156 000 Da glucosyltransferase. Hydrolysis may occur in two stages. During the first stage A and the ends of B-chains in the exterior part of the molecule may be removed at random to form a soluble glucan with

elongated B-chains. Thereafter the same enzyme could remove interior A-chains to form insoluble amyloglucan with elongated B-chains that carry fewer A-chains in both the exterior and interior part of the molecule (Chapter 3, Table 3.13). This proposal is supported by the increased yield of the longer B-chains when amyloglucan by digested by isoamylase (Chapter 3, Figure 3.13).

Glucose and other small oligosaccharides were also formed from amylopectin. These sugars would not be released by glucosyltransferases because this enzyme will not act on the 1,4-linkages in amylopectin (Chassy, 1983). However, the starch hydrolysing enzyme of 120 000 Da is an alpha-amylase that hydrolyses 1,4-linkages. This enzyme may act primarily on the released chains to yield glucose, maltose and other small oligosaccharides. More work would be necessary to establish the identity of both these enzymes.

# 5 CONCLUSION

It is apparent that the starch hydrolyzing oral viridans streptococci can adapt to growth on amylose and amylopectin. However, some isolates adapted more readily than others. These differences could be caused by factors in the growth medium, transport systems in the cells and different enzymes produced by these isolates. Almost half the isolates possessed constitutive enzymes that degraded both amylose and amylopectin. However, the growth patterns in these two starches varied which suggests that different enzymes were involved in the hydrolysis of amylose and amylopectin.

All cultures produced a slight change in the glucose content of the broths. These changes suggest that glucose was utilized for growth as soon as it was produced. In contrast almost half the isolates produced more than 22 mg glucose oligomers in 24 hours. This accumulation of oligosaccharides suggests these isolates were unable to degraded these sugars.

The final pH of amylose and amylopectin broths was significantly lower than yeast extract, but there was no difference when organisms from carious lesions and healthy teeth were compared. The lowest pH was 5,4 but most cultures did not reduce the pH to below 6 which is above the pH for the decalcification of teeth. The final pH of the mixtures was even higher, the lowest being 6,4. This gives a clearer indication of the situation in the mouth in so far as all microbial communities in the oral cavity are mixed. Plaque containing a mixture of starch hydrolysing streptococci

would probably not decrease the pH of plaque sufficently to decalcify enamel.

Acetic acid predominated i.. 60 percent of the cultures grown on yeast extract. Acid production was probably influenced by the limited amount of carbohydrate in the medium because lactic acid was produced by most of these isolates when amylose and amylopectin was added. Lactic acid was also produced by isolates which grew vigorously. This was probably the result of efficient sugar transport into the cell.

Succinic acid was detected in small amounts in most cultures but it predominated in 3 cultures from subject 3 who had healthy teeth. None of the cultures produced formic acid. The reason is that the broths were aerated during incubation and the presence of oxygen probably inhibited the enzyme pyruvate formate-lyase which is responsible for the production of formic acid.

The main contribution of the present study has been the observation that two glucans are produced by the modification of the amylopectin molecule. The first is a non-adherent soluble glucan whose exterior chain lengths have increased. The second amyloglucan, is a highly insoluble polymer that adheres readily to glass. It is has interior and exterior chains that are longer than amylopectin. The glucose units in both polysaccharides are alpha-1,4-linked with branch points involving alpha-1,4,6-linkages.

These glucans were produced by enzymes that have been isolated from S. sanguis 1 204 and S. mitis 101. The enzymes are capable of

metabolising starch, producing glucose, oligosaccharides, amyloglucan and soluble glucan from amylopectin and dextran from sucrose. At least two enzymes are involved, one is a glucosyltransferase-like enzyme and the other an alpha-amylase.

This investigation has shown that strains of oral streptococci can degrade starch to acids. Furthermore, they can use this carbohydrate for the production of polysaccharides. Thus starch is similar to sucrose because both carbohydrates are metabolized to acids and polysaccharides. Both these products may be implicated in the carious process.

Finally, a comparison of organisms from carious lesions with isolates from healthy teeth showed they did not grow more vigorously, produce a lower pH and more acids. Nor was there a difference in glucan production. These findings are similar to a study reported by Drucker and Melville (1968) who found there was no differences between cariogenic and non-cariogenic streptococci when fermentation end-products and acid production were compared. The similarity between organisms from carious lesions and isolates from healthy teeth suggests that diet and not a characteristic flora may be primarily implicated on the carious process. This hypothesis is summed up by Levine who stated in 1977 that 'Thus it would appear that a cariogenic flora is encouraged by a cariogenic diet. The idea that caries susceptible people are those who are smitten with a particularly virulent organism is baseless and from no part of the present problem'.

# CHAPTER 5

## REFERENCES


Abbe, K, Takahashi, S & Yamada, T (1983) Purification and properties of pyruvate kinase from Streptococcus sanguis and activator specificity of pyruvate kinase from oral streptococci. Infection and Immunity, 39, 1007 - 1014.

Abdullah, M, Fleming, I D, Taylor, P M & Whelan, W J, (1913) Substrate specificity of the amyloglucosidase of Aspergillus niger Biochemical Journal, 89, 35 - 36.

Abdullah, M & French, D (1970) Substrate specificity of pullulanase, Archives of Biochemistry and Biophysics, 137, 483 - 493.

Adams, R L P , Burdon,R H , Campbell, A M , Leader, D P & Smellie, R M S (1981) In The Biochemistry of the Nucleic Acids 9th ed. Ch. 13. London: Chapman & Hall.

Ahmed, M & Lelievre, J (1978) Effect of various drying procedures on the crystallinity of starch isolated from wheat grains. Die Starke, 30, 78 - 79.

Akai, H, Yokobayashi, K, Misaki, A & Harada, T (1971) Structural analysis of amylopectin using Pseudomonas isoamylase. Biochimica et Biophysica Acta, 252, 427-431.

Alsberg, C L (1938) Structure of the starch granule. Plant Physiology, 13, 295 - 330.

Anderson, D M W, Greenwood, C T & Hirst, E L (1955) Physiochemical studies on starches. Part II. The oxidation of starches by potassium metaperiodate, Journal of the Chemical Society, 225 - 231.

Asem, K G, Kenney, A C & Cole, J A (1986) Origin and function of the multiple extracellular glucosyltransferase species from cultures of a serotype c strain of Streptococcus mutans . Archives of Biochemistry and Biophysics, 244, 607-618.

Bacon, J S D (1979) Factors limiting the action of polysaccharide degrading enzymes. In Microbial Polysaccharides and Polysaccharases Eds. Berkeley, R C W & Ellwood, D C, Ch. 11. London : Academic Press.

Badenhuizen, N P (1969) The biogenesis of starch granules in higher plants New York : Appleton Century Crofts.

Bahl, O P & Smith, F (1966) Studies on the anomalous linkages in glycogen and amylopectin. The periodate oxidation evidence. Journal of Organic Chemistry, 31, 2915 - 2920.

Bailey, J M & French D (1956) The significance of multiple reactions in enzyme-polymer systems. Journal of Biological Chemistry, 266, 1 - 14.

Baldwin, R R, Bear, R S & Rundle, R E (1944) The relation of starch-iodine absorption spectra to the structure of starch and starch components. Journal of the American Chemical Society, 66, 111 -

Balls, A K, Walden, M K & Thompson, R R (1948) A crystalline beta-amylase from sweet potatoes. Journal of Biological Chemistry, 173, 9 - 19.

Banks, W & Greenwood, C T (1959) Physiochemical studies on starches. Part XX. The existence of an anomalous amylopectin in starch. Journal of the Chemical Society, 3436 - 3439.

Banks, W & Greenwood, C T (1966) The fine structure of amylose. The action of pullulanase as evidence of branching. Archives of Biochemistry and Biophysics, 117, 674 - 675.

Banks, W & Greenwood, C T (1967) Physiochemical studies on starches. XXXII. The incomplete beta-amylolysis of amylose. A discussion of its cause and implications. Die Starke, 19, 197 - 206.

Banks, W & Greenwood, C T (1968) A noval method of determining the number-average degree of polymerization of linear maltodextrins and amylose. Carbohydrate Research, 6, 177 - 183.

Banks, W, Greenwood, C T & Thomson, J (1959a) Effect of oxygen-treatment on the beta-amylolysis of amylose. Chemistry and Industry, 928.

Banks, W, Greenwood, C T & Thomson, J (1959b) Properties of amylose as related to the fractionation and subfractionation of starch. Makromolecular Chemistry, 31, 197 - 213.

Barker, S A, Bourne, E J & Peat, S (1949) The enzymic synthesis and degradation of starch, Part IV. The purification and storage of the Q-enzyme of the potato. Journal of the Chemical Society, 1705 - 1717.

Barker, S A, Bourne, E J, Stacey, M & Whiffen, D H (1954) Infra-red spectra of carbohydrates, Part I. Some derivatives of D-glucopyranose. Journal of the Chemical Society, 171 - 176.

Barnham, D & Trinder, P (1972) An improved colour reagent for the determination of blood glucose by the oxidase system. Analyst, 97, 142 - 145.

Bates, F L, French, D & Rundle, R E (1943) Amylose and amylopectin content of starches determined by their iodine complex formation. Journal of the American Chemical Society , 65, 142 - 148.

Bathgate, G N & Manners, D G (1966) Multiple branching in glycogens. Biochemical Journal, 101, 3C - 5C.

Baum, H A & Gilbert, G A (1954) The anaerobic fractionation of potato starch. Chemistry and Industry, 490.

Bender, H (1981) A bacterial glucoamylase EC 3.2.1.3 degrading cyclodextrins partial purification and properties of the enzyme from a flavobacterium species. European Journal of Biochemistry , 115, 287 -292.

Bender, H, Siebert, R & Stadler-Szoke, A (1982) Can cyclodextrin glycosyl transferase EC 2.4.1.19 be useful for the investigation of the fine structure of amylopectins? Characterization of highly branched clusters isolated from digests with potato and maize starches. Carbohydrate Research, 110, 245 - 259.

Bender, H & Wallenfels, K (1961) Untersuchungen an pullalan II spezifischer abbau durchein baklerielles enzyme. Biochemische Zeitschrift , 334, 79-95.

Bernfeld, P (1951) Enzymes of starch degradation and synthesis Advances in Enzymology, 12, 379 - 428.

Bernfeld, P & Gurtler, P (1948) 22 Recherches sur l'amidon XXXVIII Methode perfectionnee de degradation beta-amylatique de l'amylose et de l'amylopectine. Helvetica Chimica Acta, 31, 106 - 108.

Bernfeld, P & Studer-Pecha, H (1947a) Studies on starch. 35. Degradation of amylose by alpha-amylase. Helvetica Chimica Acta , 30, 1895 - 1903.

Bernfeld, P & Studer-Pecha, H (1947b) Sur les enzymes amylolytiques II L'affinite entre enzyme et substratum pour quelques alpha-amylases. Helvetica Chimica Acta, 30, 1904 - 1910.

Birkhed, D Edwardsson, S Ahlden, M-L & Frostell, G (1979) Effects of 3 month frequent consumption of hydrogenated starch hydrolysate (Lycasin, maltitol, sorbitol and xylitol on human dental plaque. Acta Odontologica Scandinavica , 37, 103 - 115.

Birkhed, D & Skude, G (1978) Relation of amylase to starch and Lycasin metabolism in human dental plaque in vitro . Scandinavian Journal of Dental Research , 86, 248 - 25.

Birkhed, D & Tanzer J M (1979) Glycogen synthesis pathway in Streptococcus mutans strain NCTC 10449S and its glycogen synthesis-defective mutant 805. Archives of Oral Biology, 24, 67 - 73.

Blackwell J, Sarko, A & Marchessault, R H (1969) Chain conformation in beta-amylose. Journal of Molecular Biology, 42, 379 - 383.

Bourne, E J, Peters, J & Weigel, H (1964) Synthesis of sucrose labelled with carbon-14 in the fructose part. Journal of the Chemical Society, 4605 - 4607.

Bowen, W H (1969) A vaccine against dental caries. A pilot experiment in monkeys ( Macaca irus ). British Dental Journal , 126, 159 - 160.

Bowen, W H (1976) Dental Plaque and the bacteriology of Caries. In Scientific Foundations of Dentistry. Eds. Cohen, B & Kramer, I R H. London : William Heinemann Medical Books Ltd.

Bowen, W H, Ciardi, J, Gibbons, R J, Schachtele, C & Smith, D (1983) Critical needs in glucosyltransferase and dental caries research. In Proceedings Glucosyltransferases, Glucans, Sucrose and Dental Caries. Eds. Doyle, R J & Ciardi, J E. Washington D C : IRL Press.

Boyer, E W & Hartman, P A (1971) Extracellular transglucosylase and alpha-amylase of Streptococcus equinis. Journal of Bacteriology, 106, 561 - 570.

Bradford, M M (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry, 72, 248 - 254.

Brooker, B E (1979) Electron microscopy of the dextrans produced by lactic acid bacteria. In Microbial Polysaccharides and Polysaccharases. Eds. Berkley, R C W & Ellwood, D C, Ch 5. London : Academic Press.

Brown, F, Dunstan, S, Halsall, T G, Hirst, E L & Jones, J K N (1945) Application of new methods of end-group determination to structural problems in the polysaccharides. Nature, 156, 785 - 786.

Brown, F, Halsall, T G, Hirst, E L & Jones, J K N (1948) The structure of starch. The ratio of non-terminal to terminal groups. Journal of the Chemical Society, 27 - 32.

Brown, A T & Ruh, R (1977) Negative interaction of orthophosphate with glycolytic metabolism by Streptococcus mutans as a possible mechanism for dental caries production. Archives of Oral Biology. 22, 521 - 524.

Brown, A T & Wittenberger, C L (1971a) Mechanism for regulating the distribution of glucose carbon between the Emden-Meyerhof and hexose-monophosphate pathways in Streptococcus faecalis. Journal of Bacteriology, 106, 456 - 467.

Brown, A T & Wittenberger, C L (1971b) The occurrence of multiple glyceraldehyde-3-phosphate dehydrogenases in cariogenic streptococci. Biochemica et Biophysica Research Communication, 43, 217 - 224.

Brown, A T & Wittenberger, C L (1972) Fructose-1,6-diphosphate-dependent lactate dehydrogenase from a cariogenic streptococcus: purification and regulatory properties. Journal of Bacteriology, 110, 604 - 615.

Buchanan, R E & Gibbons, N E (1974) Bergey's Manual of Determinative Bacteriology , 8th ed. Baltimore : The Williams and Wilkins Company.

Buehrer, E A & Miller, C H (1983) Growth of oral bacteria on amylase treated starch. Journal of Dental Research , 62, 231.

Builder, J E & Walker, G J (1970) Metabolism of the reserve polysaccharide of Streptococcus mitis. Properties of glycogen synthetase. Carbohydrat Research, 14, 35 -51.

Burchall, J J , Niederman, R A & Wolin, M J (1964) Amino group formation and glutamate synthesis in Streptococcus bovis. Journal of Bacteriology, 88, 1038 - 1044.

Burchard, W, & Huseman, E (1961) A comparitive structure analysis of cellulose and amylose tricarbanilates in solution. Makromolecular Chemistry, 44 - 46, 358 - 387.

Burnett, G W , Scherp, H W & Schuster, G S (1976) In Oral Microbiology and Infectious Disease, Ch. 17, 18. Baltimore: The Williams & Wilkins Company.

Caldwell, R C & Bibby, B G (1958) The effect of foodstuffs on the pH of dental caries. The Journal of the American Dental Association, 685 - 692.

Carlsson, J (1970) Chemically defined medium for growth of Streptococcus sanguis . Caries Research , 4, 297.

Carlsson, J & Griffith, C J (1974) Fermentation products and bacterial yields in glucose-limited and nitrogen-limited cultures of streptococci. Archives of Oral Biology, 19, 1105 - 1109.

Carlsson, J, Iwami, Y & Yamada, T (1983) Hydrogen peroxide excretion by oral streptococci and effect of lactoperoxidase-thiocyanate-hydrogen peroxide. Infection and Immunity, 40, 70 - 80.

Casu, B & Reggiani, M (1964) Infrared study of amylose and its oligomers. Journal of Polymer Science, C7, 171 -

Casu, B & Reggiani, M (1966) Conformation of amylose and its derived products I. Infrared spectra of amylose and its oligomers in the amorphous solid phase and in solution. Die Starke, 18, 218 - 229.

Ceska, M, Granath, K, Guggenheim, B & Norman, B (1972) Structural and enzymatic studies on glucans synthesized with glucosyltransferase of some strains of cariogenic streptococci. Acta Chemica Scandinavica, 26, 2223 - 2230.

Chassy, B (1983) Sucrose metabolism and glucosyltransferase activity in oral streptococci In Proceedings Glucosyltransferases, Glucans, Sucrose and Dental Caries. Eds. Doyle, R J & Ciardi, J E. Washington D C : IRL Press.

Chassy, B M & Porter, E V (1982) Sucrose-6-phosphate hydrolase from Streptococcus mutans. Methods in Enzymology, 90, 556 - 559.

Ciardi, J E (1983) Purification and properties of glucosyltransferase from Streptococcus mutans. A Review. In Proceedings Glucosyltransferases, Glucans, Sucrose and Dental Caries. Eds. Doyle, R J & Ciardi, J E Washington D.C.: IRL Press.

Ciardi, J E, Hageage, G J & Wittenberger, C L (1976) Multicomponent nature of the glucosyltransferase system of Streptococcus mutans. Journal of Dental Reserach, 55, C 87.

Collison, R (1968) Swelling and gelation of starch. In Starch and its Derivatives Ed. Radley, J A 4th ed. Ch 5. London : Chapman & Hall Ltd.

Coogan, M M (1986) A code for the identification of the 'viridans' streptococci. Journal of the Dental Association of South Africa, 41, 129 - 133.

Cori, G T & Cori, C F (1939) The activating effect of glycogen on the enzymic synthesis of glycogen from glucose-1-phosphate. Journal of Biological Chemistry, 131, 397 - 398.

Cowie, J M G & Greenwood, C T (1957) Physiochemical studies on starches Part VIII Further observations on the fractionation of potato starch. Journal of the Chemical Society, 4640 - 4644.

Critchley, P , Wood, J M , Saxton, C A & Leach, S A (1967) The polymerization of dietary sugars by dental plaque. Caries Research, 1, 112 - 129.

Crow, V L & Pritchard, G G (1977) The effect of monovalent and divalent cations on the activity of Streptococcus lactis C10 pyruvate kinase. Biochemica et Biophysica Acta, 481, 105 - 114.

Cruickshank, R (1962) Machie and McCartney's Handbook of bacteriology , 10th ed. Ch VI. Edinburgh and London : E & S Livingstone Limited.

Davis, B D , Dulbecco, R , Eisen, H N & Ginsberg, H S (1980) In Microbiology Including Immunology and Molecular Genetics, 3rd ed. Ch 5. London : Harper and Row.

Denham, W S & Woodhouse, H (1913) CLXXXVI. The methylation of cellulose. Journal of the Chemical Society, 103, 1735 - 1745.

de Stoppelaar, J D, van Houte, J & Backer-Dirks, D (1970) The effect of carbohydrate on the presence of Streptococcus mutans, Streptococcus sanguis and iodophilic polysaccharide-producing bacteria in dental plaque. Caries Research, 4, 114 - 123.

Dewar, M D (1977) The enzymic synthesis and degradation of extracellular and intracellular polysaccharide by oral streptococci. MSc Thesis, University of Sydney.

Dewar, M D & Walker, G J (1975) Metabolism of the polysaccharides of human dental plaque. I Dextranase activity of streptococci, and the extracellular polysaccharides synthesized from sucrose. Caries Research, 9, 21 - 35.

Distler, W , Hagemann, D & Kronke, A (1982) Starch degradation by streptococci and actinomyces. Journal of Dental Research , 61, 568.

Distler, W & Kroncke, A (1983) The acid pattern in human dental plaque. Journal of Dental Research, 62, 87-91.

Ditson, S L & Mayer, R M (1984) Dextransucrase : The direction of chain growth during autopolymerization. Carbohydrate Research , 126, 170-175.

Doelle, H W (1969) In Bacterial metabolism Ch. 4. New York: Academic Press.

Doelle, H W (1975) In Bacterial metabolism 2nd ed, Ch 9. New York: Academic Press.

Doi, A, Doi, K & Nikuni, Z (1964) ADP-D-glucose: alpha-1,4 glucan alpha-4-glucosyltransferase in spinach chloroplasts. Biochemica et Biophysica Acta, 92, 628-630.

Doty, P, Wada, A, Yang, J T & Blout, E R (1951) Polypeptides. VIII. Molecular configurations of poly-L-glutamic acid in water-dioxane solution. Journal of Polymer Science, 23, 851-861.

Drucker, D R & Melville, T H (1968) Fermentation end products of cariogenic and non cariogenic streptococci. Archives of Oral Biology, 13, 553-570.

Dube, S K & Nordin, P (1961) Isolation and properties of sorghum alpha-amylase Archives of Biochemistry and Biophysics, 94, 121-127.

Dubois, M, Gilles, K A, Hamilton, J K, Rebers, P A & Smith, F (1956) Colorimetric method for determination of sugars and related substances. Analytical Chemistry, 28, 350-356.

Duguid, R (1985) In vitro acid production by the oral bacterium Streptococcus mutans 10449 in various concentrations of glucose, fructose and sucrose. Archives of Oral Biology , 30, 319-324.

Dunican, L K & Seeley, H W (1962) Starch hydrolysis by Streptococcus equinus . Journal of Bacteriology , 83, 264-269.

Dutton, G G S (1974) Applications of gas-liquid chromatography to carbohydrates Part II. Advances in Carbohydrate Chemistry, 30, 9-110.

Edgar, W M , Bibby, B G , Mundorff, S & Rowley, J (1975) Acid production in plaques after eating snacks : modifying factors in foods. Journal of the American Dental Association , 90, 418-425.

Edwardsson, S , Birkhed, D & Mejare, B , (1977) Acid Production from Lycasin, maltitol, sorbitol and xylitol by oral streptococci and lactobacilli. Acta Odontologica Scandinavica , 35, 257-263.

Ehrlich, J, Stivala, S S, Bahary, W S, Garg, S K, Long, L W & Newbrun, E (1975) Levans. I Fractionation, solution viscosity and chemical analysis of levan produced from Streptococcus salivarius. Journal of Dental Research, 54, 290-297.

Ellwood, D C & Hamilton, I R (1982) Properties of Streptococcus mutans Ingbritt growing on limiting sucrose in a chemostat: Repression of the phophoenol-pyruvate phosphotransferase transport system. Infection and Immunity, 36, 576-581.

Ellwood, D C , Hunter, J R & Longyear, V M C (1974) Growth of Streptococus mutans in a chemostat. Archives of Oral Biology, 19, 659 - 664.

Enevoldsen B S (1980) Page IL4 : Abstracts Xth International Symposium on Carbohydrate Chemistry. Sydney, Australia.

Englard, S, Soraf, S & Singer, T P (1951) Intramolecular nature of the oxidative inactivation of crystalline beta-amylase. Journal of Biological Chemistry, 189, 217 - 226.

Evans, C G T , Yeo, R G & Ellwood, D C (1979) Continuous culture studies on the production of extracellular polysaccharides by Xanthomonas juglandis . In Microbial polysaccharides and polysaccharases. Eds. Berkley, R C W & Ellwood, D C, Ch 3. London : Academic Press.

Everett, W W, & Foster, J F (1959) The conformation of amylose in solution. Journal of the American Chemical Society, 81, 3464 - 3469. Facklam, R R (1977) Physiological differentiation of viridans streptococci. Journal of Clinical Microbiology, 5, 184 - 201.

Facklam, R.R. (1984) The major differences in the American and British Streptococcus taxonomy schemes with special reference to Streptococcus milleri . European Journal of Clinical Microbiology , 3, 91 - 93.

Featherstone, J D B & Rodgers, B E (1981) Effect of acetic, lactic and other organic acids on the formation of artificial carious lesions. Caries Research, 15, 377 - 385.

Flehn, N E & Moe, D (1983) Alpha-amylase activity in supragingival dental plaque in humans. Scandanavian Journal of Dental Research , 91, 365-370.

Fischer, E H & Stein, A E (1960) Alpha-amylases in The enzyme, Eds. Boyer, P D, Lardy, H & Myrback, K, Vol 4, p 345 London : Academic Press.

Fisher, E H & Stein, E A (1961) Alpha-amylase from human saliva. Biochemical Preparations, 8, 27 - 33.

Fleming, I D (1968) Amyloglucosidase In Starch and its Derivatives, 4th ed, Ed. Radley, J, Ch. 16. London : Chapman & Hall Ltd.

Fogarty, W M & Kelly, C T (1980) Amylases, amyloglucosidases and related glucanases In Economic Microbiology Volume 5, Microbial enzymes and bioconversions Ed. Rose A H Ch 3. London : Academic Press.

Foster, J F (1965) Physical properties of amylose and amylopectin in solution. In Starch chemistry and Technology, , Eds. Whistler, R L & Paschall, E T Ch. 15. London: Academic Press.

Foster, J F & Hixon, R M (1943) Solution viscosities of the amylose components of starch. Journal of the American Chemical Society, 65, 618 - 622.

Foster, J F & Hixon, R M (1944) Relationship between solution viscosity and molecular weight in the amylose series. Journal of the American Chemical Society, 66, 557 - 560.

Freedman, M, Birkhed, D, Coykendall, A & Rizzo, D (1979) Linkage analyses of extracellular glucans from Streptococcus sanguis and Streptococcus mitior . Infection and Immunity, 23, 907 - 909.

French, D (1957a) The Schardinger dextrins. Advances in Carbohydrate Chemistry , 12, 189 - 260.

French, D (1957b) Recent developments and theoretical aspects of amylase action. Bakers Digest, 31, 24 - 36.

French, D (1973) Chemical and physical properties of starch. Journal of Animal Science, 37, 1048 - 1061.

French, D (1981) Amylases : enzymatic mechanisms. Basic Life Sciences , 18, 151-182.

French, D (1984) Organization of starch granules in Starch Chemistry and Technology 2nd ed. Eds. Whistler, R L, Bemiller, J N & Paschall, E F. Ch VII. New York: Academic Press.

French, D, Levine, M L, Pazur, J H & Norberg, E (1950) Studies on the Schardinger dextrins. IV. The action of soy bean beta amylase on amyloheptaose. Journal of the American Chemical Society, 72, 1746 - 1748.

French, D & Wild, G M (1953) Primer specificity of potato phosphorylase. Journal of the American Chemical Society, 75, 4490 - 4492.

French, D & Youngquist, R W (1963) The mode of action of beta-amylase on starch oligosaccharides. Die Starke, 15, 425 - 431.

Freudenberg, K & Boppel, H (1940) The position at which branching occurs in the starch chain. Naturwissenschaften, 28, 264.

Frostell, G & Baer, P N (1971a) Effects of sucrose, starch and a hydrogenated starch derivative on dental caries in man. Acta Odontologica Scandinavica , 29, 253 - 259.

Frostell, G & Baer, P N (1971b) Caries experience of rats fed various starches for study of experimental calculus formation. Acta Odontologica Scandinavica , 29, 401 - 413.

Frostell, G & Birkhed, D (1978) Acid production from Swedish Lycasin (candy quality) and French Lycasin (80/55) in human dental plaques. Caries Research , 12, 256 - 263.

Frostell, G , Blomlof, L , Blomquist, I , Dahl, G M , Edward, S , Fjellstrom A, Hendrikson, C O , Larje, O , Nord, C E , and Nordenvall, K J (1974). Substitution of sucrose by Lycasin (R) in candy. 'The Roslagen study'. Acta Odontologica Scandinavica , 32, 235 - 254.

Frydman, R B (1963) Starch synthetase of potatos and waxy maize. Archives of Biochemistry and Biophysics, 102, 242 - 248.

Frydman,R B & Cardini, C E (1964a) Biosynthesis of phytoglycogen from adenosine diphosphate D-glucose in sweet corn. Biochemical and Biophysical Research Communications, 14, 353 - 357.

Frydman, R B & Cardini, C E (1964b) Soluble enzymes related to starch synthesis. Biochemical and Biophysical Research Communications, 17, 407 - 411.

Garszczynski, S M & Edwards, J R (1973) Synthesis of a broth levan by a cell bound levansucrase from Streptococcus salivarius (SS2). Archives of Oral Biology, 18, 239 - 251.

Geddes, D A M (1972) The production of L (+) and D (-) lactic acid and volatile acids by human plaque and the effect of plaque buffering and acidic strength on pH. Archives of Oral Biology, 17, 537 - 545.

Ghosh, H P & Preiss, J (1965) Biosynthesis of starch in spinach chloroplasts. Biochemistry, 4, 1354-1361.

Gibbons, G C & Boissonnas, R A (1950) Nature de la laison d'embrunchement du glycogene et de l'amylopectine. Recherches sur l'amidon. Helvetica Chimica Acta, 33, 1477 - 1481.

Gibbons, R J (1983) Importance of glucosyltransferase in the colonization of oral bacteria. In Glucosyltransferases, Glucans, Sucrose and Dental Caries. Eds. Doyle, R J & Ciardi, J E, Washington D C : IRL Press.

Gibbons, R J & Banghart, S B (1967) Synthesis of extracellular dextran by cariogenic bacteria and its presence in human dental plaque. Archives of Oral Biology, 12, 11 - 24.

Gibbons, R J & Banghart, S B (1968) Variation in extracellular polysaccharide by cariogenic streptococci. Archives of Oral Biology, 13, 697 - 701.

Gibbons, R J , Berman, K S , Knoettner, P & Kapsimalis B (1966) Dental caries and alveolar bone loss in gnotobiotic rats infected with capsule forming streptococci of human origin. Archives of Oral Biology, 11, 549 - 560.

Gibbons, R J & Fitzgerald, R (1969) Dextran-induced agglutination of Streptococcus mutans and its potential role in the formation of microbial dental plaques. Journal of Bacteriology, 98, 341 - 346.

Gibbons, R J & Keyes, P H (1969) Inhibition of insoluble dextran synthesis, plaque formation and dental caries in hamsters by low molecular weight dextran. Archives of Oral Biology , 14, 721 - 724.

Gibbons, R J & Nygaard, M (1968) Synthesis of insoluble dextran and its significance in formation of gelatinous deposits by plaque forming streptococci. Archives of Oral Biology 13, 1249 - 1262.

Glor, E B, Miller, C H & Spandau, D F (1988) Degradation of starch and its hydrolytic products by oral bacteria. Journal of Dental Research, 67, 75 - 81.

Gottschalk, G (1979) In Bacterial Metabolism. Ch. 7,8. New York: Springer-Verlag.

Grahame, D A & Mayer, R M (1984) The origin and composition of multiple forms of dextransucrase from Streptococcus sanguis. Biochemica et Biophysica Acta, 786, 42 - 48.

Gray, G M, Lally, B C & Conklin, K A (1979) Action of intestinal sucrose-isomaltase and its free monomer on an alpha-limit dextrin. Journal of Biological Chemistry, 254, 6038 - 6043.

Green, J W (1980) Oxidative reactions and degradations. In The Carbohydrates Chemistry and Biochemistry. 3rd ed. Eds. Pigman, W & Horton, D Ch 24. London: Academic Press.

Greenwood, C T (1956) Aspects of the physical chemistry of starch. Advances in Carbohydrate Chemistry, 11, 335 - 393.

Greenwood, C T (1960) Physiochemical studies on starches. XXII The molecuclar properties of the components of starches. Starke, 12, 169 - 174.

Greenwood, C T (1970) Starch and glycogen. In The Carbohydrates Chemistry and Biochemistry, 2nd ed. Eds. Pigman, W & Horton, D Vol II B, Ch 38. New York: Academic Press.

Greenwood, C T (1976) Advances in Cereal Science and Technology 1, 119- (From Shannon and Garwood, 1984).

Greenwood, C T & Milne, E A (1968) Starch degrading and synthesizing enzymes: A discussion of their properties and action pattern. Advances in Carbohydrate Chemistry , 23, 280 - 367.

Greenwood, C T & Robertson, J S M (1954) Physiochemical studies on starches Part I. The characterization of the starch present in the seeds of the rubber tree, Hevea brasiliensis . Journal of the Chemical Society, 3769 - 3778.

Greenwood, C T & Thomson, J (1960) Observations on starch of high amylose-content; the properties of the amylopectin component. Chemistry and Industry, 1110 - 1111.

Greenwood, C T & Thomson, J (1962) Physiochemical studies on starches. Part XXXIV. The fractionation and characterization of starches of various plant origins. Journal of the Chemical Society, 222 - 229.

Grenby, T H (1965) The influence of cooked and raw wheat starch on dental caries in rats. Archives of Oral Biology, 10, 433 - 438.

Grenby, T H (1967) Investigations in experimental animals on the cariogenicity of diets containing sucrose and/or starch. Caries Research , 1, 208 - 221.

Griffin, G J L (1985) Starch granules, their properties and industrial applications. In New Approaches to Research on Cereal Carbohydrates, Eds. Hill, R D & Munck, L, Vol 1, p 201 - 210. Amsterdam, : Elsevier Science Publishers.

Guggenheim, B (1970) Enzymatic hydrolysis and structure of water-insoluble glucan produced by glucosyl transferases from a strain of Streptococcus mutans. Helvetica Odontologica Acta, 14, 89 - 108.

Guggenheim, B & Newbrun E (1969) Extracellular glucosyltransferase activity of an HS strain of Streptococcus mutans. Helvetica Odontologica Acta, 13, 84 - 97.

Guggenheim, B & Schroeder, H E (1967) Biochemical and morphological aspects of extracellular polysaccharides produced by cariogenic streptococci. Helvetica Odontologica Acta, 11, 131 - 152.

Gunja-Smith, Z, Marshall, J J, Mercier, C, Smith, E E & Whelan, W J (1970a) A revision of the Meyer-Bernfeld model of glycogen and amylopectin. Febs Letters, 12, 101 - 104.

Gunja-Smith, Z, Marshall, J J, Smith, E E & Whelan, W J (1970b) A glycogen-debranching enzyme from capnocytophaga Febs Letters, 12, 96 - 100.

Hakomori, S (1964) A rapid permethylatin of glycolipid, and polysaccharide catalyzed by methylsulfinyl carbanion in dimethyl sulfoxide. Journal of Biochemistry (Tokyo) 55, 205 - 208.

Hall, R S & Manners, D J (1980) The structural analysis of some amylodextrins. Carbohydrate Research , 83, 93-101.

Halsall, T G, Hirst, E L, Jones, J K N & Roudier, A J (1947) Structure of starch: Mode of attachment of the side-chains in amylopectin. Nature, 160, 891 - 900.

Hamada, S, Horikoshi, T, Minami, T, Okahashi, N & Koga, T (1989) Purification and characterization of cell-associated glucosyltransferase synthesizing water-insoluble glucan from serotype c Streptococcus mutans. Journal of General Microbiology, 135, 335 - 344.

Hamilton, I R (1976) Intracellular polysaccharide synthesis by cournalariogenic microorganisms. In Microbial Aspects of Dental Caries, Eds. H M Stiles, Loesche, W J & O'Brien, T C, Vol 3, p 683 - 701. Washington D C : Information Retrieval Inc.

Hamilton, I R , Phipps, P J & Ellwood, D C (1979) Effect of growth rate and glucose concentration on the biochemical properties of Streptococcus mutans Ingbritt in continuous culture. Infection and Immunity, 26, 861 - 869.

Hamilton, I R & St Martin, E J (1982) Evidence for the involvement of proton motive force in the transport of glucose by a mutant of Streptococcus mutans strain DR 0001 defective in glucose-phosphoenolpyruvate phosphotransferase activity. Infection and Immunity, 36, 567 - 575.

Hamilton, J K & Smith, F (1956) Reduction of the products of periodate oxidation of carbohydrates. II. A new method for the end-group assay of amylopectin. Journal of the American Chemical Society, 78, 5907 - 5909.

Hancock, R , Marshall, K & Weigel, H (1976) Structure of the levan elaborated by Streptococcus salivarius strain 51 : An application of chemical-ionization mass-spectrometry. Carbohydrate Research, 40, 351 - 360.

Hanes, C S (1937) The action of amylases in relation to the structure of starch and its metabolism in the plant. Parts IV - VIII. The New Phytologist, 31, 189 - 239.

Hanes, C S (1940) The reversible formation of starch from glucose-1-phosphate catalysed by potato phosphorylase. Proceedings of the Royal Society, London, 129B, 174 - 208.

Hanraham, V M & Caldwell, M L (1953) A study of the action of taka amylase Journal of the American Chemical Society, 75, 2191 - 2197.

Hansen, S (1975) Thin layer chromatographic method for identification of oligosaccharides in starch hydrolysates. Journal of Chromatography , 105, 388 - 390.

Harada, T, Misaki, A, Akai, H, Yokobayashi, K & Sugimoto, K (1972) Characterization of pseudomonas isoamylase by its actions on amylopectin and glycogen : comparison with aerobacter pullulanase Biochemica et Biophysica Acta, 268, 497 - 505.

Harada, T, Yokobayaski, K & Misaki, A (1968) Formation of isoamylase by pseudomanas. Applied Microbiology, 16, 1439 -1444.

Hare, M D , Svensson, S & Walker, G J (1978) Characterisation of the extracellular water-insoluble alpha-D-glucans of oral streptococci by methylation analysis and enzymatic synthesis and degradation. Carbohydrate Research, 66, 245 - 264.

Harris, P J, Henry, R J, Blakeney, A B & Stone, B A (1984) An improved procedure for the methylation analysis of oligosaccharides and polysaccharides. Carbohydrate Research , 127, 59-73.

Hassid, W Z & McCready, R M (1941) The molecular constitution of enzymatically synthesized starch. Journal of the American Society, 63, 2171 - 2173.

Hassid, W Z & McCready, R M (1943) The molecular constitution of amylose and amylopectin of potato starch. Journal of the American Chemical Society, 65, 1157 - 1161

Haworth, W N (1915) A new method of preparing alkylated sugars. Journal of the Chemical Society (London) 107, 8 - 16.

Haworth, W N (1929) The constitution of the sugars p 94. London : Arnold.

Haworth, W N, Heath, R L & Peat, S (1942) Constitution of the starch synthesized in vitro by the agency of potato phosphorylase. Journal of th Chemical Society, 55 - 58.

Haworth, W N, Hirst, E L & Isherwood, F A (1937) Polysaccharides. Part XXIII Determination of the chain length of glycogen. Journal of the Chemical Society, 577 - 581.

Haworth, W N, Hirst, E L & Webb, J I (1928) CCCLIV Polysaccharides Part II. The acetylation and methylation of starch. Journal of the Chemical Society, 2681 - 2690.

Haworth, W N, Peat, S & Bourne, E J (1944) Synthesis of amylopectin. Nature, 154, 236.

Heller, J & Schramm, M (1964) Alpha-limit dextrins of high molecular weight obtained from glycogen. Biochimic et Biophysica Acta, 81, 96 - 100.

Hestrin, S (1949) Action patterns of crystalline muscle phosphorylose. Journal of Biological Chemeistry, 179, 943 - 955.

Higashihara, M & Okada, S (1974) Studies on beta-amylase of Bacillus megaterium strain No. 32. Agricultural and Biological Chemistry, 38, 1023 - 1029.

Hirst, E L, Jones, J K N & Roudier, A J (1948) Structure of acorn starch. Journal of the Chemical Society, 1779 - 1783.

Hirst, E L, Plant, M M T & Wilkinson, M D (1932) Polysaccharides. Part XIV. The molecular structure of amylose and of amylopectin. Journal of the Chemical Society, 2375 - 2383.

Hizukuri, S (1985) Relationship between the distribution of the chain length of amylopectin and the crystalline structure of starch granules. Carbohydrate Research, 141, 295 - 306.

Hizukuri, S (1986) Polymodal distribution of the chain lengths of amylopectins, and its significance. Carbohydrate Research, 147, 342 - 347.

Hizukuri, S, Takeda, Y, Yasuda, M & Suzuki, A (1981) Multi-branched nature of amylo- nd the action of debranching enzymes. Carbohydrate Research, 205 - 213.

Hobson, P M, Whelan, W J & Peat, S (1951) The enzymic synthesis and degradation of starch. Part XIV. R-enzyme. Journal of the Chemical Society, 1451 - 1459.

Holdeman, Lilian V , Cato, Elizabeth P & Moore, W E C (1977) Anaerobe Laboratory manual , 4th ed. Blacksburg Virginia: V P I Anaerobe Laboratory.

Hollo, J & Szejtli, J (1968) The reaction of starch with iodine. In Starch and its derivatives 4th ed. Ed. Radley, Ch 7, p 203 - 246. London : Chapman and Hall.

Hughes, R C (1959) Ph.D Dissertation University of London.

Hughes, R C, Smith, E E & Whelan, W J (1963) Alpha-limit dextrin. Biochemical Journal, 88, 63P.

Hyun, H H & Zeikus, J G (1985) General biochemical characterization of thermostable pullulanase EC 3.2.1.41 and glucoamylase EC. 3.2.13 from Clostridium thermohydrosulfuricum . Applied and Environmental Microbiology , 49, 1168-1173.

Irvine, J C & Macdonald, J (1926) The constitution of polysaccharides. Part X. The molecular unit of starch. Journal of the Chemical Society, 1502 - 1518.

Iverson, W G & Mills, N F (1974) A method for the detection of starch hydrolysis by bacteria. Journal of Applied Bacteriology , 37, 443 - 446.

Iwami, Y, & Yamada, T (1980) Rate-limiting steps of the glycolytic pathway in the oral bacteria Streptococcus mutans and Streptococcus sanguis and the influence of acidic pH on the glucose metabolism. Archives of Oral Biology, 25, 163 - 169.

Iwami, Y , Yamada, T & Araya, S (1975) Glycolytic intermediates in Streptococcus mutans PK 1. Archives of Oral Biology, 20, 695 - 697.

Jackson, E L & Hudson, C S (1936) The oxidation of alpha-methyl d-manno-pyranoside. Journal of the American Chemical Society, 58, 378 - 379.

Jackson, E L & Hudson, C S (1937) Application of the cleavage type of oxidation by periodic acid to starch and cellulose. Journal of the American Chemical Society, 59, 2049 - 2050.

Jackson, E L, & Hudson, C S (1938) The structure of the products of the periodic acid oxidation of starch and cellulose. Journal of the American Chemical Society, 60, 989 - 991.

Janda, W M & Kuramitsu, H K (1976) Regulation of extracellular glucosyltransferase production and the relationship between extracellular and cell-associated activities in Streptococcus mutans. Infection and Immunity, 14, 191 - 202.

Jane, J L & Robyt, J F (1984) Structure studies of amylose-V complexes and retrograded amylose by action of alpha-amylases and a new method for preparing amylodextrins. Carbohydrate Research, 132, 105 - 118.

Jeanes, A & Hudson, C S (1955) Preparation of meso-erythritol and D-erythronic lactone from periodate-oxidized starch. Journal of Organic Chemistry, 20, 1565 - 1568.

Johnson, M C, Bozzola, J J, Scheckmeister, I L & Shklair, I L (1977) Biochemical study of the relationship of extracellular glucan to adherence and cariogenicity in S.mutans and an extracellular polysaccharide mutant. Journal of Bacteriology, 129, 351 - 357.

Joklik, W K , Willett, H P & Amos, D B (1984) Energy metabolism In Zinsser Microbiology, 18th ed. Ch. 4., Norwalk Connecticut: Appleton - Century Crofts.

Jorgensen, B B & Jorgensen, O B (1960) Investigations on the molecular weight of aqueous solutions of potato amylose by osmotic pressure. Acta Chemica Scandinavica, 14, 2135 - 2138.

Kainuma, K, Kobayashi, S, Ito, T & Suzuki, S (1972) Isolation and action pattern of maltohexaose producing amylase from Aerobacter aerogenes. Febs Letters, 26, 281 - 285.

Kainuma, K, Kobayashi, S & Harada, T (1978) Action of Pseudomonas isoamylase on various branched oligo- and poly-saccharides. Carbohydrate Research, 61, 345 - 357.

Kainuma, K, Wako, K, Kobayashi, S, Nogami, A & Suzuki, S (1975) Purification and some properties of a novel maltohexaose-producing exo-amylase from Aerobacter aerogenes. Biochemica et Biophysica Acta, 410, 333 - 346.

Kakiuchi, K, Hamacuchi, K & Isemura, T (1965) Association and dissociation of Bacillus subtilis alpha-amylase molecule. III. The effects of pH and salts on the monomer-dimer transformation. The Journal of Biochemistry, Tokyo, 57, 167 - 175.

Kanapka, J A & Hamilton, I R (1971) Fluoride inhibition of enolase activity in vivo and its relationship to the inhibition of glucose-6-phosphate formation in Streptococcus salivarius. Archives of Biochemistry and Biophysics, 146, 167 - 174.

Kelly, J J & Alpers, D H (1973) Properties of human intestinal glucoamylase. Biochimica et Biophysica Acta, 315, 113 - 120.

Kemp, C W , Robrish, S A , Curtis, M A , Sharer, S A & Bowen, W H (1983) Application of a competition model to the growth of Streptococcus mutans and Streptococcus sanguis in binary continuous culture. Applied and Environmental Microbiology, 45, 1277 - 1282.

Keyes, P H (1968) Research in dental caries. Journal of the American Dental Association, 76, 1357 - 1373.

Kneen, E (1950) The amylases. Properties and production In Chemistry and Industry of Starch , Ed. Kerr, R W, Ch 15. New York : Academic Press.

Koepsel, H J, Tsuchiya, H M, Hellman, N N, Kazenko, A, Hoffman, C A , Sharpe, E S & Jackson, R W (1953) Enzymatic synthesis of dextran. Acceptor specificity and chain initiation. Journal or Biological Chemistry, 200, 793 - 801.

Koga, T & Inoue, M (1979) Effects of dextranases on cell adherence, glucan-film formation and glucan synthesis by Streptococcus mutans glucosyltransferase. Archives of Oral Biology, 24, 191 - 198.

Kondo, H , Nakatani, H , Hiromi, K & Matsuno, R (1978) Multiple attack in porcine pancreatic alpha-amylase-catalyzed hydrolysis of amylose studied with a fluorescent probe. Journal of Biochemistry (Tokyo) , 84, 403 - 417.

Kreitzman, S N (1978) New Perspectives on Diet and Dental Caries. Journal of Preventative Dentistry , 5, 12 - 17.

Kuramitsu, H K (1974) Characterisation of cell-associated dextransucrase activity from glucose-grown cells of Streptococcus mutans, Infection and Immunity, 10, 227 - 235.

Kvernheim, A L (1987) Methylation analysis of polysaccharides with butyllithium in dimethyl sulfoxide. Acta Chemica Scandinavica B 41, 150 - 152.

Laemmli, U K (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227, 680 - 685.

Lanke, L S (1957) Influence of different carbohydrate foodstuffs on the sugar content of the saliva. Influence on salivary sugar of certain properties of foodstuffs and individual oral conditions. Acta Odontologica Scandinavica, 15, Supplement 23, 39 - 62.

Lansky, S, Kooi, M & Schoch, T J (1949) Properties of the fractions and linear subfractions from various starches. Journal of the American Chemical Society, 71, 4066 - 4075.

Larson, B L , Gilles, K A & Jenness, R (1953) Amperometric method for determining the sorption of iodine by starch. Analytical Chemistry, 25, 802 - 804.

Le Blanc, D J , Crow, V L , Lee, L N & Garon, C F (1979) Influence of the lactose plasmid on the metabolism of galactose by Streptococcus lactis. Journal of Bacteriology, 137, 878 - 884.

Lee, E Y C, Mercier, C & Whelan, J J (1968) A method for the investigation of the fine structure of amylopectin. Archives of Biochemistry and Biophysics, 125, 1028 - 1030.

Lee, E Y C & Whelan W J (1966) Enzymic methods for the micro determination of glyucogen and amylopectin, and their unit-chain length. Archives of Biochemistry and Biophysics, 116, 162 -167.

Lehninger, A L (1975) Biochemistry , 2nd ed. Ch 10. New York : Worth Publishers Inc.

Levine, R S (1977) The aetiology of dental caries - an outline of current thought. International Dental Journal, 27, 341 - 348.

Lindberg, B (1981) Haworth memorial lecture Structural studies of polysaccharides. Chemical Society Reviews, 10, 409 - 434.

Loewus, F A & Briggs, D R (1957) A potentiometric study of the change in iodine binding capacity of amylose while retrograding in dilute solution. Journal of the American Chemical Society, 79, 1494 - 1497.

Luzio, G A, Graham, DA & Mayer, R M (1982) The dissociation of aggregate forms of dextransucrase. Archives of Biochemistry and Biophysics, 216, 751 - 757.

MacWilliam, I C & Percival, E G V (1951) The constitution of barley starch. Journal of the Chemical Society, 2259 - 2267.

Malaprade, L (1934) Action of polyalcohols on periodic acid and alkaline periodates. Bulletin of the Society of Chemistry, (5) 1, 833 - 852.

Manners, D J (1985a) Some aspects of the structure of starch. Cereal Foods World, 30, 461-467.

Manners, D J (1985b) Some aspects of the metabolism of starch. Cereal Foods World, 30, 722-727.

Manners, D J (1985c) Structural analysis of starch components by debranching enzymes. In Progress in Biotechnology Volume 1. New Approach to Research on Cereal Carbohydrates. Eds. Hill, R D & Munck, L. pp 45 - 54. Amsterdam : Elsevier.

Manners, D J (1989) Recent developments in our understanding of amylopectin structure. Carbohydrate Polymers, 11, 87 - 112.

Manners, D J & Matheson, N K (1981) The fine structure of amylopectin. Carbohydrate Research, 90, 99 - 110.

Manning, G B & Campbell, L L (1961) Thermostable alpha-amylase of Bacillus stearothermophilus. 1. Crystallization and some general properties. Journal of Biological Chemistry, 236, 2952 - 2957.

Marchessault, R H & Deslandes, Y (1981) Crystalline conformation of homo-and regular heteroglucan chains. Carbohydrate Polymers, 1, 31 - 38.

Marshall, J J (1974) Application of enzymic methods to the structural analysis of polysaccharides. Part I. In Advances in Carbohydrate Chemistry and Biochemistry , 30, 257 - 333. Ed. Wolfram, M L & Tipson, R S. New York : Academic Press.

Marshall, J J (1975) Starch-degrading enzymes, old and new. Die Staerke, 27, 377 - 383.

Marshall, J J & Whelan, W J (1970) Incomplete conversion of glycogen and starch by crystalline amyloglucosidase and its importance in the determination of amylaceous polymers. Febs Letters, 9, 85 - 88.

Marshall, J J & Whelan, W J (1974) Multiple branching in glycogen and amylopectin. Archives of Biochemistry and Biophysics, 161, 234 - 238.

McCabe, M M & Hamelik, R M (1983) An enzyme from Streptococcus mutans forms branches on dextran in the absence of sucrose. Biochemical and Biophysical Research Communications, 115, 287 - 294.

McCabe, R M , Keyes, P H & Howell, A (1967) An in vitro method for assessing the plaque forming ability of oral bacteria. Archives of Oral Biology, 12, 1653 - 1656.

McCabe, M M, Koga, T, Inoue, M, Freedman, M L & Hamelik, R M (1983). Glucosyltransferase isoenzymes from Streptococcus mutans. In Proceedings Glucosyltransferases, Glucans, Sucrose and Dental Caries. Special supplement to Chemical Senses, Eds. Doyle, R J & Ciardi, J E. Washington DC : IRL Press.

McCready, R M & Hassid, W Z (1943) The separation and quantitative estimation of amylose and amylopectin in potato starch. Journal of the American Chemical Society, 65, 1154-1157.

McDonald, J L & Stookey, G K (1977) Animal studies concerning the carciogenicity of dry breakfast cereals. Journal of Dental Research , 56, 1001 - 1006.

McHugh, W D (1970) Introduction In Dental plaque A symposium held in the University pf Dundee 22-24 September 1969. London : E S Livingstone.

Mercier, C (1973) Fine structure of corn starches of various amylose percentage. Waxy, normal and amylomaize. Die Starke, 25, 78 - 83.

Meyer, K H (1950) In Physical Chemistry of high polymeric systems. 2nd ed. Eds. Mark, H and Tobolsky, A V. p 456 - 489. New York : Interscience Publishers Inc.

Meyer, K H (1952) The past and present of starch chemistry. Experientia, 8, 405 - 444.

Meyer, K H & Bernfeld, P (1940) Recherches sur l'amidon. VII Sur la structure fine du grain d'amidon et sur les phenomenes du gonflement. Helvetica Chemica Acta, 23, 890 - 897.

Meyer, K H & Bernfeld, P (1941) Recherches sur l'amidon XIV. La reaction coloree a l'iode de l'amidon et du glycogene. Helvetica Chimica Acta , 24, 389 - 393.

Meyer, K H, Bernfeld, P & Wolff, E (1940) Recherches sur l'amidon III Fractionnement et purification de l'amylose de mais naturel. Helvetica Chimica Acta, 23, 854 - 864.

Meyer, K H, Fischer, E H, Bernfeld, P (1947) 10. Sur les enzymes amylolytiques (1) L'isolement de l'alpha-amylase de pancreas. Helvetica Chimica Acta, 30, 64 - 70.

Meyer, K H, Fischer, E H & Piquet, A (1951) Purifications et cristallisation de la beta-amylase de malt. Sur les enzymes amylolytiques XVI. Helvetica Chimica Acta, 34, 316 - 324.

Meyer, K H & Gonon, W F (1951) La degradation de l'amylose par les alpha-amylases. Rescherches sur l'amidon 50. Helvetica Chimica Acta, 34, 294 - 307.

Meyer, K H & Mark, H (1950) In Makromolekulare Chemie 2nd ed. p 1023. Leipsig: Geest & Portig.

Meyer, K H, Spahr, P F & Fisher, E H (1953) Purifications, cristallisation et proprietes de la beta-amylase de ble. Sur les enzymes amylolytiques 22. Helvetica Chimica Acta, 36, 1924 - 1936.

Meyer, K H, Wertheim, M & Bernfeld, P (1940) 106. Recherches sur l'amidon IV Methylation et determination des groups terminaux d'amylose et d'amylopectine de mais. Helvetica Chimica Acta, 23, 865 - 875.

Meyer, K H, Wertheim, M & Bernfeld, P (1941) Starch. 13. Potato starch. Helvetica Chimica Acta, 24, 378 - 389.

Meynell, G G & Meynell, E (1970) In Theory and Practice in Experimental Bacteriology. 2nd ed. Ch 1. Cambridge : Cambridge University Press.

Mickelson, M N (1967) Aerobic metabolism of Streptococcus agalactiae, Journal of Bacteriology, 94, 184 - 191.

Mickelson, M N (1972) Glucose degradation, molar growth yields, and evidence for oxidative phosphorylation in Streptococcus agalactiae. Journal of Bacteriology, 109, 96 - 105.

Miles, M J, Morris, V J, Orford, P D & Ring, G (1985) Recent observations on starch retrogradation in New Approaches to Research on Cereal Carbohydrates, Eds. Hill, R D & Munck, L. pp. 109 - 114. Amsterdam : Elsevier Science Publishers.

Miller, W D (1890) The Microorganisms of the Human mouth , Basel : S Karger 1973. Fascimile reprint.

Miyasaki, K T & Newbrun, E (1983) Inhibition of adherence of Streptococcus mutans by acarbose. In Proceedings Glucosyltransferases, Glucans, Sucrose and Dental Caries. A Special Supplement to Chemical Senses . Eds. Doyle, R J & Ciardi, J E. pp 201-210. Washington D C : IRL Press.

Montgomery, E M, Sexson, K R & Senti, F R (1961) High amylose corn starch fractions. Die Starke, 13, 215 - 222.

Montville, T J, Cooney, C L & Sinskey, A J (1978) Streptococcus mutans, A review in Proceedings Glucosyltransferases, Glucans, Sucrose and Dental Caries, Special supplement to Chemical Senses, Eds. Doyle, R J & Ciardi, J E pp 51 - 64. Washington DC : IRL Press.

Morgan, F J , Adams, K R & Priest, F G (1979) A cultural method for the detection of pullulan - degrading enzymes in bacteria and its application to the genus bacillus. Journal of Applied Bacteriology , 46, 291 - 294.

Mormann, J E & Muhlemann, H R (1981) Oral starch degradation and its influence on acid production of dental plaque. Caries Research , 15, 166 - 175.

Mormann, J E , Schmid, R & Muhlemann, H R (1983) Effect of alpha-amylase and alpha-glucosidase inhibitors on caries incidence and plaque accumulation in rats. Caries Research , 17, 353 - 356.

Mukasa, H (1986) Properties of Streptococcus mutans glucosyltransferases. In Molecular Microbiology and Immunobiology of Streptococcus mutans. Eds. Hamada, S, Michalek, S M, Kiyono, H, Menaker, L & McGhee, J R. pp 121 - 132. Amsterdam : Elsevier Science Publishers BV. (Biochemical Division),

Mukasa, H, & Slade H D (1974) Mechanisms of the adherence of Streptococcus mutans. to smooth surfaces. III Purification and properties of the enzyme complex responsible for adherence. Infection and Immunity, 10, 1135 - 1145.

Muus, J, Brockett, F P & Connelly, C C (1956) Effect of various ions on the stability of crystalline salivary amylase in solution. Archives of Biochemistry and Biophysics, 65, 268 - 277.

Myrback, K (1926) Compounds of some enzymes with inactivating substances II. Zeitschrift fur Physikalische Chemie, 159, 1 - 84.

Myrback, K (1948) Products of the enzymic degradation of starch and glycogen. Advances in Carbohydrate Chemistry , 3, 251 - 310.

Myrback, K & Nycander G (1942) XV Dextrinic acids and their cleavage by the amylases. Biochemische Zeitschrift, 311, 234 - 241.

Nalbandian, J, Freedman, M L, Tanzer, J M & Lovelace, S M (1974) Ultrastructure of mutants with reference to agglutination, adhesion and extracellular polysaccharides. Infection and Immunity, 10, 1170 - 1179.

Nakayama, S & Amagase, S (1963) An improved method for the purification of sweet potato beta-amylase. Journal of Biochemistry (Tokyo), 54, 375 - 377.

Neely, W B (1961) Optical rotatory dispersion studies on polysaccharides. III Amylose, amylopectin and methylcellulose. Journal of Organic Chemistry, 26, 3015 - 3016.

Newbrun, E (1982) Sucrose in the dynamics of the carious process. International Dental Journal , 32, 13 - 23.

Newbrun, E, Lacy, R & Christie, T M (1971) The morphology and size of the extracellular polysaccharides from oral streptococci. Archives of Oral Biology, 16, 863 - 872.

Nisizawa, T , Imai, S , Akada, H , Hinoide, M & Araya, S (1976) Extracellular glucans produced by oral streptococci. Archives of Oral Biology, 21, 207 - 213.

Niven, C F, Smiley K L & Sherman, J M (1941) The polysaccharides synthesized by Streptococcus salivarius and Streptococcus bovis. Journal of Biological Chemistry, 140, 105 - 109.

Nordin, P & French, D (1958) 1-phenyl-flavazole derivatives of starch dextrins. Journal of the American Chemical Society, 80, 1445 - 1447.

Norman, B E (1979) The application of polysaccharide degrading enzymes in the starch industry. In Microbial Polysaccharides and Polysaccharases Eds. Berkeley, R C W & Ellwood, D C. Ch 15. London : Academic Press.

Ohba, R & Ueda, S (1975) Some properties of crystalline extra and intra-cellular pullulanase from Aerobacter aerogenes . Agricultural and Biological Chemistry , 39, 967 - 972.

Pacsu, E & Mullen, J W (1941) Separation of starch into its two constituents Journal of the American Chemical Society, 63, 1168 - 1169.

Palmer, T N , Woeber, G & Whelan, W J (1973) The pathway of exogenous and endogenous carbohydrate utilization in Escherichia coli : A dual function for the enzyme of the maltose operon. European Journal of Biochemistry , 39, 601 - 612.

Pazur, J H (1972) Methods in Enzymology, Ed. Ginsburg, V. Vol 28, 931. New York : Academic Press.

Pazur, J H, French, D & Knapp, D W (1950) Mechanism of salivary amylase action. Proceedings of the Iowa Academy of Science, 57, 203 - 209.

Pazur, J H & Okada, S (1967) Properties of the glucoamylase from Rhizopus delemar. Carbohydrate Research, 4, 371 - 379.

Peat, S, Pirt, S J & Whelan, W J (1952) Enzymic synthesis and degradation of starch. Part. XV Beta-amylase and the constitution of amylose. Journal of the Chemical Society, 705 - 713.

Peat, S, Whelan, W J & Thomas G J (1956) The enmzymic synthesis and degradation of starch Part XXII. Evidence of multiple branching in waxy-maize starch. A correction. Journal of the Chemical Society, 3025 - 3030.

Perlin, A S (1958) Radiochemical evidence for heterogeneity in wheat starch. Canadian Journal of Chemistry, 36, 810 - 813.

Peticolas, (1963) Helix-coil transmission and electronic conductivity of the amylose-iodine complex. Nature, 197, 898 - 899.

Pfanne Muller, B (1967) Natural and synthetic amylose. XXIX The kinetics in amylose decomposition by means of beta-amylase and phosphorylase. Die Starke 19, 27 - 33.

Piquet, A & Fischer, E H (1952) Properties de la beta-amylase de malt. Sur les enzymes amylolytiques 21. Helvetica Chimica Acta, 35, 257 - 262.

Posternak, T (1951) On the phosphorous of potato starch, Journal of Biological Chemistry , 188, 317 - 325.

Potter, A L & Hassid, W Z (1948a) Starch I. End-group determination of amylose and amylopectin by periodate oxidation. Journal of the American Chemical Society, 70, 3488 - 3490.

Potter, A L & Hassid, W Z (1948b) Starch II. Molecular weights of amyloses and amylopectins from starches of various plant origins. Journal of the American Chemical Society, 70, 3774 - 3777.

Preiss, J & Walsh, D A (1981) The comparative biochemistry of glycogen and starch. In Biology of Carbohydrates , Eds., Ginsburg, V & Robbins, P. Ch 5. New York : John Wiley & Sons.

Price, J C (1968) Pullulanase. In Starch and its Derivatives , Ed. Radley, J A. 4th ed, Ch 17. London : Chapman & Hall Ltd.

Protzman, T F, Wagoner, J A & Young, A H (1967) U.S. Patent 3, 344, 216.

Purdie, T & Irvine, J C (1903) C. The Alkylation of Sugars. Journal of the Chemical Society, (London) 83, 1021 - 1037.

Radley, J A (1968) General survey of starch chemistry to 1950 In Starch and its derivatives 4th ed. Ed. Radley, J A. Ch 1. London : Chapman & Hall Ltd.

Rao, V S R & Foster, J F (1963) On the conformation of the D-glucopyranose ring in maltose and in higher polymers of D-glucose. Journal of Physical Chemistry, 67, 951 - 952.

Rao, V S R, Sundararajan, P R, Ramakrishnan, C & Ramachandran, G N (1967) Conformation studies of amylase. In Conformation of Biopolymers. Vol 2, Ed. Ramachandra, G N. London: Academic Press.

Recondo, E & Leloir, L F (1961) Adenosine diphosphate glucose and starch synthesis Biochemical and Biophysical Research Communications, 6, 85 - 89.

Reese, E T, Maguire, A H & Parrish, F W (1967) Glucosidases and exoglucanases. Canadian Journal of Biochemistry, 46, 25 - 34.

Robin, J P, Mercier, C, Charbonniere, R & Guilbot, A (1974) Lintnerized starches. Gel filtration and enzymic studies of insoluble residues from prolonged acid treatment of potato starch. Cereal Chemistry, 51, 389 - 406.

Robyt, J F (1962) Ph D Dissertation, Iowa State University U S A.

Robyt, J F (1983) Synthesis of glucans from the reducing end by insertion mechanisms. In Proceedings Glucosyltransferases, Glucans, Sucrose and Dental Caries. A Special Supplement to Chemical Senses . Eds. Doyle, R J & Ciardi, J E pp 123-134. Washington D C : IRL Press.

Robyt, J F (1984) Enzymes in the hydrolysis and synthesis of starch. In Starch Chemistry and Technology 2nd ed. Eds. Whistler, R L, Bemiller, J N & Paschall, E F. London : Academic Press.

Robyt, J F & Ackerman, R J (1971) Isolation, purification and characterization of a maltotetraose producing amylase from Pseudomonas stutzeri . Archives of Biochemistry and Biophysics. 145, 105 - 114.

Robyt, J F & French, D (1963) Action pattern and specificity of an amylase from Bacillus subtilis. Archives of Biochemistry and Biophysics, 100, 451 - 467.

Robyt, J F & French, D (1964) Purification and action pattern of an amylase from Bacillus polymyxa, Archives of Biochemistry and Biophysics, 104, 338 - 345.

Robyt, J F & Whelan, W J (1968a) The alpha-amylases. In Starch and its derivatives. 4th ed. Ed. Radley, J A. Ch 14. London : Chapman & Hall Ltd.

Robyt, J F & Whelan, W J (1968b) The beta-amylases. In Starch and its derivatives. 4th ed. Ed. Radley, J A. Ch 15. London : Chapman & Hall Ltd.

Rolla, G , Ciardi, J E , Eggen, K H , Bowen, W H & Afseth, J (1983) Free glucosyl-and fructosyltransferase in human saliva and adsorption of these enzymes to teeth in vivo, In Proceeding Glucosyltransferases, Glucans, Sucrose and Dental Caries. Eds. Doyle, R J & Ciardi, J E. Washington D C : IRL Press.

Ruby, J D & Gerencser, V F (1974). Amylase activity of bacterial origin from human dental plaque. Journal of Dental Research, 53, 498.

Rundle, R E (1947) The configuration of starch in the starch-iodine complex. 5. Fourier projections from X-ray diagrams. Journal of the American Chemical Society, 69, 1769 - 1772.

Russell, R R B (1976) Classification of Streptococcus mutans strains by SDS gel electrophoresis. Microbios letters, 2, 55 - 59.

Russell, R R B (1979) Glycosyltransferases of Streptococcus mutans strain Ingbritt. Microbios, 23, 135 - 146.

Samec, M (1953) Uber wasserstoff-bindungen in der starke. Die Starke, 5, 105 - 108.

Samec, M (1954) Neure Beitrage zum problem der Alterungserscheinungen von Starke-Wasser systemen. Starke, 8, 107 - 109.

Samec, M (1957) Some recent physiochemical studies of starch. Journal of Polymer Science, 23, 801 - 807.

Sandford, P A & Conrad, H E (1966) The structure of Aerobacter aerogenes A 3. (S1) polysaccharide 1. A reexamination using improved procedures for methylation. Biochemistry, 5, 1508 - 1517.

Sandstedt, R M & Abbott, R C (1965) A comparison of methods for investigation of starch pasting. Cereal Science Today, 9, 13-26.

Sandstedt, R M, Strahan, D, Ueda, S & Abbot, R C (1962) The digestibility of high-amylose corn starches compared to that of other starches. The apparent effect of the ae gene on susceptibility to amylase action. Cereal Chemistry, 39, 123 - 131.

Sarko, A & Biloski, A (1980) Crystal structure of the KOH-amylose complex Carbohydrate Research, 79, 11 - 21.

Sarko, A & Marchessault, R H (1967) The crystalline structure of amylose triacetate 1. A stereochemical approach. Journal of the American Chemical Society, 89, 6454 - 6462.

SAS Institute (1982) Statistical Analysis Institute. Cary: SAS Institute Inc.

Schachtele, C F (1975) Glucose transport in Streptococcus mutans: preparation of cytoplasmic membranes and characteristics of phosphotransferase activity. Journal of Dental Research, 54, 330 - 338.

Schachtele, C F & Leung, W L S (1975) Effect of sugar analogues on growth sugar utilization, and acid production by Streptococcus mutans. Journal of Dental Research, 54, 433 - 440.

Schachtele, C F & Mayo J A (1973) Phosphoenolpyruvate-dependent glucose transport in oral streptococci. Journal of Dental Research, 52, 1209 - 1215.

Schwimmer, S (1950) Kinetics of malt alpha-amylase action. Journal of Biological Chemistry, 186, 181 - 193.

Seeley, H W & Dain, J A (1960) Starch hydrolyzing streptococci. Journal of Bacteriology , 79, 230 - 235.

Senti, F R & Witnauer, L P (1948) Struture of alkali amylose. Journal of the American Chemical Society, 70, 1438 - 1444.

Severson, G M (1950) Modification of starch by enzymes. In Chemistry and Industry of Starch , Ed. Kerr, R.W., Ch. 16. New York : Academic Press.

Shaw, J H & Ivimey, J K (1972) Caries in rats with starch as the only dietary carbohydrate. Journal of Dental Research , 51, 1507.

Shimamura, A, Tsumori, H & Mukasa, H (1982) Purification and properties of Streptococcus mutans extracellular glucosyltrasnferase. Biochemica et Biophysica Acta, 702, 72 - 80.

Shimamura, A, Tsumori, H & Mukasa, H (1983) Three kinds of extracellular glucosyltransferases from Streptococcus mutans 6715 (serotype g). FEBS Letters, 157, 79 - 83.

Shinke, R, Kunimi, Y & Nishira, H (1975a) Microbial beta-amylases II Production and some properties of beta-amylases of Bacillus species BQ 10. Journal of Fermentation Technology, 53, 693 - 697.

Shinke, R, Kunimi, Y & Nishira, H (1975b) Microbial beta-amylases III Production and some enzymatic properties of beta-amylase of Pseudomonas species BQ 6. Journal of Fermentation Technology, 53, 698 - 702.

Sidebotham, R L , Weigel, H & Bowen, W H (1971) Studies on dextrans and dextranases Part IX Dextrans elaborated by cariogenic organisms. Carbohydrate Research, 19, 151 - 159.

Silness, J & Loe, H (1964) Periodontal disease in pregnancy II Correlation between oral hygiene and periodontal condition. Acta Odontologica Scandinavica , 24, 747 - 759.

Slee, A M & Tanzer, J M (1979) Phosphoenol-pyruvate-dependent sucrose phosphotransferase activity in Streptocuccus mutans NCTC 10449. Infection and Immunity, 24, 821 - 828.

Snow, P & O'Dea, K (1981) Factors affecting the rate of hydrolysis of starch in food. American Journal of Clinical Nutrition , 34, 2721 - 2727.

Sonju, T, Christensen TB, Kornstad, L & Rolla, G (1975) Electron microscopy, carbohydrate analysis and biological activities of the protein adsorbed in two hours to tooth surfaces in vivo . Caries Research, 8, 113 - 122.

Spandau, D F , Miller, C H , Tellman, W C & Beuhrer, E A (1983) A starch degrading enzyme from Actinomyces viscosus, Journal of Dental Research, 62, Special Issue, 231.

Stamer, J R & Stoyla, B O (1967) Growth response of Lactobacillus brevis to aeration and organic catalysts. Applied Microbiology, 15, 1025 - 1030.

Stanier, R Y , Doudorff, M & Adelberg, E A (1971) General microbiology 3rd ed. Ch. 6, 9. London: Macmillan & Company.

Staudinger, H & Husemann, E (1937) Highly polymerized compounds CL. The constitution of starch. Annalen, 527, 195 - 236.

Stavn, A & Granum, P E (1979) Purification and physiochemical properties of an extracellular cycloamylose (cyclodextrin) glucanotransferase from Bacillus macerans . Carbohydrate Research , 75, 243 - 250.

Stein, E A (1957) Structure of B.subtilis alpha-amylase. Federation Proceedings, 16, 254.

Stein, E A, & Fischer, E H (1958) The resistance of alpha-amylases towards proteolytic attack. Journal of Biological Chemistry, 232, 867 - 879.

Stein, E A, Hsui, J & Fischer, E H (1964) Alpha-amylase as calcium-metalloenzymes. I. Preparation of calcium-free apoamylases by chelation and electrodialysis. Biochemistry, 3, 56 - 61.

Steinkraus, K H ,Bibby, B G & Gilmour, M N (1969) Acids produced by oral streptococci on cereal substrates. Journal of Dental Research , 48, 928 - 931.

Stent, G S & Calender, R (1971) In Molecular Genetics an Introductory Narative. 2nd ed. Ch 20. San Francisco : W.H. Freeman & Company.

St-Jacques, M, Sundararajan, P R, Taylor, J K & Marchaassault, R H (1976) Nuclear magnetic resonance and conformational studies on anylose and model compounds in dimethyl sulfoxide solution. Journal of the American Chemical Society, 98, 4386 - 4391.

St. Martin, E J, & Wittenberger, C L (1979) Characterization of a phosphotnolpyruvate-dependent sucrose phosphotransferase system in Streptocccus mutans. Infection and Immunity, 24, 865 - 868,

Stoddart, R W (1984) The Biosynthesis of Polysaccharides . Ch 3. London : Croom Helm.

Sutherland, I W (1979) Microbial exopolysaccharides: Control of synthesis and acylation In Microbial Polysaccharides and Polysaccharases . Eds. Berkeley, R C W and Ellwood, D C, Ch 1. London : Academic Press.

Takahashi, S, Abbe, K & Yamada, T (1982) Purification of pyruvate formate-lyase from Streptococcus mutans and its regulatory properties. Journal of Bacteriology, 149, 1034 - 1040.

Takeda, Y, Shirasaka, K & Hizukuri, S (1984) Examination of the purity and structure of amylose by gel-permeation chromatography. Carbohydrate Research, 132, 83 - 92.

Takehara, T & Inoue, M (1981) Inhibitory effects of endo-alpha-1,3 gluconase on the glucan film formation and glucan synthesis by the glucosyltransferase of the oral bacterium Streptococcus mutans. Archives of Oral Biology, 26, 217 - 222.

Tanret, C H (1915) Plurality of starches. Bulletin of the Society for Chemistry, 17, 83 - 97.

Tanzer, J M, Freedman, M L, Woodiel, F N, Eifert, R L & Rinehimer, L A (1976) Association of Streptococcus mutans virulence with synthesis of intracellular polysaccharide. In Microbial aspects of dental caries, A special supplement to Microbiology Abstracts, Vol. 3 Eds. H M Stiles, Loesche, W J & O'Brien, T C p 597 - 616. Washington DC: Information Retrieval, Inc.

Tanzer, J M, Krichevsky, M I & Keyes, P H (1969) The metabolic fate of glucose metabolized by a washed stationary phase caries-conducive streptococcus. Caries Research, 3, 167 - 177.

Tatevossian, A (1982) Hydrolysis of some carbohydrate substrates by enzymes of pooled human dental plaque fluid. Archives of Oral Biology, 27, 39 - 43.

Thachet, T C (1972) Relative titratable acidity from potato starch, maltose, sucrose and glucose produced by saliva. Journal of Dental Research , 51, 667.

Thoma, J A · Trench, D (1960) The starch-iodine interaction. Part I. Spectrophotometric investigations. Journal of the American Chemical Society, 82, 4144 - 4147.

Thomas, T D, Ellwood, D C & Longyear, V M C (1979) Change from homo- to heterolactic fermentation by Streptococcus lactis resulting from glucose limitation in anaerobic chemostat cultures. Journal of Bacteriology, 138, 109 - 117.

Thompson, J & Chassy, B M (1981) Uptake and metabolism of sucrose by Streptococcus lactis. Journal of Bacteriology, 147, 543 - 551.

Toors, F A & Herczog, L L B (1978) Acid production from a nonsugar licorice and different sugar substitutes in Streptococcus mutans monoculture and pooled plaque - saliva mixtures. Caries Research, 12, 60 - 68.

Tosiki, N, Imai, S, Akada, H, Hinoide M & Araya, S (1976) Extracellular glucans produced by oral streptococci. Archives of Oral Biology, 21, 207 - 213.

Trautner, K, Birkhed, D & Svensson, S (1982) Structure of extracellular glucans synthesized by Streptococcus mutans serotypes a-e in vitro. Caries Research, 16, 81-89.

Trautner, K & Felgenhauer, B (1979) Fractionation of glucans synthesized by Streptococcus mutans, in vitro. Caries Research, 13, 1 - 8.

Trautner, K, Gehring, F & Lohmann, D (1978) Extracellular glucans synthesized by strains of two types of Streptococcus mutans in vitro. Archives of Oral Biology, 23, 175 - 181.

Tsumori, H, Shimamura, A & Mukasa, H (1983) Comparative study of Streptococcus mutans extracellular glucosyltransferases by isoelectric focusing. Journal of General Microbiology, 129, 3261 - 3269.

Tsumori, H, Shimamura, A & Mukasa, H (1985) Purification and properties of extracellular glucosyltransferase synthesizing 1,3-alpha-D-glucan from Streptococcus mutans serotype a. Journal of General Microbiology, 131, 553 - 559.

Tsumuraya, Y & Misaki, A (1979) Structure of the water-insoluble alpha-D-glucan of Streptococcus salivarius HHT. Carbohydrate Research, 74, 217 - 225.

Vallee, B L, Stein, E A, Summerwell, W M & Fischer, E H (1959) Metal content of alpha-amylases of various origins. Journal of Biological Chemistry 234, 2901 - 2905.

van Dyk, J W & Caldwell, M L (1956a) Spectrophotometric method for quantitative evaluation of early stages of hydrolases of branched components of starches by alpha-amylases. Analytical Chemistry, 28, 318 - 320.

Van Dyk, J W & Caldwell, M L (1956b) Influence of the extent of hydrolysis upon the determination of the initial velocity of alpha-amylase action. Journal of the American Chemical Society, 78, 3343 - 3344.

van Dyk, J W & Caldwell, M L (1956C) A study of the kinetics of the hydrolysis of waxy maize starch by crystalline pancreatic amylose from swine. Journal of the American Chemical Society, 78, 3345 - 3350.

van Houte, J (1980) Bacterial specificity in the etiology of dental caries. International Dental Journal, 30, 305 - 326.

Vratsanos, S M , Abelson, D C & Mandel, I D (1979) Plaque acidogenesis and caries resistance. Journal of Dental Research, 58, Special Issue A. 1340.

Walker, G J (1965a) The cell-bound alpha-amylases of Streptococcus bovis, Biochemical Journal, 94, 289 - 295.

Walker, G J (1965b) A transglucosylase of Streptococcus bovis. Biochemical Journ [illegible] 308.

Walker, G J [illegible] the reserve polysaccharide of Streptococcus mitis [illegible] a transglucosylase. Biochemical Journal ,

Walker, G J (1968) Me[illegible]f [illegible]eserve polysaccharide of Streptococcus mitis. So[illegible]s of a pullulanase Biochemical Journal, 108, 33 - 40.

Walker, G J & Builder, J E (1971) Metabolism of the reserve polysaccharide of Streptococcus mitis. Properties of branching enzyme, and its effect on the activity of glycogen synthetase. European Journal of Biochemistry, 20, 14 - 21.

Walker, G J & Hope, P M (1964) Degradation of starch granules by some amylolytic bacteria from the rumen of sheep. Biochemical Journal , 90, 398 - 408.

Walker, G & Jacques, N A (1987) Polysaccharides of oral streptococci. In Sugar Transport and Metabolism in Gram-positive Bacteria. Ed. Reitzer, J & Peterkofsky, A , Ch. 2. Chichester: Ellis Horwood Limited.

Walker, G J , Morrey-Jones, J G , Svensson, S & Taylor C (1983) Effect of variation in growth conditions on the activity of D-glucosyltransferases and the synthesis of alpha-D-glucans by Streptococcus mutans OM2 176. In Proceedings Glucosyltransferases, Glucans, Sucrose and Dental Caries. Eds. Doyle, R J & Ciardi, J E Washington D.C.: IRL Press.

Walker, G J , Pulkownik, A & Morrey-Jones, J G (1981) Metabolism of the polysaccharides of human dental plaque. Release of dextranase in batch cultures of Streptococcus mutans. Journal of General Microbiology, 127, 201 - 208.

Walker, G J & Whelan, W J (1960) The mechanism of carbohydrase action. 8. Structures of the muscle-phosphorylase limit dextrins of glycogen and amylopectin. Biochemical Journal, 76, 264 - 268.

Wang, H L , Swain, E W & Hesseltine, C W (1984) Glucosmylase of Amylomyces rouxii . Journal of Food Science , 49, 1210 -1211.

Whelan, W J (1960) The action patterns of alpha-amylases. Die Starke, 12, 358 - 364.

Whelan, W J & Roberts, P J P (1952) Action of salivary alpha-amylase on amylopectin and glycogen. Nature, 170, 748 - 749.

Whistler, R L (1953) In Starch and its derivatives eds. Radley, J A, Vol 1, p 213. London: Chapman and Hall.

Whistler, R.L. (1973) Solubility of polysaccharides and their behavious in solution. In Carbohydrates in Solution. Advances in Chemistry Series 117 pp 242 - 255. Ed. Gould, R.F. Washington D.C.: America Chemical Society.

Whistler,R L & Daniel, J R (1984) Molecular structure of starch. In Starch chemistry and technology, Eds. Whistler, R L, BeMiller, J N & Paschall, E F 2nd ed. Ch VI. New York: Academic Press.

Whistler, R L & Doane, W M (1961) Characterization of intermediary fractions of high-amylose corn starches. Cereal Chemistry, 38, 251 - 255.

Whistler, R L & Johnson, C (1948) Effect of acid hydrolysis on the retrogradation of amylose. Cereal Chemistry, 25, 418 - 424.

Whistler, R L & Schweiger, R (1959) Oxidation of amylopectin with hydrogen peroxide at different hydrogen ion concentrations. Journal of the American Chemical Society, 81, 3136 - 3139.

White, C A & Kennedy, J F (1986) Oligosaccharides. In Carbohydrate analysis a practical approach Ch 2, Eds Chaplin, M F & Kennedy, J F. Oxford : IRL Press.

Williams, J M (1968) The chemical evidence for the structure of starch. In Starch and its derivatives, 4th ed. Ed., Radley, J A, Ch. 4. London: Chapman & Hall Ltd.

Wilson, G S & Miles, A (1975) The growth of bacteria In Topley and Wilson's Principles of Bacteriology, Virology and Immunity, 6th ed. Vol. I, Ch. 4. London: Edward Arnold.

Windish, W W & Mhatre, N S (1965) Microbial amylases (commercial use) In Advances in Applied Microbiology , 7, 273 - 304. New York : Academic Press.

Wittenberger, C L & Angelo, N (1970) Purification and properties of a fructose-1,6-diphosphate-activated lactate dehydrogenase from Streptococcus faecalis. Journal of Bacteriology, 101, 717 - 724.

Wittenberger, C L, Beaman, A J & Lee, L N (1978) Tween 80 effect on glucosyltransferase synthesis by Streptococcus salivarius. Journal of Bacteriology, 133, 231 - 239.

Wober, G (1976) Pullulanase is a characteristic of many Klebsiella species and functions in the degradation of starch. European Journal of Applied Microbiology , 3, 71 - 80.

Wolin, M J (1964) Fructose-1,6-diphosphate requirement of streptococcal lactic dehydrogenase. Science, 146, 775 - 777.

Wood, J M & Critchley, P (1966) The extracellular polysaccharide produced from sucrose by a cariogenic streptococcus. Archives of Oral Biology, 11, 1039 - 1042.

Wood, W I (1976) Tables for the preparation of ammonium sulphate solutions. Analytical Chemistry, 73, 250 - 257.

World Health Organization (1971) Oral Health Surveys . Geneva: World Health Organization.

Wu, H-C H & Sarko, A (1978a) The double helical structure of crystalline beta-amylose. Carbohydrate Research , 61, 7-25.

Wu, H-C H & Sarko, A (1978b) The double helical molecular structure of crystalline A-amylose. Carbohydrate Research , 61, 27 - 40.

Yakushiji, T, Inoue, M & Koga, T (1984) Inter-serotype comparison of polysaccharides produced by extracellular enzymes from Streptococcus mutans. Carbohydrate Research, 127, 253 - 266.

Yamada, T & Carlsson, J (1973) Phosphoenolpyruvate carboxylase and ammonia metabolism in oral streptococci. Archives of Oral Biology, 18, 799 - 812.

Yamada, T & Carlsson, J (1975a) Regulation of lactate dehydrogenase and change of fermentation products in streptococci. Journal of Bacteriology, 124, 55 - 61.

Yamada, T & Carlsson, J (1975b) Glucose-6-phosphate-dependent pyruvate kinase in Streptococcus mutans. Journal of Bacteriology, 124, 562 - 563.

Yamada, T & Carlsson, J (1976) The role of pyruvate formate-lyase in glucose metabolism of Streptococcus mutans. In Proceedings: Microbial Aspects of Dental caries, a special supplement to Microbiology Abstracts. Eds. Stiles, H M, Loesche, W J & O'Brien, T C. Vol 3, p 809 - 819. Washington DC : Information Retrieval, Inc.

Yamamoto, T (1955) Alpha-amylase-destroying enzyme 1. Occurrence and some properties of the enzyme. Bulletin of The Agricultural Chemical Society of Japan, 19, 22 - 28.

Yamashita, Y, Hanada, N & Takehara, T (1988) A novel glucosyltransferase from Streptococcus mutans produces oligoisomaltosaccharides. Biochemical and Biophysical Research Communications, 150, 687 - 693.

Yamashita, Y, Hanada, N & Takehara, T (1989) Purification of a fourth glucosyltransferase from Streptococcus sobrinus. Journal of Bacteriology, 171, 6265 - 6270.

Yokobayashi, K, Akai, H, Sugimoto, T, Hirao, M, Sugimoto, K & Harada, T (1973) Comparison of the kinetic parameters of Pseudomonas isoamylase and Aerobacter pullulanase. Biochimica et Biophysica Acta, 293, 197 - 202.

Yokobayishi, K, Misaki, A & Harada, T (1969) Specificity of pseudomanas isoamylase. Agricultural and Biological Chemistry, 33, 625 - 627.

Yokobayashi, K, Misaki, A & Harada, T (1970) Purification and properties of pseudomonas isoamylase. Biochimica et Biophysica Acta, 212, 458 - 469.

Young, A H (1984) Fractionation of starch In Starch Chemistry and Technology . Eds. Whistler, R L, BeMiller, J N & Paschall, E F, 2nd ed. Ch. VIII. New York: Academic Press.

Yovanovitch, O (1961) Infra-red absorption bands of starches with different water contents. Comptes-Rendus, 252, 2884 - 2886.

# APPENDIX

All the glassware in this study was cleaned thoroughly before use by soaking overnight in Extran MA 02 Neutral, then rinsing in running tap water followed by distilled water.

## MEDIA USED IN THIS STUDY

### HYDROLYSIS OF STARCH

#### Basal medium

25g nutrient broth No. 2 (Oxoid)
3g yeast extract
12g agar
870 ml distilled water.

The agar was melted in the distilled water. Nutrient broth and yeast extract were added.

#### Starch solution

50g starch
1000 ml distilled water

The starch was dissolved in distilled water and 130 ml was added to 870 ml of basal medium. The mixture was autoclaved at 115°C for 20 minutes.

### STARCH MEDIUM

10 g yeast extract
0,5 g sodium chloride
5 g starch (amylose or amylopectin)
1000 ml phosphate buffered saline adjusted to pH7,6.

The starches that were used were amylose type 111 (Sigma No A-0512) and amylopectin (Sigma No A-8515) both from potato.

### TODD HEWITT BROTH

10 g nutrient broth (Oxoid)
20 g tryptone (Oxoid L42)
2 g dextrose (Merck)
2 g sodium chloride (Merck)
2 g sodium bicarbonate (Merck)
0,4 g sodium phosphate (Na2 HP04)
1000 ml distilled water

The ingredients were dissolved in distilled water and autoclaved at 115°C for 20 minutes.

CHEMICALLY DEFINED MEDIUM (Jandra and Kuramitsu, 1976)

Salts and bases

| | |
|---|---|
| 3 g | ammonium sulphate |
| 0,15 g | adenine |
| 0,05 g | ferrous sulphate |
| 0,1 g | guanine |
| 0,05 g | manganous sulphate |
| 1,125 g | trisodium citrate |
| 30 g | sodium acetate |
| 0,05 g | sodium chloride |
| 15,75 g | disodium hydrogen phosphate |
| 10,25 g | sodium dihydrogen phosphate |
| 0,15 g | uracil |
| 2,2 g | potassium dihydrogen phosphate |
| 1,5 g | dipotassium hydrogen phosphate |
| 25 g | casein hydrolysate |
| 5000 ml | distilled water |

The salts and bases were dissolved in the distilled water and autoclaved at 115°C for 20 mins.

Vitamins

| | |
|---|---|
| 4 mg | riboflavin |
| 0,5 mg | biotin |
| 0,1 mg | folic acid |
| 8 mg | pantothenate |
| 0,1 mg | para amino benzoic acid |
| 4 mg | thiamine |
| 20 mg | nicotinamide |
| 8 mg | pyridoxamine |
| 20 ml | distilled water |

The vitamins were dissolved in the distilled water and autoclaved at 115°C for 20 minutes. The solution was stored at 4°C.

Final medium

| | |
|---|---|
| 1000 ml | salts and bases |
| 1 ml | vitamins |
| 10 ml | 50 % glucose |
| 1 ml | 20 % magnesium sulphate |
| 0,25 g | cysteine HCl |
| 2,2 g | sodium carbonate |

The final medium was prepared by mixing the above solutions under sterile conditions. The glucose and magnesium sulphate were prepared separately and autoclaved at 115°C for 20 minutes. The cysteine hydrochloride and sodium carbonate were prepared fresh before use. They were mixed in a small quantity of distilled water and sterilized by passing through a filter with a pore size of 0,2 µm.

REAGENTS USED FOR CHEMICAL TESTS.

ELECTROPHORESIS

Reagents for one dimentional sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

BUFFERS

1.5 M Tris buffer pH 8.7

45 g Tris was dissolved in 200 ml distilled water, the pH was adjusted to 8.7 with HCl and made up to 250 ml with distilled water.

Running buffer (concentrated)

144 g glycine
10 g SDS
30 g Tris

The ingredients were dissolved in 1 litre of distilled water. The buffer was diluted 1 in 10 in distilled water before use.

Sample buffer

2 g SDS
10 ml glycerol
5 ml beta-mercaptoethanol
0,76 g Tris

The ingredients were dissolved in 80 ml of distilled water and adjusted to pH 6.8 with HCl. A 0,2 per cent solution of bromophenol blue was made up in ethanol, 0,6 ml was added to the suspension and the solution was made up to 100 ml.

Incubation buffer for glucosyltransferases

1 g Triton X-100
10 mg dextran T10
10 mg ampicillin
50 µg nystatin
100 ml 0,05 M phosphate buffer pH 6.5

The carbohydrates were added to this buffer i.e. either 5 g sucrose or raffinose

Incubation buffer for amylolytic enzymes

0,5 g amylopectin
2 ml ethanol
2 ml 10 percent sodium hydroxide
1 g Triton X-100
10 mg dextran T10
10 mg ampicillin
50 µg nystatin
100 ml 0,05 M phosphate buffered saline

The amylopectin was moistened with the ethanol and sodium hydroxide and 10 ml of the buffer was added. The mixture was stirred until the amylopectin dissolved. The remainder of the buffer was added and the pH was adjusted to 6,4 with 10 percent hydrochloric acid. The remaining ingredients were dissolved in this solution.

GELS

Stacking gel

10 g acrylamide
0,16 g bisacrylamide
0,6 ml TEMED
0,2 g SDS
3 g Tris
4 ml glyc

The ingredients were dissolved in 150 ml water and adjusted to pH 6.8 with HCl. The solution was made up to 200 ml with water. To prepare the gel 4 ml of the solution was mixed with 20 µl 10 per cent ammonium persulphate

Separating gel

0,125 g amylopectin
0,5 ml ethanol
0,5 ml 10 per cent sodium hydroxide
6,2 ml 1,5 M Tris pH 8.7
6,6 ml distilled water
6,7 ml 30 per cent acrylamide
2,2 ml 1 per cent bisacrylamide
250 µl 10 per cent SDS
10 µl Temed
100 µl 10 per cent ammonium persulphate

The amylopectin was moistened with the ethanol. Sodium hydroxide and 2,5 ml of the buffer was added and the mixture was stirred until the amylopectin dissolved. The remaining 3,7 ml buffer was added, the pH adjusted to 8.7 with 10 per cent HCl and the solution made up to 13,8 ml with distilled water. Acrylamide and bisacrylamide were added and the solution was degassed. SDS, Temed and ammonium persulphate were added and the gel was poured.

GEL STAINING SOLUTIONS

Silver stain

Stain

0,8 g silver nitrate in 4 ml distilled water
1,89 ml 1 M sodium hydroxide
1,4 ml concentrated ammonium hydroxide

The sodium hydroxide and ammonium hydroxide were mixed and the silver nitrate solution was added dropwise while stirring. The mixture was diluted to 100 ml with distilled water and used within 5 minutes.

Developer

0,025 g citric acid
0,25 ml concentrated formaldehyde
500 ml distilled water

The citric acid and formaldehyde were dissolved in the water. This solution was made up fresh before use.

The gel was soaked in a few changes of 30 per cent ethanol, left overnight in this solution, stained with the silver solution for 15 minutes and washed in distilled water for 5 minutes with constant gentle agitation. The silver stain was developed for 10 to 15 minutes by soaking the gel in the developer until the bands appeared. The gel was washed in distilled water and placed in 50 per cent methanol to stop stain development.

Periodic Acid Schiff stain

Schiff's reagent

1 g basic fuchsin
2 g sodium metabisulphite
2 ml concentrated HCl
0.2 g activated charcoal
200 ml distilled water

The water was boiled and the basic fuchsin was added. The mixture was cooled to 50 °C and the metabisulphite was added. The solution was cooled to room temperature, the HCl was added and the solution was allowed to stand overnight in the dark. The charcoal was added and the solution was mixed and filtered. The solution was stored in the dark at 4°C. The reagent was diluted 1 in 20 in distilled water before use.

Periodic acid solution

0,7 g periodic acid
14 ml glacial acetic acid
86 ml distilled water

Metabisulphite solution

0,2 g sodium metabisulphite
14 ml glacial acetic acid
86 ml distilled water

The amylopectin gel was fixed in 75 per cent alcohol for 30 minutes. The gel was stained by agitation in periodic acid solution for 1 hour, shaken for a further hour in metabisulphite solution with several changes and placed in Schiffs reagent diluted 1 in 20 for 30 seconds. After staining the gel was placed in acetic acid:ethanol:water::10:45:45 to remove the excess stain and allowed to stand overnight to allow the colour to develop.

PROTEIN DETERMINATION

Reagent

100 mg Coomassie Brilliant Blue G
50 ml 95 per cent ethanol
100 ml 85 per cent phosphoric acid

The Coomassie Blue was dissolved in ethanol and phosphoric acid was added to the solution. The mixture was made up to 250 ml with water and diluted 1 in 4 before use.

Standard

0,50 mg per ml serum albumin

A protein assay was performed by pipetting 5-50 µg protein into a final volume of 0,1 ml and adding 2,5 ml of reagent. The solution was mixed and read after 2 and within 60 minutes at 595 nm against a reagent blank.

FEHLINGS TEST

Reagent A

6,93 g Copper sulphate
1000 ml distilled water.

The copper sulphate was dissolved in distilled water and the solution was made up to 1 litre.

Reagent B

20g sodium hydroxide
35g potassium sodium tartrate
100 ml distilled water

The sodium hydroxide was dissolved in one third of the water. The potassium sodium tartrate was added and the solution was made up to 100 ml.

Phosphomolybdic acid

70 g ammonium heptamolybdate
10 g sodium tungstate
400 ml 10 per cent sodium hydroxide
400 ml distilled water.

The ingredients were boiled in a beaker for 40 mins to remove the ammonia. The mixture was cooled, transfered to a 1 litre volumetric flask and the volume was made up to approximately 600 ml. 250 ml of an 89 per cent solution of orthophosphoric acid was added and the solution was made up to 1 litre.

IODINE STAINING

10 mg starch
1,1 ml ethanol
1 ml distilled water
0.2 ml 10 per cent sodium hydroxide

The starch was moistened with ethanol and the water and sodium hydroxide were added. The mixture was stirred until the starch dissolved. The solution was diluted in 20 ml distilled water and 1 drop of 6 N hydrochloric acid was added. One ml of a 0,2 per cent solution of iodine in 2 per cent potassium iodide was added. A tenfold dilution of the starch iodine mixture was read on a spectrophotometer at wavelengths from 250 mμ to 600 mμ.

**Author: Coogan Maeve M.**
**Name of thesis: The Degradation Of Starch By The Oral Streptococci.**

***PUBLISHER:***
**University of the Witwatersrand, Johannesburg**
**©2015**

***LEGALNOTICES:***

**Copyright Notice:** All materials on the University of the Witwatersrand, Johannesburg Library website are protected by South African copyright law and may not be distributed, transmitted, displayed or otherwise published in any format, without the prior written permission of the copyright owner.

**Disclaimer and Terms of Use:** Provided that you maintain all copyright and other notices contained therein, you may download material (one machine readable copy and one print copy per page)for your personal and/or educational non-commercial use only.

The University of the Witwatersrand, Johannesburg, is not responsible for any errors or omissions and excludes any and all liability for any errors in or omissions from the information on the Library website.