GLUCOSE TRANSPORT BY *STREPTOCOCCUS MUTANS*

by

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Abstract

Streptococcus mutans is a principal etiological agent of dental caries, the dimineralization of the tooth surface caused by end-products of carbohydrate metabolism. This study examined the first step in glucose metabolism by S. mutans Ingbritt, namely, glucose transport into the cell. S. mutans Ingbritt is known to transport a variety of carbohydrates including glucose via the phosphoenolpyruvate phosphotransferase system (PTS), a group translocation system whereby glucose is simultaneously transported across the cell membrane and phosphorylated at the expense of phosphoenolpyruvate (PEP). Continuous culture studies have indicated that the PTS is under genetic regulation and is repressed by a variety of conditions including excess glucose, elevated growth rates and growth at low pH. However, glucose transport by cells grown under these conditions is greater than can be accounted for by the repressed PTS alone, suggesting the existence of a second, non-PTS glucose transport system. The sensitivity of glycolysis in S. mutans Ingbritt grown in PTS-repressed conditions to various ionophores and metabolic inhibitors led to the proposal of an alternative, protonmotive force (PMF)-linked glucose transport in symport with protons.

The first part of the study involved a closer examination of the effect of ionophores such as valinomycin, nigericin and gramicidin and metabolic inhibitors such as fluoride on

glycolysis and on glucose transport in S. mutans Ingbritt grown in continuous culture in conditions that optimize PTS activity or lead to its repression. For example, glycolysis was far more sensitive than glucose transport to the effects of valinomycin, regardless of the growth conditions of the cells. With the other ionophores, it was difficult to separate direct effects on transport from secondary effects due to metabolic inhibition. These results did not conclusively support or refute the PMF-linked model of transport. However, it was possible to demonstrate that PTS-repressed cells actively transport glucose, which supports the presence of a second, non-PTS glucose transport system. Moreover, with increasing PTS repression, ATP became the preferred phosphoryl donor to glucose which would be expected with the non-PTS transport. Studies on the kinetics of glucose transport indicated the presence of a non-PTS glucose transporter with a K_m of 65 μM. A non-PTS glucose transporter with similar K_m had previously been identified in S. sobrinus 6715.

The definitive test for PMF-linked glucose transport would be the demonstration of glucose transport in response to the establishment of artificial electrical or pH gradients. Transport studies were performed with S. mutans Ingbritt using the non-metabolizable glucose analogue 6-deoxyglucose. Because the analogue is not transported via the PTS, its use was critical for these studies. Despite imposition of electrochemical gradients of sufficient magnitude to produce

a 15-fold accumulation of 6-deoxyglucose, the analogue was not concentrated in the cells but merely equilibrated across the cell membrane. Thus, it was conclusively proven that the PMF does not drive glucose transport in S. mutans Ingbritt.

A technique was developed for the isolation of right-side out cell membrane vesicles. Vesicles offer several advantages over whole cells for transport studies. Because the cytoplasm is removed, vesicles cannot metabolize glucose. Therefore, the use of glucose analogues is not required. Also, the lack of metabolism removes the need for the use of metabolic inhibitors. The PTS is effectively eliminated, as the soluble components essential for PTS are also absent in vesicles. Vesicles derived from S. mutans Ingbritt demonstrated a glucose counterflow phenomenon. Glucose counterflow was the transient accumulation of radiolabelled glucose in vesicles previously loaded with unlabelled glucose due to the entry of labelled glucose into the vesicles and the inhibition of its efflux by unlabelled glucose. This was the first direct proof of a non-PTS, specific glucose transporter in S. mutans Ingbritt. Counterflow was detected in vesicles from cells with high PTS activity and very low PTS activity, indicating that expression of the non-PTS glucose transporter is constitutive.

A model of phosphate-bond driven glucose transport in S. mutans Ingbritt, derived from the data obtained, is proposed. It would allow for active transport in cells with excess glucose and explain the lack of active transport in de-

energized cells. The demonstrated sensitivity of glucose transport to various inhibitors and ionophores is explained as it related to this model.

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Chapter 1

Literature review

Historical perspective

Dental caries has plagued humans since Paleolithic times and continues to be one of the most common human diseases (Loesche, 1986). Caries is the outcome of demineralization of the tooth enamel by acid end products of carbohydrate metabolism by acidogenic bacteria (Hamada and Slade, 1980; van Houte, 1986). Aristotle and others made the connection between "putrification of certain food, soft sweet figs" and tooth damage, but it was not until the 1830's that the agent or chemical factor was identified as acid by Robertson and Regnart, when they showed that dilutions of inorganic acids could corrode enamel and dentin (Newbrun, 1989). A century earlier, Anthony van Leeuwenhoek had described "little worms" present in a decayed tooth, but it was Lebet and Rottenstein (1872) who suggested a microbial role in the caries process proposing that microbes enlarged holes in the tooth, facilitating acid diffusion. Pasteur had shown that acids resulted from carbohydrate fermentation by bacteria, although it was Maginot who first demonstrated <u>in vitro</u> tooth dissolution brought about by microbial fermentation of sugars.

Miller (1889) extended the previous studies by showing that acids were present along with bacteria in caries lesions. He demonstrated that certain foods mixed with saliva resulted in sufficient acid production to dissolve the crown of a tooth

and identified the acid as lactate. He further showed that various isolates from the mouth were acidogenic enough to produce caries and cited 'gelatinous plaque' as being involved in caries (Miller, 1889). Plaque refers to the salivary film consisting of host-derived components and oral bacteria associated with the tooth surface. Miller did not distinguish between the different bacteria present on the tooth surface so his observations led to what has been called by Loesche (1986) the 'non-specific plaque hypothesis'. This hypothesis states that plaque bacteria in general are causal agents of caries and that the disease can be controlled only by their eradication from the oral cavity. Unfortunately, while careful tooth-brushing and professional cleaning at very frequent intervals is effective in reducing bacteria, this method of caries control is not feasible for large populations. Thus, it would appear that caries is a disease that must be accepted as part of the human condition.

In contrast to the theory described above, the 'specific plaque hypothesis' (Loesche, 1986) states that certain bacteria present in plaque are responsible for caries. Therefore, caries prevention would not require the impossible task of plaque removal, but the elimination of specific bacteria from the mouth. In the past sixty years, much attention has been directed at comparing bacteria on healthy teeth with those on carious teeth in order to identify etiological agents of caries. In 1924, Clarke described pleomorphic, Gram-positive cocci he called Streptococcus

mutans in association with caries (Clarke, 1924). *Lactobacillus* species were later implicated in tooth decay by the work of Enright (1932) and others. Today, the etiology and progression of caries is recognized as a complex process dependent on many factors in the oral ecosystem.

Oral flora and environment

The microbiology of the adult human mouth is complex and "the variety of bacteria is ... legion" (Kligler, 1915). This complexity reflects the range of potential habitats in the mouth, each with distinct environmental characteristics, available to colonizing bacteria. Bacteria are not uniformly distributed over oral surfaces but are localized in micro-communities associated with either the soft tissues of the tongue and oral mucosa, or the hard, non-shedding surface of the tooth (Loesche, 1986). Early studies did not always demonstrate the variety of bacteria present (Bibby, 1940), but with improvements in techniques for sampling, culturing and bacterial identification, it is now recognized that the mouth harbors over 25 genera and more than 150 bacterial species (Hamilton and Bowden, 1992) (Table 1). The mouth provides a specialized environment as demonstrated by the fact that bacteria such as *Corynebacterium matruchottii*, *Porphyromonas gingivalis*, *Rothia dentocariosa*, *Micrococcus mucilagenosus* and some of the *Actinomyces* species are found uniquely in the oral cavity (Marsh and Martin, 1992).

Table 1. Bacterial genera isolated from the oral cavity

Streptococcus	Prevotella
Leptotrichia	Selenomonas
Fusobacterium	Eubacterium
Actinomyces	Neisseria
Lactobacillus	Veillonella
Rothia	Peptococcus
Propionibacterium	Bacteroides
Bifidobacterium	Peptostreptococcus
Treponema	Porphyromonas
Haemophilus	Micrococcus

[a] From Bowden et al., 1979; Hamilton and Bowden, 1992

Different bacteria have evolved to meet the needs defined by their niche in a specific environment. To survive in the oral environment, bacteria must be able to colonize the mouth. To maintain a specific niche, an organism must be better suited to the parameters of the niche than any other bacteria. The presence of bacteria at a site can be modified by competition with other bacteria and host factors such as diet, pH and saliva. The two basic criteria used to measure the efficiency with which an oral microbe occupies its niche are: its presence in the environment and the proportion of the total community that it comprises. It is fundamental to survival of the organism, that it must be able to reproduce in a densely populated environment with limited nutrients. Ultimately, the oral bacteria can be described as microbial commensals or parasites. The host provides the physical habitat which includes antimicrobial barriers that must be overcome to allow successful colonization by bacteria.

Bacterial colonization of surfaces of the oral cavity

Communities of oral bacteria develop on surfaces and together with the local environment form small localized ecosystems. Bacteria have a wide choice of oral surfaces available for colonization. The surfaces can be broadly divided into two types: the soft tissue and the tooth. Distinct florae are found on the two surfaces reflecting differences in environmental features such as oxygen content,

pH, nutrient source and epithelial desquamation (Marsh and Martin, 1992).

Soft tissues

The soft tissue surfaces of the mouth can be divided into several types including the epithelial lining of the mouth and the tongue. The lining of the hard palate and the gingiva, known as the masticatory mucosa, is keratinized epithelium and is subject to the most friction, while most of the remaining mucosa consists of non-keratinized epithelium (Dahlén et al., 1992). The flora of the epithelium is relatively simple, consisting mainly of aerobic and facultative anaerobes averaging 5-25 bacteria per epithelial cell. The tongue has a highly irregular structure made up of numerous crypts and four different types of papillae and has a higher number of bacteria (approximately 100 bacteria per epithelial cell). Oxygen and nutrient availability to bacteria can vary widely resulting in a greater diversity of bacteria associated with the tongue, ranging from aerobes to strict anaerobes. Colonization is largely dependent on specific interactions and bacteria in these areas tend to display a high affinity for the epithelial cell surface. The greatest obstacle to bacterial accumulation on the soft tissue surface is epithelial desquamation which results in the constant removal of bacteria along with the shedding of epithelial cells. Saliva bathes the epithelium washing away epithelial cells and any attached bacteria as well as

providing a source of antibodies that may agglutinate bacteria facilitating their removal or block the binding of bacteria to epithelial cells.

Teeth

Teeth are composed of crystalline hydroxyapatite and are unique in that they are the only hard, non-shedding, external surface of the body. The non-shedding nature of the tooth surface encourages the development of complex bacterial communities called dental plaque. Dental plaque is defined as the salivary film consisting of host-derived components and the bacteria associated with the tooth surface that cannot be readily removed by moderate washing. The bacterial component of plaque can consist of up to 10^{11} cells per gram wet weight and is associated with a fluid phase containing a variety of components of either host or bacterial origin. Plaque can be divided into at least three types: fissure, subgingival and supragingival. Each type of plaque possesses a characteristic flora, depending on its location on the tooth (Table 2).

Deep pits and fissures on the occlusal surfaces of teeth offer a harsh environment to a few hardy bacteria. This area is usually completely anaerobic because oxygen cannot diffuse deep into the sites, and for the same reason, has very low nutrient availability. The limited access also means that the bacteria present are protected from mechanical and biological antimicrobial defences. Electron microscope

Table 2.

Bacteria commonly found in different types of plaque[a].

Bacteria	Supragingival	Subgingival	Fissure
Actinomyces	++++	+	-
Streptococcus	+++	+	++++
Neisseria	+	-	-
Lactobacillus	+	-	-
Bacteroides	++	+++	-
Haemophilus	++	-	+
Fusobacterium	+	++	-
Campylobacter (Vibrio)	+	++	-
Treponema/ Borrelia	+/-	++	-
Other Gram -ve anaeobes	+	++	-
Streptococcus mutans	+	-	+++
Streptococcus sanguis	++	-	+

[a] Hamilton and Bowden, 1992
[b] (+) denotes occurence and isolation frequency at site,
(-) denotes usually absent from site.

studies have suggested that many of the bacteria in the site are non-viable (Guggenheim, 1976).

Supragingival plaque includes the bacteria that accumulate at and above the gingival margin (the interface between the teeth and gums) and are responsible for dental caries. This plaque is aerobic at its surface but has increasingly reduced oxygen tension closer to the tooth. Nutrients derived from saliva and the host diet diffuse into the plaque and many of the bacteria located here metabolize carbohydrates (Hamilton and Bowden, 1992). The bacteria present in supragingival plaque have developed various mechanisms including a high capacity for adherence to surfaces to withstand the protective, cleansing effects of saliva. The supragingival plaque shows greater diversity in bacteria than that of fissure plaque. In addition, the bacterial composition of supragingival plaque varies depending on whether the lingual, buccal or interproximal facet of the tooth is examined (Loesche, 1986).

The third type of plaque is the subgingival, which is located in the gingival sulcus, the small space or pocket found between the tooth and gum below the gingival margin. Subgingival plaque displays the most bacterial diversity since this site has access to nutrients in saliva and gingival crevicular fluid. The latter fluid, which diffuses through the epithelium lining into the sulcus, is derived from serum. Thus, bacteria in this site are provided with a protein-rich environment which supports the growth of more

fastidious and proteolytic organisms than are found in supragingival plaque. This site is relatively inaccessible and is, therefore, protected from the mechanical disruption and flow of saliva encountered by the subgingival plaque.

Saliva

One of the most important host factors modulating the oral environment is saliva. Saliva performs a myriad protective functions through a combination of physical and chemical properties. Salivary flow is variable, with the highest rates normally observed following meals and the lowest during sleep (Dawes, 1987; Birkhed and Heintze, 1989). Flow rates for whole saliva vary widely between individuals, with a range of 0.01-1.9 ml per min (Pearce, 1991). The major source of saliva is from submandibular, sublingual and parotid glands and the flow rate and properties of the saliva from individual glands differs. As a consequence, contents and flow rates for whole saliva do not reflect the observed differences encountered at different sites in the mouth (Mandel, 1987; Dawes, 1987).

The flow of saliva regularly eliminates loosely-adhering bacteria, limits bacterial growth by removing food debris that can serve as potential bacterial substrates and clears harmful bacterial products of metabolism, such as acid, from the mouth. Abelson and Mandel (1981) demonstrated the importance of salivary flow by showing that in plaque sites isolated from saliva, the pH fall following a sucrose rinse

was large and the acidic pH maintained for long periods. By stimulating the salivary flow rate in "caries-resistant" individuals, no pH fall was seen following a sucrose rinse, provided the plaque sites were freely accessible to saliva.

It has been estimated that the salivary film is approximately 0.1 mm or less in thickness (Collins and Dawes, 1987) and that salivary film velocities for unstimulated saliva range from a low of 0.8 mm per min in the upper anterior buccal site to a high of 7.6 mm per min in the lower anterior lingual site (Dawes et al., 1989). Upon stimulation, salivary flow rates increases up to 40-fold with values of 1.3 and 350 mm per min reported for the two sites, respectively. As a result, the mechanical protection afforded by saliva will differ depending on the location in the mouth. Longer clearance times for harmful bacterial products such as acid will be encountered with lower saliva velocities. This observation is of importance when one considers the biological and biochemical protection mechanisms within saliva. Areas with the faster film velocities will have fastest clearance times of harmful products, but will have more restricted diffusion of protective salivary constituents into plaque.

Salivary glycoproteins and mucins lubricate the mucous membranes, keep the mouth moist, facilitate the chewing and swallowing of food by softening it and reduce attrition of the hard tooth surface (Birkhed and Heintze, 1989). Saliva alone can meet the nutritional demands of many oral bacteria

(de Jong et al., 1986), which explains why individuals fed by intubation maintain a diverse oral flora. In the absence of dietary carbohydrates, glycoproteins present in saliva can be broken down to provide carbohydrates for oral bacteria and allow growth at rates comparable to those of bacteria in hosts fed diets high in carbohydrate (de Jong et al., 1986).

Another significant role for saliva concerns its ability to effect repair of teeth through the buffer system. Saliva contains three buffer systems that counteract pH alterations induced by food or its metabolism by oral bacteria: (a) the carbonic acid-bicarbonate system, (b) the phosphate system, and (c) proteins (Birkhed and Heintze, 1989). The pH of saliva varies among individuals but is usually maintained between pH 6.0 and pH 7.0 (Kleinberg et al., 1976). It has been estimated that the carbonic acid-bicarbonate system accounts for about 60% of the buffering capacity of unstimulated saliva and 90% in saliva following stimulation by food. The remaining buffering activity of saliva is divided between the phosphate system and presumably the proteins, although there is some dispute as to the importance of the latter system (Birkhed and Heintze, 1989).

Saliva is supersaturated with calcium and phosphate which is essential to prevent the spontaneous dissolution of the hydroxyapatite structure of the tooth (Hay and Moreno, 1979). With the abundance of calcium and phosphate in solution, spontaneous precipitation of enamel salts from saliva should occur, but is not seen except in some pathological states

such as cystic fibrosis (Blomfield et al., 1974). The absence of precipitation of calcium phosphates from saliva had suggested that saliva was not actually supersaturated but in a "potential state of supersaturation" (Birkhed and Heintze, 1989). This anomaly was later resolved by the identification of several inhibitors of calcium phosphate precipitation in saliva such as statherin, proline-rich proteins and histidine-rich proteins.

Statherin is a small salivary protein consisting of 43 amino acids that has been shown to inhibit calcium phosphate precipitation in solutions supersaturated to the same extent as saliva (Hay and Moreno, 1979). It selectively adsorbs to the tooth surface preventing spontaneous precipitation. Along with statherin, a family of proteins, collectively known as the proline-rich proteins (PRP), has been identified (Hay and Moreno, 1979). Although the PRP do not seem effective at inhibiting the primary crystallization of calcium phosphates, they seem important in inhibition of enamel crystal growth. A third inhibitor of crystallization is the family of the histidine-rich proteins now individually isolated and named histatins 1-12 (Oppenheim et al., 1988). These proteins are similar to statherin and PRP's in that they adhere to the tooth surface and help prevent crystal growth but differ in that they exhibit strong antimicrobial and antifungal activity.

Saliva contains a range of other components whose primary function is antimicrobial. Most secretions including saliva

contain lysozyme, a protein produced by salivary glands and also epithelial cells. The predominant mode of action of lysozyme is as a muramidase disrupting the cell wall of susceptible bacteria. It also can act to lyse membranes and activate autolytic bacterial enzymes and can potentiate the lysis effect of salts or detergents against bacteria resistant to its muramidase activity. The peroxidase system in saliva consists of peroxidase enzymes, hypothiocyanate and hydrogen peroxidase that act in a complex manner to attack bacteria and to protect from potentially harmful effects of host and bacteria-generated hydrogen peroxide. In addition, lactoferrin chelates free iron, an essential requirement of bacteria, resulting in inhibition of microbial activity. The iron-chelating property is bacteriostatic, but when free of iron, lactoferrin has been shown to have a direct bactericidal effect by a mechanism not yet known (Birkhed and Heintze, 1989). Several salivary glycoproteins, including mucins, have been identified that have the ability to induce agglutination of bacteria blocking their adherence and facilitating their elimination from the oral cavity.

Diet

The role of diet in the oral environment tends to be viewed in light of the cariogenicity of particular foods. Ingestion of foods having a high content of refined sugar can promote the growth or dominance of acidogenic bacteria in the mouth. The link between caries, bacteria and diet was first shown in

animal studies. While caries was absent in germ-free animals regardless of their diet, a diet of fermentable carbohydrate was essential for caries formation in animals carrying 'normal' flora (Orland et al., 1954). The results of this study were confirmed by epidemiological studies, comparing caries rates with sugar consumption in human populations. Tukeuchi (1961) found that the caries rate in Japanese children was related to sugar consumption and that caries incidence reached its lowest level around 1946 when sugar restriction also reached an all time low of 0.2 kg per person per year. However, caries increased in subsequent years when sugar consumption increased. In comparing caries incidence in 12-year olds from 47 different countries, Sreebny (1982) found a positive relationship between sugar consumption and caries. One criticism of these studies is that it is very difficult to factor in variables such as oral hygiene in evaluating cause and effect.

While caries and diet are related, plaque formation is not dependent on dietary components, as demonstrated by the presence of plaque in patients who have undergone tube-feeding for prolonged periods of time (Littleton et al., 1967; Bibby, 1979). However, when plaque is removed from intubated individuals and incubated in vitro with glucose or sucrose, it is found that little acid is produced, suggesting that the proportion of acidogenic bacteria in the plaque is reduced (Littleton et al., 1967). Others have reported that restriction of dietary carbohydrates can reduce or eliminate

certain bacteria from dental plaque but this is not a universal finding (Bibby, 1979).

The most immediate effect of dietary carbohydrate is the generation of metabolic acid by acidogenic plaque bacteria on the tooth surface. Stephan (1944) was the first to plot the plaque pH in individuals following a sucrose rinse and found that: (a) initial pH was much lower in individuals with extensive caries experience and (b) the pH following the sucrose rinse decreased to a greater extent and stayed at the low levels longer with increasing caries experience . The characteristic "Stephan curve", a rapid initial pH fall followed by a gradual return to a higher resting level with time, has been seen with various foodstuffs and is related to the carbohydrate content of the food. The number of cycles of Stephan curve that occur daily will reflect the individual's diet including the amount of fermentable food and the frequency of its consumption (Geddes, 1991).

Most studies have indicated only a weak association between diet composition and caries incidence. Bibby (1979) points out that food is normally cleared from the mouth within 30 minutes, therefore, in order to have an effect, it would have to compete with salivary components with greater oral retention times. Edgar and Higham (1991) and Geddes (1991) have reviewed the many problems in determining the relationship between diet, the flora of the oral cavity and caries. For example, in clinical studies, it can be difficult to manipulate diet alone and large sample

populations would be required to counteract the variability seen between individuals. The composition and effect of diet is often related to oral hygiene practices and dental care, making evaluations of single factors impossible. In vitro studies have the drawback that they cannot take into account the effect of saliva, its flow rate and components, and the differential responses of diverse oral microbial communities to food. At present, it can best be said that diet is one of several factors in the oral environment which influences the oral microbial community.

Immune status

The immune system operates in the oral cavity with both the antibody-reactive and the cell-mediated components active in modulating the flora (Bowden et al., 1979; Smith and Taubman, 1991). The humoral component includes immunoglobulin production by lymphocytes associated with salivary glands and the oral mucosa, and transfer of serum antibody into the mouth via the gingival margin. In the gingival sulcus, elements of cellular immunity, such as lymphocytes, monocytes and macrophages, as well as polymorphonucleocytes (PMNs) can be detected. The fact that a rich microbial community exists indicates that the immune system functions to control rather than to eliminate flora from the mouth.

Humoral immunity in the oral cavity is derived from B-lymphocytes producing predominantly three classes of immunoglobulin: IgA, IgG and IgM. There is a relative

abundance of IgA-producing lymphocytes in secretory sites and the salivary glands are no exception (Brandtzaeg, 1989). It is estimated that 95% of the IgA found in the mouth is derived from lymphocytes associated with salivary ducts, while far fewer lymphocytes producing IgG and IgM are found at this site. Because of the paucity of IgG- and IgM-producing lymphocytes found in association with the salivary gland, it is believed that most of the salivary IgG and IgM is derived from serum where more than 50% is located extravascularly and passively diffuse into the oral cavity through the gingival margin. This explains, in part, the difference in ratios of IgG:IgM in the various body fluids, with a ratio of 4:1 in serum and a ratio of 1:14 in saliva (Brandtzaeg, 1989).

Following secretion by B-lymphocytes, many of the IgM and IgA antibodies are modified by their association with the secretory piece, a glycoprotein produced by epithelial cells. The secretory IgA (SIgA) and IgM (SIgM) molecules are resistant to most bacterial and host proteases that may attack immunoglobulin molecules (Brandtzaeg, 1989). The secretory piece also stabilizes the IgA molecule making it more resistant to acid degradation (Smith, 1992).

The immune environment in the oral cavity can be divided into two categories: the saliva and the gingival crevicular fluid. In the saliva, SIgA is the predominant antibody and it functions to neutralize viruses, block adherence of bacteria to plaque or epithelium, and, by interacting with

mucins, force agglutination of bacteria resulting in their elimination from the oral cavity (Smith and Taubman, 1991). Antibodies to bacterial enzymes such as glucosyltransferases alter the colonization patterns of plaque bacteria (Arnold, 1986). IgA also induces loss of bacterial plasmids that code for antibiotic resistance and adherence-associated molecules (Smith and Taubman, 1991). It has also been demonstrated that PMNs contain receptors for some SIgA molecules that allow opsonization, which is the preferential destruction of antibody-coated cells by phagocytic host cells. Most of the IgM present in the oral cavity is SIgM and is believed to be very important after birth when little IgA is synthesized. SIgM performs many of the functions of SIgA and compensates for lack of IgA in individuals with specific IgA deficiency (Brandtzaeg, 1971). Unlike IgA, IgM is also capable of interacting with complement to enhance bacterial phagocytosis and lysis (Smith, 1992).

In the gingival crevice, immunity is predominantly derived from serum with IgG as the dominant antibody. Antibodies to many of the organisms found in subgingival plaque are present and fulfill similar functions as antibodies in saliva. In addition, other components of immunity such as PMNs and complement are present at higher concentrations than are found in saliva and act in concert with IgG and IgM (Smith, 1992). Complement is a family of peptides that exert anti-bacterial effects in a variety of ways that can include bacterial lysis, as well as increased vascular

permeabilization resulting in increased local concentrations of immune components and the formation of chemotactic gradients for accelerated migration of phagocytic cells (Smith, 1992). The majority of phagocytic cells in the gingival crevice are PMNs accounting for approximately 90% of cells with the remainder consisting of B- and T-lymphocytes and monocytes.

Oral bacteria are able to elude immune defences through a variety of mechanisms. Some bacteria, such as Streptococcus sanguis, and Treponema denticola produce peptidases that cleave antibodies, protecting themselves and other susceptible bacteria. Porphyromonas gingivalis also produces proteases that attack immunoglobulins and complement (Slots and Rams, 1991). Streptococci are thought to modify their surface components in a process called antigenic shift so that antibodies originally targeted at them are no longer effective. The oral pathogen, Actinobacillus actinomycetemcomitans, binds the antibody site that is recognized by phagocytic cells preventing opsonization and produces specific leucotoxins that can remove lymphocytes from the gingival crevice. Capnocytophaga and other bacteria release toxins that interfere with chemotaxis, impeding cellular responses to bacteria. Bacteria can also shed antigens which bind and remove antibodies that could react with the surface of intact bacterial cells.

Despite the presence of bacterial-specific immune functions in the oral cavity, a rich microflora flourishes, suggesting

that immunity controls rather than eliminates bacteria from the mouth. Immunosuppressed or immuno-deficient individuals have elevated rates of caries and other oral infectious disease demonstrating the protective role of the immune system (Slots and Rams, 1991; Scully, 1992). For example, it is believed that oral immunity plays a critical role during colonization of the oral cavity. Smith and co-workers (1989, 1990) compared the humoral responses of predentate children to various streptococcal antigens and found differences in the development of responses and the specificity of antibodies formed between children. Some of these differences were probably due to strain and antigenic load differences among the bacteria, as well as variation in the immune response between children. It is reasonable to assume that these variations would translate into differences in plaque colonization patterns. In the healthy individual, the immune system is important in determining the colonization of the mouth and limiting the growth of bacteria.

Formation of dental plaque

Adherence of bacteria to surfaces

Plaque is complex, both in chemical and microbial composition. The high variability seen in plaque samples from different individuals, or even between adjacent sites on the same tooth, reflects the variety of host and bacterial factors that contribute to its formation. Today, with an

increased understanding of the properties of oral bacteria and host-bacterial interactions, the molecular mechanisms involved in plaque development are being elucidated. Bacteria colonizing the oral cavity display specificity in their choice of site, with some bacteria preferentially colonizing soft tissues, such as the association between S. salivarius and the tongue. Other bacteria, including S. sanguis, S. mutans, and A. naeslundii, do not colonize the mouth until teeth are present and disappear from the mouth upon the removal of teeth, (Ellen, 1985). This observation suggests that colonization involves specific recognition of surfaces by bacteria.

Tooth eruption creates a disturbance in the oral environment that results in the introduction of a new habitat and establishment of the plaque bacteria in the mouth. Dental plaque consists of 70-80% bacteria and 20-30% intercellular material of bacterial and host origin (Schroeder and De Boever, 1970). The formation of plaque is initiated upon tooth emergence and its structure and complexity increases with time. As with any other ecosystem, the bacterial communities become progressively more diverse and provided that the flora is not disrupted by mechanical or chemical action, a "climax" community is approached that is relatively resistant to change. The most stable communities are found in protected sites, such as fissures and approximal areas located between adjacent teeth (Bowden et al., 1979).

The first step in plaque formation is the selective deposition of salivary proteins onto the tooth surface. Collectively known as the acquired enamel pellicle, it can be found within minutes of exposure of a cleaned tooth surface to the oral environment and usually reaches a final thickness of 0.3-1.0 μm within 90 minutes (Sonju and Rölla, 1973). Pellicle composition varies between individuals but has been reported to contain up to 10 different proteins including mucins, proline-rich proteins, histatins, statherin, immunoglobulins, albumin and lysozyme (Örstavik and Kraus, 1974; Rölla et al., 1983; Kishimoto et al., 1990). Statherin, proline-rich proteins and histatins in pellicle stabilize and maintain the crystal structure of the tooth, confer selective permeability to the enamel and help guard against acid attack (Meckel, 1965). In addition, pellicle also protects the tooth surface by providing lubrication and reducing attrition. Immunoglobulins present in pellicle may deter colonization by some bacteria while other early colonizers bind IgA and other proteins found in the pellicle. The carbohydrate and protein components of pellicle also serve as substrates for plaque bacteria.

Colonization of the tooth surface is a two step process: the first step involving attachment of the bacteria, the second step is their growth at this site. Bacteria in plaque can attach to constituents of pellicle, other bacteria already present at the site, or bacterial and host components in plaque. Attachment of bacteria can be further divided

into two stages; an initial, readily-reversible interaction that may lead to a second stage which involves high-affinity binding with specific recognition of attachment sites by bacterial receptors known as adhesins (Gibbons, 1979).

In the early stage of colonization, adhesion is mediated by non-specific, non-covalent interactions between the bacteria and the tooth surface. This interaction can be described by the application of the DLVO theory named after the researchers Derjaguin, Landau, Verwey and Overbeek (DLVO)(Friberg, 1977). According to the DLVO theory, the interactive energy between a surface and a particle, in this case between the bacteria and the pellicle-coated tooth, will be the sum of attractive and repulsive forces. The charges on both surfaces will be modified by the adsorption of ions, called counterions, in the surrounding solution, so that the net surface charge will actually be the charge of the counterion, which for bacteria is positive since the unmodified bacterial surface is negatively-charged. The attractive energy includes electromagnetic energy between adjacent molecules due to their fluctuating electrical charges including dipoles, induced dipoles and nonpolar effects collectively known as van der Waal's forces. Hydrophobic and electrostatic forces also contribute to non-covalent binding between bacteria and teeth (Cowan et al., 1987; Simonsson et al., 1987). Repulsion occurs because of the overlapping electric double layers. While the attractive energy is greater than the repulsive energy, bacteria will be

loosely held on the surface and an equilibrium will form between the surface bacteria and those in suspension. Thus, the number of bacteria held on a surface is related to the ionic strength and concentration of bacteria in the suspension. Loosely-held bacteria may be released back into the suspension while others may proceed to the second stage of strong, irreversible adherence involving specific recognition that is able to withstand mechanical stress in the oral environment.

This second stage is characterized by recognition of receptors or ligands by adhesin molecules on bacteria (Gibbons, 1989). Ligands are components of the macromolecular structure of pellicle, the surface of other bacteria or components of the plaque matrix. The array of adhesin-ligand reactions possible suggests that there is a variety of mechanisms and that a single bacterium may possess a repertoire of adhesins recognizing different ligands, and that considerable variation can exist in the complement of adhesins within species. Streptococci are among the pioneer organisms colonizing plaque and remain a dominant component in mature plaque (Ritz, 1967). Consequently, more is known about the relationships of streptococcal surface polymers to adherence than those of other oral bacteria. For example, Murray and co-workers (1992) recently examined the adherence of streptococci to salivary glycoproteins and found a variety of relationships. Some strains of S. sanguis tested recognized a specific sialic acid-containing structure or

epitope on a particular purified mucin protein and substitution of alternative, but related, sialic acid-containing glycoproteins would not allow adherence. Other streptococci, such as strains of S. sanguis, S. gordonii, and S. oralis, bound several different proteins including proline-rich proteins and mucins suggesting an epitope common to multiple proteins. In some cases, the adhesin has been identified as SSP-5, a surface protein of S. sanguis that has been shown to bind sialic acid residues on human salivary agglutinin (Delmuth et al., 1990).

Proline-rich proteins are salivary glycoproteins which readily adsorb onto the tooth surface and contain ligands for diverse bacteria including streptococci, A. naeslundii, Por. gingivalis and Pre. loescheii (Gibbons et al., 1991; Gibbons and Hay, 1989, Murray et al., 1992). Their importance in determining colonization patterns can be recognized by the fact that they have been shown to compete with statherin for binding on enamel suggesting that the efficiency with which they bind to the tooth will later effect the adherence of plaque flora. Bacterial binding to salivary glycoproteins could be a mixed blessing since, when the proteins have adhered to the tooth surface, they facilitate bacterial colonization. However, these same proteins when in solution in saliva, remove bacteria from the oral environment. Gibbons and Hay (1988) proposed that cryptitopes, ligands that may be hidden but are exposed following some modification of the protein, accounted for the conundrum.

Proof of this came with the identification of a S. gordonii isolate that readily bound proline-rich proteins adsorbed to hydroxyapatite but did not recognize the same protein in solution, suggesting that a conformational alteration in the protein accompanied adsorption, exposing previously hidden epitopes (Gibbons et al., 1991).

A distinct cryptitope has been proposed for the recognition of human salivary agglutinin by the P1 adhesin on the mutans streptococci S. mutans and S. sobrinus (Brady et al., 1992). Aggregation of these bacteria occurs when the P1 protein is in solution. However, adhesion occurs when human salivary agglutinin is adsorbed to hydroxyapatite. Panels of monoclonal antibodies raised against P1 protein were tested for their ability to block adherence and aggregation. They displayed different inhibitory capacities depending on the form of the agglutinin suggesting that the adhesin reacts in different ways to the agglutinin in solution as opposed to the hydroxyapatite-bound form.

Other proposed cryptitopes are ligands exposed following enzyme treatment of surfaces (Gibbons, 1989). A. naeslundii, F. nucleatum, E. corrodens and Por. gingivalis bind poorly to unmodified surfaces, but binding occurs following cleavage of terminal sialic residues by neuraminidase exposing galactosyl residues on ligands. As neuraminidase levels are elevated in the saliva of individuals with poor oral hygiene or oral disease, such findings are of clinical relevance.

Adhesins can also recognize ligands found in molecules other than those derived from saliva. One of the most important is glucan, a carbohydrate polymer formed by the action on dietary sucrose of glucosyltransferases, enzymes associated with oral streptococci (see section Glucosyltransferases). Streptococci, particularly those grouped as the mutans streptococci (see section Taxonomy of Streptococcus mutans) bind glucans and increased levels of glucans in plaque are associated with increased accumulation of these bacteria (Hamada and Slade, 1980; Tanzer et al., 1985). Glucosyltransferases are usually secreted enzymes but may also be found associated with the cell surface of bacteria and adhere to glucans (Hamada and Slade, 1980). In mutans streptococci, cell-associated glucan-binding proteins have been identified which lack any enzymatic function, and in the case of S. sobrinus, it has been suggested that they result from proteolytic degradation of glucosyltransferases (Mooser and Wong, 1988). The binding of various strains of S. mutans and S. sobrinus to experimental pellicle containing salivary proteins, or salivary proteins and glucans, was measured (Schilling and Bowen, 1992). Some of the bacteria would not bind significantly to pellicle unless glucans were present, while others bound to both glucans and proteins in pellicles indicating distinct adhesins on the bacterial surface. Glucans in pellicle appeared to block some of the salivary protein ligands suggesting that glucans in pellicle may facilitate mutans streptococci binding, but interfere

with binding by other bacteria. Others have demonstrated that interbacterial adherence of S. sobrinus can be mediated through glucans (Rölla et al., 1985). The extracellular glucans of both A. naeslundii and S. mutans have been shown to promote homologous intercellular adhesion which is the binding of a specific bacterium to another of the same species.

Surface adhesins can also recognize ligands on unrelated bacteria, resulting in coaggregation between bacteria (McIntire, 1985). For example, microscopic examination has identified distinct structures within plaque called "corn-cobs" which are filamentous bacteria either Propionibacterium matruchotii or F. nucleatum coated with streptococci (Mouton et al., 1977). Metabolic requirements may be more readily met by associations between bacteria such as the growth of the lactate-consuming Veillonella with lactate-producing streptococci. Veillonellae do not adhere well to host tissues, but are among early isolates from tissue and plaque which could be explained by their adherence to bacteria that do bind host tissue (Hughes et al., 1992). Veillonella distribution follows that of its coaggregation partners, with strains isolated from the mucosa coaggregating preferentially with S. salivarius and strains from subgingival plaque coaggregating with other subgingival bacteria (Hughes et al., 1988).

Two distinct mechanisms of coaggregation have been identified, one is lactose-inhibitable while the other is not

(Hughes et al., 1992). The properties of the two mechanisms are sufficiently different to suggest that they involve distinct adhesins. Lactose-sensitive coaggregation is common and has been seen with Pre. loescheii binding to S. oralis, A. naeslundii binding to S. sanguis and S. mitis and Capnocytophaga ochracea binding to A. naeslundii, A. israelii, and S. sanguis (McIntire, 1985; Hughes et al., 1992; Jenkinson, 1992). Socransky et al. (1988) examined the associations between various bacteria in subgingival plaque and found that the isolation of certain bacteria in a site could predict the presence or absence of other bacteria in the same site. For example, if Actinomyces or Prop. acnes was found in a plaque sample, the chances of isolating Por. gingivalis, Peptostreptococcus micros and several other bacteria were reduced. S. intermedius was positively associated with S. mitis and S. sanguis, but negatively associated with Bacteroides forsythus. Many of these associations could be mediated through adhesin reactions between bacteria. Host proteins can interfere with coaggregation between bacteria and possibly affect colonization of the oral cavity by bacteria. For example, certain histatins and lysozyme inhibit coaggregation between certain Por. gingivalis and S. mitis with one possible mechanism being through competition for adhesins or receptors (Murakami et al., 1991).

Colonization of the oral cavity

The fetal mouth is sterile and while passage through the birth canal leads to the transient appearance of candida and lactobacilli, these organisms are not maintained in the mouth (Socranksy and Mangiello, 1971). By 10 hours, however, a wide variety of bacteria may be isolated including streptococci, veillonella, and Neisseria. Zinner and Jablon (1969) detected S. salivarius in 80% of 1-day-old infants and this organism persists on oral soft tissues throughout life. The flora present in the infant mouth comprises aerobes and facultative anaerobes that usually possess receptors for soft tissue, allowing them to avoid clearance by the flushing action of saliva (Socranksy and Mangiello, 1971; Ellen, 1985). The origin of these bacteria, many of which reside uniquely in the oral cavity, is believed to be from inoculation from other family members, especially the mother. As the infant ages, other bacteria, including Rothia, Actinomyces and Veillonella are routinely isolated. Bacteria not normally associated with soft tissues, such as S. mutans and S. sanguis, sporadically appear in low numbers, suggesting that while they contaminate the mouth, they cannot establish in the absence of their usual habitat, the tooth (Ostrom et al., 1977).

Streptococci are among the first bacteria to be isolated from a cleaned tooth surface. In vivo experiments have shown that 80% of isolates found within 4 hours following implantation of test enamel in the mouth were Streptococcus (Nyvad and Killian, 1987; Macpherson et al., 1991). In these

studies, the first species observed were S. salivarius , S. sanguis , S. oralis, and S. mitis. The number of S. salivarius rapidly decreased in the plaque over time. S. sanguis and S. mutans have an inverse relationship in plaques surveyed from various subjects. That is, in plaque with higher proportions of S. sanguis, S. mutans is reduced and the converse holds (Staat et al., 1975). S. sanguis, S. mitis, and S. salivarius do not compete with each other for binding of pellicle suggesting that each has a distinct binding site (Lilemark and Schauer, 1977). The remaining bacteria colonizing the tooth tend to be Gram-positive rods with most being Actinomyces and other isolates including Arachnia, Corynebacterium, Rothia and Propionibacterium (Nyvad and Kilian, 1987).

If left undisturbed, the bacterial mass of plaque stops increasing after about 2 days, although changes occur in the relative proportions of bacterial species (Macpherson et al., 1991). The numbers of anaerobic bacteria found in plaque increase at the expense of aerobic flora (Ritz, 1967). At this time, plaque at different sites begins to acquire a distinctive flora. In subgingival plaque, spirochetes appear after the first week. In supragingival plaque, streptococci dominate followed by Actinomyces. Neisseria decreases as plaque ages and an increase in anaerobes like Veillonella is seen and Gram-negative bacteria such as Fusobacterium, Bacteroides and Treponema are more commonly isolated. It takes approximately 14 days for undisturbed plaque to

approach a climax community that is resistant to change in the absence of a physical or chemical disturbance.

Etiology of Caries

Caries Process

As observed over 100 years ago, fermentation of carbohydrates by oral bacteria results in acid production that, in turn, may result in enamel dissolution known as dental caries (Miller, 1889). The size and duration of the pH drop in plaque is determined by the microflora present in plaque, the buffering capacity and flow rate of saliva, and the type and availability of carbohydrate (Stephan, 1944). The pH of plaque can be as high as 8.6 dropping to as low as 4.0 during fermentation in fissures and caries lesions (Stephan, 1944). Buffering by saliva is limited in deep caries lesions and fissures where the pH minimum can be maintained even after a 9-hour fast (Dirksen et al., 1962). Salivary buffering is important for enamel homeostasis since it can not only restrict the acid dissolution of enamel but it is essential for the remineralization of tooth enamel. The pH of the plaque ecosystem is an important factor in regulating the nature of the microflora since it can select for bacteria best able to survive acidic conditions. Thus, within plaque, different microenvironments exist with their own characteristic bacterial communities, some of which

facilitate the caries process (Hardie and Bowden, 1976; Loesche, 1986).

Teeth are composed of the crystal hydroxyapatite. An equilibrium forms between the crystals and the surrounding aqueous environment that can be described in a simplified form as:

$$Ca_{10}(OH_2)(PO_4)_6 \longleftrightarrow 10\ Ca^{+2} + 6PO_4^{-3} + 2OH^- \quad (1)$$

The ion concentrations in the aqueous phase determine whether the apatite dissolves. When crystal dissolution is in equilibrium with reprecipitation of crystal, the ion activity product is referred to as the solubility product. If this value is exceeded, the aqueous phase is supersaturated with respect to the ions and crystal precipitation will occur. If the activity is less than the solubility product, dissolution of the apatite occurs until equilibrium is again reached. Saliva is always saturated or supersaturated with calcium, phosphate and fluoride (Hay and Moreno, 1979), although values for individual ions can vary widely among subjects and are dependent on the flow rate and the source of the saliva (Pearce, 1991; Ferguson, 1989).

Acidification of the aqueous phase results in a decrease of the hydroxyl concentration and conversion of the phosphate ion present into more hydrogenated forms:

$$Ca_{10}(OH_2)(PO_4)_6 \xrightarrow{H^+} 10\ Ca^{+2} + 6\ PO_4^{-3} + 2\ OH^- \quad (2)$$

As there is an apparent decrease in the ion concentrations of the hydroxyl and phosphate ions, a tendency to return to

equilibrium will drive reaction (2) to the right and the hydroxyapatite will dissolve. The concentrations of calcium and phosphate species present will determine the pH at which the aqueous phase is saturated with the ions and is termed the "critical pH". Clinically, this pH has been found to range between 5.2 and 5.5 for hydroxyapatite (Pearce, 1991). When the plaque pH falls below the critical pH for hydroxyapatite there is a net loss of crystal from the tooth due to acid dissolution.

In most people in developed countries, the composition of the tooth is modified due to exposure to fluoride. Bibby (1942) first demonstrated the anti-caries effects of fluoride in clinical studies and its widespread use means that most individuals encounter it in drinking water, fluoridated dentifrices or topical treatment by the dentist. It acts in several ways to combat caries but in this section, its role in enamel will be highlighted. Fluoride modifies the tooth structure so that fluoride ions replace the hydroxyl ions in the structure as follows:

$$\underset{\text{(Hydroxyapatite)}}{Ca_{10}(OH_2)(PO_4)_6} + 2F^- \longrightarrow \underset{\text{(Fluorapatite)}}{Ca_{10}F_2(PO_4)_6} \qquad (3)$$

Fluorapatite is more acid-resistant than hydroxyapatite because it has a larger, more stable crystal structure that presents a smaller surface area per unit volume for acid dissolution and it is estimated to have a "critical pH" around pH 4.5 (Pearce, 1991). The difference between these two critical pH values mean that when the plaque pH is

reduced to 5.0 as a result of bacterial metabolism, the hydroxyapatite may start to dissolve, however, the fluorapatite will not. However, should the pH drop further to values below 4.5, net loss of both apatites will occur. As the acid is cleared or buffered, the pH increases and remineralization occurs. The earliest detectable caries lesion is called a "white spot" lesion and signals acid damage of the tooth but precedes the actual disruption of the surface layer. Backer-Dirks (1966) reported that nearly half the "white-spot" or early caries lesions found on the erupting first molars of 8-year-old children recalcified and were undetectable when examined five years later.

Flora associated with caries

Pathogenicity or the ability to cause disease is usually ascribed to a microorganism provided it fulfills Koch's postulates (Boyd and Sheldon, 1980). The suspected pathogen must be present when the disease is occurring and its elimination must result in cessation of disease. The suspected pathogen, isolated in pure culture on artificial media, must produce the disease when inoculated into a suitable host and be re-isolated from the diseased host. These criteria are readily applied to diseases involving bacterial invasion of normally sterile body sites, but do not easily apply to diseases in the oral cavity, where under normal circumstances, a complex microbial community exists. Based largely on experimental evidence using animal models

and epidemiological surveys, various odontopathogens in humans have been identified belonging to the genera: Lactobacillus, Actinomyces and Streptococcus (Bowden, 1991).

Lactobacillus

Lactobacilli were the first bacteria to be named as a possible etiological agent of caries. Kligler (1915) had observed an association between caries and lactobacilli and Enright and co-workers (1932) reported that elevated salivary counts of Lactobacillus preceeded clinical diagnosis of caries. Lactobacilli possess many of the properties that would be expected of a caries agent and most research conducted until the early 1960's centered on members of this genus. They were routinely isolated in high numbers from caries lesions and were often among the dominant flora at this site (Kligler, 1915; Enright et al., 1932).

The early interest in Lactobacillus species stemmed from the fact that these organisms were not only acidogenic, but also aciduric, continuing to grow at pH values as low as pH 4.0 (Enright et al., 1932). The lactobacilli in the mouth are divided into three biochemical types: (a) homofermentative species which convert sugars to lactate, (b) heterofermentative which may produce a variety of acids from sugars, (c) facultative heterofermentors which have the combined sugar metabolism of the two (Carlsson, 1986). While all types are found in the oral cavity, the homofermenters

(L. acidophilus, and L. fermentum), and facultative heterofermenters (L. casei and L. plantarum), outnumber heterofermenters in the mouth and are usually associated with caries (Bowden, 1991).

The shift in interest away from the lactobacilli as etiological agents of caries stemmed from animal experiments demonstrating that compared to other bacteria, namely streptococci, they had more limited cariogenicity. Lactobacillus species produced caries in some animal models under specific conditions but most commonly in the protected area of fissures (Fitzgerald, 1963, Fitzgerald et al., 1981). It was observed that lactobacilli did not adhere well to teeth and constituted only a modest proportion of normal plaque (van Houte and Green, 1974, Gibbons, 1964; van Houte, 1980; Boyar and Bowden, 1985). Therefore, it was argued that their contribution to acid present in plaque and, by extension to caries formation, must be limited. Much of the evidence suggesting their prominent role in caries came from cross-sectional studies where the microflora present in caries lesions was compared with that of plaque from healthy tooth surfaces (Enright et al., 1932; Shovlin and Gillis, 1969). In caries lesions, lactobacilli were detected at high numbers but upon restoration of the tooth, their numbers declined to the very low levels associated with healthy plaque.

While cross-sectional studies can demonstrate gross differences in the microflora between healthy and diseased

state, they do not enable one to separate bacteria which cause a lesion from those that colonize after lesion formation, ie. cause and effect. Progressive diseases require the evaluation of the flora at the earliest stage of disease to avoid ascribing pathogenicity to a bacterium that may be present as a consequence rather than an etiological agent of the disease. Thus, only when longitudinal studies were conducted, aided by caries detection with X-rays, was the role of the lactobacilli revealed. In such studies, the flora of initially healthy sites on the tooth were monitored over time until the formation of caries lesion. The results of such studies indicated that while lactobacilli could in some cases increase in plaque at sites before caries formation, increases in their numbers were more commonly associated with later stages of disease and cavitation of the tooth (Ikeda et al., 1973; Loesche and Straffon; 1979; Fitzgerald et al., 1981; Boyar and Bowden, 1985).

Current evidence suggests that the native habitat of the oral lactobacilli is in the caries lesion or fissure, protected from mechanical trauma and that they can compete successfully with other residents of plaque flora in the acidic environment (van Houte, 1980; Bowden, 1991). The ability to metabolize sugar in acid conditions means that while lactobacilli may not commonly initiate caries, they may be critical for the progression of the disease.

Actinomyces

Members of the genus Actinomyces normally comprise a high proportion of plaque flora ranking as the second most common isolate in plaque after the streptococci (Ritz, 1967; Thomson et al., 1980). They readily colonize teeth and will produce acid from sugar although at a lower rate than streptococci and lactobacillus (van Houte, 1980; Carlsson, 1986). Actinomyces naeslundii is comprised of several serovars, of which, A. naeslundii serovar 2 is regarded as a potential odontopathogen (Bowden, 1991). Some evidence exists which associate it with root surface caries, a less common type of caries associated with cementum exposed following the loss of gingival tissue in elderly individuals (Syed et al., 1975).

Streptococcus

Although streptococci make up the largest proportion of the plaque population, they were largely ignored in the search for a causal agent of caries until the early 1960's (Fitzgerald, 1973). Orland and co-workers (1954) had pioneered the use of gnotobiotic animals to determine the pathogenicity of specific bacteria by infection under controlled conditions and demonstrated that bacteria were a requirement for the development of caries.

Fitzgerald and Keyes (1960) first identified a Gram-positive Streptococcus that met Koch's postulates for pathogenicity. In their study, penicillin-treatment was used to suppress flora of hamsters and after several generations, hamsters born of antibiotic-treated dams were caries-free

when fed high-sucrose diets. However, these hamsters developed caries if they were housed with caries-active hamsters or their feces, or inoculated with plaque material derived from the caries-active hamsters. Further study showed that the caries activity could be conferred through the implantation of a specific Gram-positive bacterium alone. Thus, the organism, which did not match any of the streptococci described in Bergey's manual at the time, was shown to meet the transmissibility requirements of an etiological agent.

With this report, attention in caries research was focussed on oral streptococci. Organisms were isolated from human plaque that were cariogenic in animals and also possessed the serological, biochemical and morphological characteristics of the organism isolated by Fitzgerald and Keyes (Zinner et al., 1965; Gibbons et al., 1966). The organism was identified as Streptococcus mutans by both Carlsson (1967) and Edwardsson (1968) because of its similarity to an organism named by Clarke in 1924. Clarke (1924) had reported the presence of "unusual" streptococci in association with caries lesions, which he called S. mutans because of their ovoid appearance. They adhered strongly to teeth and could produce caries if inoculated onto teeth kept in glucose broth (Clarke, 1924). No cultures of Clarke's S. mutans were stored, however, a Type strain of S. mutans had been deposited in the National Collection of Type Cultures in Great Britain by Sims in the late 1950's. This organism was similar to the highly

cariogenic animal and human isolates studied by Fitzgerald (1973).

In humans, S. mutans is generally present in higher proportion in carious versus healthy plaque, and has also been demonstrated to occur in higher amounts in pre-caries plaque than in plaque found on caries-free tooth surfaces (Ikeda et al., 1973; Huis in 't Veld et al., 1979; Hamada and Slade, 1980; van Houte, 1980; Loesche, 1986). Caries-free individuals develop caries after acquisition of S. mutans and the caries occurs preferentially on S. mutans-infected tooth surfaces (Hamada and Slade, 1980; van Houte, 1980). Cross-sectional studies (Loesche and Syed,1973; Loesche et al., 1975) and longitudinal studies (Ikeda et al., 1973; Bowden et al., 1979; Loesche and Straffon, 1979, Boyar and Bowden, 1985) implicate S. mutans in early caries development with Lactobacillus becoming dominant in later stages of the disease.

However, S. mutans does not always increase on tooth surfaces prior to caries as demonstrated in the longitudinal study by Hardie and co-workers (1977) and Mikkelsen and Poulsen (1976). In addition, Ikeda et al. (1973) and Loesche and Straffon (1979) identified increases in lactobacilli in association with a few of the caries lesions occurring in individuals with low levels of S. mutans.

Over the last two decades, further taxonomic studies, have subdivided the former S. mutans into seven species with S. mutans and S. sobrinus now recognized as the major human

odontopathogens (Bowden, 1991). The term mutans streptococci still occurs in the literature and refers to organisms that have not been identified to the species level. Other streptococci, including S. salivarius and S. sanguis have limited cariogenicity in gnotobiotic animals (Fitzgerald, 1968), however, their role in human disease is unclear.

General model for the microbiology of caries

Four factors must be present for caries to occur; teeth/host, cariogenic bacteria, a diet high in refined fermentable carbohydrate and time. S. mutans does not establish in the mouth until after tooth eruption, although it may persist on dentures and artificial hard surfaces applied to the mouth after tooth loss (Loesche, 1986). While S. mutans was considered the dominant cariogenic bacterium in the earlier literature, new information suggests that the presence of S. mutans does not of itself guarantee caries. For example, it is apparent that not all strains within the species have the same pathogenicity. Thus, the caries experience of an individual may be linked to the virulence of the specific odontopathogens harbored. Recently, Macpherson and co-workers (1992) compared cariogenic properties of S. mutans isolated from individuals of either high or low caries experience. The S. mutans strains isolated from the two groups differed in acidogenicity and the degree of demineralization they generated after inoculation onto test enamel slabs in glucose broth. The cariogenicity of the S.

mutans as demonstrated in vitro, correlated with the caries experience of the individuals from which the strains were isolated, with "highly cariogenic" S. mutans isolated from subjects with more extensive caries experience.

While S. mutans may be the most common odontopathogen, it is not the only one and other bacterial species, or combinations of species, may also produce caries (Loesche, 1986; Bowden, 1991). In examining the flora in plaque taken from healthy sites and early caries lesions, it was found that the proportion of mutans streptococci varied considerably ranging from 0.001%-10.0% of the cultivable flora. The caries plaque lowered the pH faster to a lower value, and tended to have a higher proportion of mutans streptococci, than did plaque from healthy sites (Van Houte et al., 1991a). However, other isolates, including S. sanguis biovars I and II, S. milleri II, S. mitis and S. morbillorum were present in the plaque and capable of acid production to pH values within 0.2 units of those found with S. mutans. These presumably contributed to the observed acidogenesis of the carious plaque (van Houte et al., 1991a). In an associated study, the most acidogenic of the non-mutans streptococci showed a concomitant increase along with the number of mutans streptococci in plaque exposed to sugar (van Houte et al., 1991b). Other bacteria along with S. mutans may produce acid in plaque and contribute to early caries activity particularly in plaque with low proportions of mutans streptococci (Boyar et al., 1989; van Houte et al.,

1991a). Thus, high counts of S. mutans may be present in individuals who have no caries, while lesions can occur in the absence of S. mutans (Ikeda et al., 1973; Hardie et al., 1977; Loesche and Straffon, 1979; Bowden et al., 1984; Loesche, 1986).

As already described, a diet high in fermentable carbohydrate contributes to the caries process (van Houte, 1980). In the absence of sugar, cariogenic bacteria may be present in plaque. However, they will be unable to produce the sustained acid environment required for enamel dissolution. Conversely, as shown in animal studies, a "cariogenic" or high sucrose diet without bacteria does not produce caries (Orland et al., 1954). Time is also needed since caries is a progressive disease that requires cariogenic conditions be met over a prolonged time. It is also possible that enamel demineralization can be reversed by remineralization prior to the development of overt lesions (Newbrun, 1989; Bowden, 1991; Marsh and Martin, 1992).

Bowden (1991) has presented a scheme for caries progression that reconciles the different experimental observations and is in keeping with the dynamic environment of the oral cavity. In this scheme, the caries process is divided into four stages (Figure 1): Stage 1 represents the plaque community present on the healthy tooth, which may or may not contain S. mutans. An event may occur (for example, a dietary change resulting in an increase in fermentable

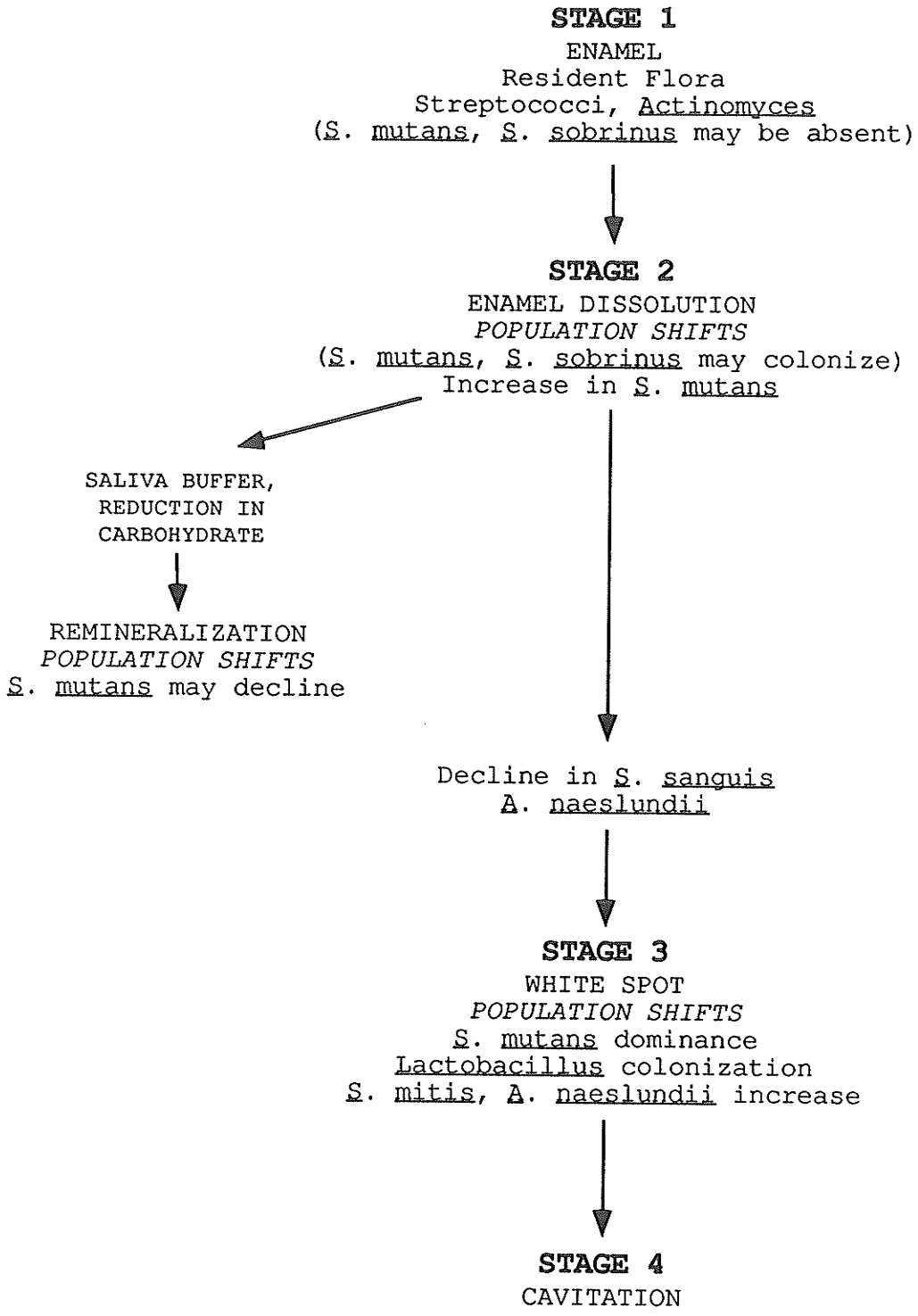

Figure 1. Microbiological Course of Dental Caries
(From Bowden, 1991)

carbohydrate) that favours the colonization of S. mutans, or its outgrowth from within the plaque community, usually at the expense of other members of the oral community (Staat et al., 1975). In Stage 2, microscopic enamel dissolution occurs, however, even at this point the disease process is completely reversible. Buffering by the saliva can allow remineralization and a concomitant restriction or reduction of the odontopathogen. If the disease is not arrested, S. mutans may increase further and the continued formation of acid leads to Stage 3, the formation of a white spot lesion, the first visible sign of caries. Other acidogenic and aciduric bacteria may become involved, either by colonization from another site or outgrowth of minor pre-existing populations, leading to Stage 4 where cavitation occurs. At this point, the environmental changes initiated by the activity of S. mutans may start to limit its growth and its viability may decline and the outgrowth of more aciduric bacteria, such as Lactobacillus species, may occur that will continue the caries process. Other bacteria increase in numbers in Stage 4, including strains of S. mitis and A. naeslundii serovar 2, while others such as A. naeslundii serovar 1 are eliminated (Bowden, 1991).

Characteristics and properties of *Streptococcus mutans*

Taxonomy and distribution of *Streptococcus mutans*

In spite of Clarke's discovery of *S. mutans* in 1924 and its 're-discovery' in 1960, it was not until 1977 that a formal proposal to elevate it to species status was made by Coykendall (1977). Many of the human and animal studies involved organisms that were termed '*Streptococcus mutans*', but the designation actually represented a heterogeneous group of organisms that were similar morphologically, but differed serologically, biochemically and genetically (Coykendall and Gustafson, 1986). There was a need to define properly *S. mutans* to allow accurate comparisons of the results of studies on different strains and eliminate the confusion resulting from the imprecise terminology. Coykendall's original proposal distinguished five species in the 'mutans streptococci': *S. mutans*, *S. sobrinus*, *S. rattus*, *S. cricetus*, and *S. ferus* (Coykendall, 1977). Since that time, investigation has led to the addition of two more species, *S. macacae* and *S. downei* (Beighton *et al.*, 1984; Whiley *et al.*, 1988). Of these species, *S. mutans* (serovars c,e, and f) and *S. sobrinus* (serovars d and g) are associated with humans, while the others are found in animals and rarely in humans (Bowden, 1991). It has been suggested that the less cariogenic strain, *S. ferus*, should not be included in the mutans streptococci and is more closely related to *S. sanguis* based on genetic analysis of several enzymes (Gilmour

et al., 1987). Some of the properties used to distinguish the mutans streptococci are presented in Table 3. Throughout this thesis, when reviewing the older literature, the term S. mutans is used interchangeably with 'mutans streptococci'.

As reviewed by Bratthall (1991), S. mutans and S. sobrinus are found in 40 -100% of the individuals sampled within different populations with the wide range attributed to the various methods employed to obtain the data. S. mutans is found with greater regularity than S. sobrinus and this is exemplified by one study conducted in Iceland in which S. mutans was isolated from 98% of schoolchildren sampled, while S. sobrinus was found in only 30% (Köhler and Bjarnason, 1987). In addition, S. sobrinus only occurred in children carrying S. mutans, usually at high levels. Contrary to these observations was a Swedish study which reported S. sobrinus was found in individuals who had no detectable S. mutans (Emilson and Thorselius, 1988).

Both species can produce bacteriocins, protein molecules produced by one strain that may have an inhibitory effect on other bacteria. In comparing S. mutans and S. sobrinus from seven individuals, it was found that S. mutans was more likely to produce bacteriocins, so this proclivity may contribute to the dominance of S. mutans noted in the Emilson and Thorselius study (Lindquist and Emilson, 1991). In vitro studies (Ikeda et al., 1988) have shown that S. mutans can kill S. sobrinus and eliminate it from the plaque of

Table 3.

Properties of *Streptococcus mutans* and other streptococci[a].

Property	*S. mutans*	*S. sobrinus*	*S. rattus*	*S. cricetus*	*S. ferus*	*S. macacae*	*S. downei*	*S. sanguis*
mol % G+C[b]	36-38	44-46	41-43	42-44	43-45	35-36	41-42	40-46
arg hydrolysis[c]	-	-	+	-	-	-	-	+
Fermn of sugars[d]								
raffinose	+	-	+	+	-	+	-	-/+
melibiose	+	-	+	+	-	-	-	-
sorbitol	+	+	+	+	+		-	-
mannitol	+	+	+	+	+	+	+	-
Prod'n of H_2O_2	-	+	-	-	-	-	-	+
Aerobic Growth	+	+	+	-	+	+	+	+
Bacit.Sens.[e]	-	-	-	+	+	+	-	+
Serotypes	c,e,f	d,g	b	a	c	c	h	H

[a] Compiled from Coykendall, 1977; Hamada and Slade, 1980; Bridge and Heath, 1982; Coykendall and Gustafson, 1986; Whiley *et al.*, 1988; Bergey's Manual, 1986.

[b] mole percent guanosine and cytosine.

[c] hydrolysis of arginine.

[d] fermentation of sugars.

[e] sensitivity to bacitracin.

experimental animals. The ecological relationship between the two species could be very significant in light of one report indicating that fresh isolates of S. sobrinus were more cariogenic than freshly isolated strains of S. mutans (de Soet et al., 1991).

To date, S. ferus and S. macacae have been found only in wild rats (Coykendall and Freedman, 1981) and monkeys (Beighton, 1984), respectively. S. cricetus and S. rattus have been found in humans, but only on rare occasions. There are isolated populations in Egypt and Sri Lanka where S. rattus is endemic, but they seem to be anomalies since no isolates can be found in surrounding populations (Bratthall, 1991). As S. downeii is a recent addition, little information on its distribution is available although preliminary reports suggest it is restricted to animals (Whiley et al., 1988).

Carbohydrate metabolism of Streptococcus mutans

In catabolism, energy is generated by the breakdown of molecules via substrate-level phosphorylation or as electron pairs in oxidation-reduction reactions and is then "stored" as adenosine triphosphate (ATP) (Lehninger, 1982). In the latter reactions, the transfer of electrons through a hydride ion occurs via the coenzymes, nicotinamide adenine nucleotide (NAD) and nicotinamide adenine nucleotide phosphate (NADP), which are alternately oxidized when coupled to an energy-requiring reaction, or reduced in a spontaneous reaction.

Uncontrolled, this mechanism could lead to cell death resulting from the build-up of protons in the cells. To counteract this, cells maintain a balance between oxidation and reduction or a redox balance (Carlsson, 1986).

Bacteria catabolize a wide range of carbohydrates via the Embden-Meyerhoff-Parnas glycolytic pathway to provide carbon and energy for the cell. This pathway involves the step-wise degradation of glucose to two molecules of pyruvate (Figure 2). Glycolysis is a metabolic pathway found in virtually all organisms from bacteria to man, although differences are seen in the metabolism of pyruvate and the regulation of the pathway (Lehninger, 1982). Glucose is primed for the subsequent energy-generating steps by the addition of two phosphates. In bacteria, the first phosphate may be donated by ATP through the action of a sugar-specific kinase or by phosphoenolpyruvate (PEP) in a group translocation process called the phosphoenolpyruvate phosphotransferase system (PTS). Further phosphorylation by ATP via phosphofructokinase (Figure 2, 1) generates fructose-1,6-bisphosphate (FBP_2) which is eventually degraded to pyruvate, generating four molecules of ATP and 2 molecules of $NADH_2$.

With respect to energy metabolism by S. mutans, the organism lacks the oxidative portion of the pentose phosphate cycle and contains no transhydrogenase activity to produce $NADH_2$ and $NADPH_2$. To overcome this, S. mutans contains two glyceraldehyde 3-P dehydrogenases, one NAD-dependent and the second NADP-dependent. The NAD-dependent glyceraldehyde-3-P

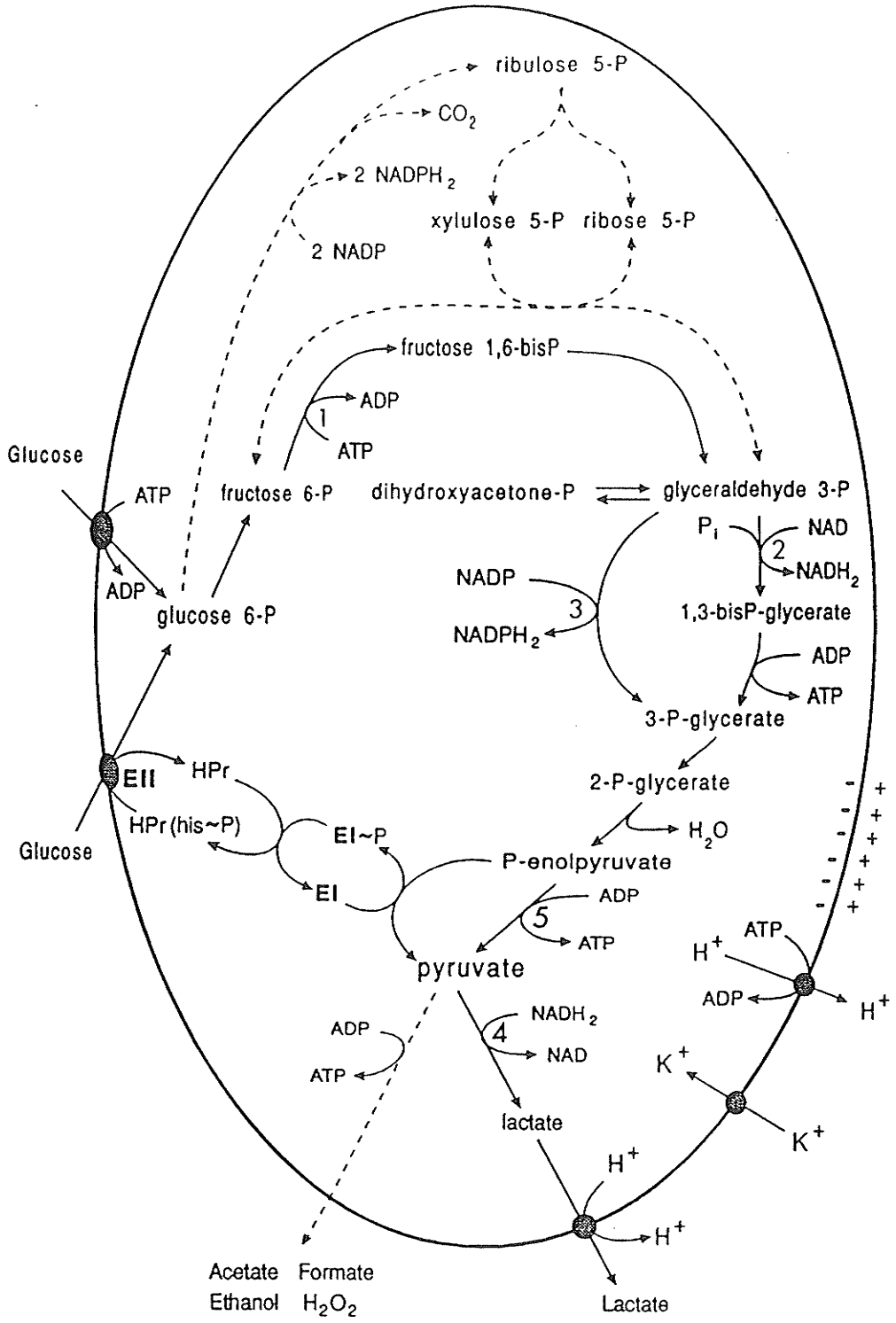

Figure 2. Glucose Metabolism in Streptococcus mutans From Carlsson, 1986.

dehydrogenase (Figure 2,2) reduces NAD to $NADH_2$ as it converts glyceraldehyde 3-P into 1,3-bisphosphoglycerate. This enzyme is inhibited by $NADH_2$, so oxidation of the coenzyme is required to avoid inhibition of glycolysis (Iwami, 1988). The NADP-dependent glyceraldehyde 3-phosphate dehydrogenase (Figure 2, 3) oxidizes glyceraldehyde 3-phosphate to 3-phosphoglycerate bypassing the ATP-generating step. $NADH_2$ and $NADPH_2$ must be reoxidized to preserve the oxidation/reduction balance in the cell (Carlsson, 1986).

The fate of the pyruvate formed by glycolysis in S. mutans is central to the bacterium's role as an oral pathogen. One of the first observations made by Clarke in 1924 regarding S. mutans was that it was capable of prodigious acid production from glucose. Research since has shown that the organism is one of the most acidogenic of the oral flora (Loesche, 1986; Hamilton and Bowden, 1991). Initially, it was thought that S. mutans was homofermentative, producing exclusively lactate from glucose. Although Friedemann demonstrated that streptococci were heterofermentative as early as 1938, Carlsson and Griffith (1974) using continuous culture, were the first to demonstrate that S. mutans could change from homo- to heterofermentation depending on the sugar concentration. For example, when cells were grown with excess glucose they generated lactate. However, a shift to glucose-limited conditions produced acetate, formate and ethanol and with much lower concentrations of lactate.

The change in fermentation patterns is a reflection of the regulation of glycolysis in S. mutans. Pyruvate in the organism can be degraded in two different directions to lactate by lactate dehydrogenase(Figure 2,4), or to ethanol, acetate and formate in the pyruvate formate lyase pathway (Figure 3, 1). The catabolism of glucose by the two divergent pathways can be summarized by the following equations:

$$\text{glucose} + 2\ \text{NAD} + 2\ \text{ATP} + 4\ \text{ADP} \longrightarrow 2\ \text{lactate} + 2\ \text{NAD} + 4\ \text{ATP} + 2\ \text{ADP} \quad (4)$$

$$\text{glucose} + 2\ \text{NAD} + 2\ \text{ATP} + 5\ \text{ADP} \longrightarrow 1\ \text{ethanol} + 2\ \text{formate} + \text{acetate} + 2\ \text{NAD} + 5\ \text{ATP} + 2\ \text{ADP} \quad (5).$$

The advantage of heterofermentation (equation 5) is the net gain of 3 mol ATP/mol glucose consumed, making it more efficient than the homofermentative pathway which yields only 2 mol ATP/mol glucose. Clearly, under glucose-excess conditions, the cellular concentration of ATP is high and the cells have a high energy charge (Atkinson, 1968). However, under conditions of a limiting carbon source, typical of dental plaque, the ability to shift to the more efficient heterofermentative pathway would be an ecological advantage. On the tooth surface, S. mutans will be faced with prolonged bouts of low carbohydrate and nutrient availability when the host is fasting between meals disrupted by sudden increases in available sugar when the host eats. This situation could pose a significant threat to acidogenic oral bacteria if

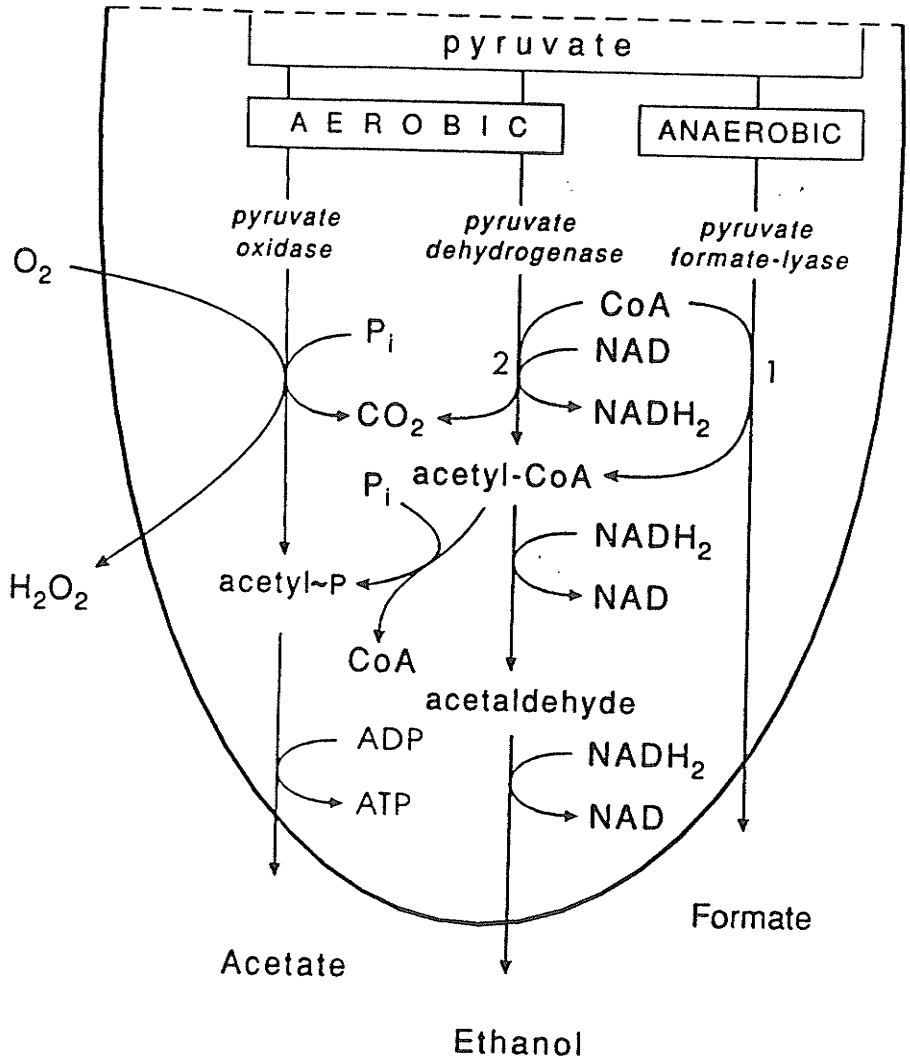

Figure 3. Pyruvate Catabolism in Streptococcus mutans From Carlsson, 1986.

these bacteria had no means of dealing quickly with the rapid fluxes in nutrients, where 10,000-fold increases in sugar concentration upon eating can be observed. The build-up of glycolytic intermediates would kill the bacteria in a process called 'substrate-accelerated death' or 'sugar killing' (Carlsson, 1986). S. mutans has developed strategies to cope with the 'feast and famine' conditions of the oral cavity, including synthesis of intracellular and extracellular polysaccharides and the rapid breakdown of pyruvate to lactate by regulation of the glycolytic pathway.

Regulation of glycolysis in Streptococcus mutans

Glycolysis in S. mutans is regulated by the cellular concentrations of critical intermediates of the pathway. When the extracellular sugar concentration is high, the intracellular level of glycolytic intermediates is high, conversely, as the sugar concentration declines, so do the levels of intermediates. The control of acid production in S. mutans is centered on the regulation of pyruvate kinase, lactate dehydrogenase, and pyruvate formate lyase (Carlsson, 1986).

The major regulatory site of glycolysis in streptococci is pyruvate kinase, an enzyme strongly activated by glucose-6-P, and to a lesser extent by ribose-5-P, and inhibited by inorganic phosphate (Yamada and Carlsson, 1975b; Iwami and Yamada, 1980; Yamada, 1987). In conditions of limiting

sugar, the level of glucose-6-P is low, resulting in low pyruvate kinase activity allowing the build-up of PEP in the cell, making it available for sugar transport via the PTS (Yamada and Carlsson,1975b). When sugar is in excess, high intracellular levels of the glycolytic intermediates coupled with a low concentration of intracellular inorganic phosphate lead to activation of pyruvate kinase. This in turn, leads to a decrease in PEP and a corresponding decrease in the activity of the PTS.

The lactate dehydrogenase of most strains of S. mutans requires activation by fructose-1,6-bisphosphate (FBP_2) and, consequently, cells grown under conditions of sugar-excess have high enzyme activity (Yamada and Carlsson, 1975a). The redox potential of the cell can affect lactate dehydrogenase activity, since NADH inhibition of glyceraldehyde-3-P dehydrogenase will further increase levels of FBP_2. The combination of increased levels of this activator and one of its substrates, $NADH_2$, enhances lactate dehydrogenase activity. On the other hand, in cells grown in limiting glucose, FBP_2 levels will be low and lactate dehydrogenase will not be fully active. Moreover, high intracellular levels of phosphate antagonise FBP_2 activation so that higher concentrations of the activator are required to achieve the same activity compared to conditions of low phosphate (Yamada and Carlsson, 1975a). The regulation is further complicated by the fact that PEP and ATP have been shown to be inhibitory (Brown and Wittenberger, 1972; Yamada and Carlsson, 1975a).

As ATP generation is the primary function of glycolysis, inhibition by this nucleotide would allow the organism to slow the rate of glucose metabolism in conditions of excess glucose when its pool of ATP is high (Brown and Wittenberger, 1972). No difference is found between amounts of lactate dehydrogenase in cells grown in limiting or excess glucose ruling out the possibility of genetic regulation (Yamada and Carlsson, 1975a). One truly homofermentative strain of S. mutans has been described (Yamada et al., 1976). S. mutans FIL possesses a lactate dehydrogenase that does not require FBP_2 for activation and lactate predominates as the end product regardless of the external glucose concentration.

At low sugar concentrations, lactate dehydrogenase is not active in oral streptococci, and a heterofermentative pattern of end-products occurs through the action of pyruvate formate lyase. To maintain the redox balance, formate, ethanol and acetate are produced in a ratio of 2:1:1(Yamada and Carlsson, 1975b.;Yamada, 1987). Pyruvate formate lyase is inhibited by glyceraldehyde-3-P and to a lesser extent, dihydroxyacetone-P, compounds elevated in cells exposed to high concentrations of sugar (Yamada and Carlsson, 1976). Consequently, with excess sugar, lactate dehydrogenase is active and pyruvate formate lyase is inactive. Aside from allosteric control, it has been suggested that further regulation is occurring at the genetic level and synthesis of pyruvate formate lyase is repressed in conditions of glucose excess (Yamada, 1987).

Dissacharides such as lactose and sucrose are metabolized more slowly by S. mutans than monosaccharides such that glycolytic intermediates do not build up to the same extent. Thus, in cells grown anaerobically on these sugars, lactate dehydrogenase is not fully activated and pyruvate formate lyase is not completely inhibited, so combinations of lactate, acetate, formate and ethanol are produced (Yamada, 1987). With respect to sugar alcohols such as mannitol and sorbitol, $NADH_2$ is generated during metabolism since subsequent to their entry into the cell via the PEP-phosphotransferase system, they are acted on by specific dehydrogenases:

$$\text{sorbitol-6-P} + \text{NAD} \xrightarrow{\text{sorbitol-6-P dehydrogenase}} \text{fructose-6-P} + NADH_2 \qquad (6)$$

$$\text{mannitol-1-P} + \text{NAD} \xrightarrow{\text{mannitol-1-P dehydrogenase}} \text{fructose-6-P} + NADH_2 \qquad (7)$$

By increasing the pyruvate degradation to ethanol at the expense of acetate formation, the extra $NADH_2$ is oxidized maintaining the redox balance of the cell (Carlsson, 1986).

The concentration and type of the extracellular sugar are not the only determinants of the fermentation products from the metabolism of sugars by S. mutans. Growth rate and pH also help dictate the fate of sugars. This was seen by examining the end-products produced from cells grown under glucose-excess and glucose-limited cultures of S. mutans Ingbritt in the chemostat at various growth rates (Hamilton,

1987). As mentioned previously, lactate was the dominant end-product of cells grown with excess glucose regardless of the growth rate, but when the growth rate of the glucose-limited cultures was increased, lactate appeared in addition to acetate, ethanol and formate. One assumes that the increased growth rate would be accompanied by increased FBP_2 levels which activate lactate dehydrogenase.

Shifts in pH from pH 6.5 to 5.5 with glucose-limited cells resulted in a shift from heterofermentation to lactate production exclusively (Hamilton, 1987). The drop in pH was accompanied by an increase in glycolytic rate so that the associated increase in the cellular concentration of glycolytic intermediates would allow for the full activation of lactate dehydrogenase. Thus, with the fluctuations in sugar concentration and pH found in dental plaque, S.. mutans will not use either pyruvate formate lyase or lactate dehydrogenase exclusively, but a combination of both pathways depending on the conditions.

From this discussion, it is evident that S. mutans possesses carbohydrate metabolism that adjusts to environmental conditions, including pH and specific carbohydrate availability. Conditions of excess carbohydrate, which would relate to high carbohydrate intake by an individual, would lead to high lactate production promoting conditions favorable to caries formation and, in part explains the observed link between diet and caries.

Aerobic metabolism of *Streptococcus mutans*

In addition to the above factors regulating the pattern of end-product formation, shifts in fermentation patterns are observed when *S. mutans* is exposed to oxygen. Pyruvate formate lyase is irreversibly inhibited after as little as a 2 minute exposure to oxygen, therefore, heterofermentation is curtailed and lactate becomes the dominant end product (Abbe *et al.*, 1982). Lactate dehydrogenase continues to be activated by levels of FBP_2 that are not appreciably altered by the shift from anaerobic to aerobic environment (Iwami *et al.*, 1975).

When pyruvate formate lyase is inactivated by oxygen, *S. mutans* induces the multi-enzyme complex, pyruvate dehydrogenase, to generate acetyl CoA (reaction 8)(Figure 3,2):

$$\text{pyruvate} + \text{NAD} + \text{CoA} \longrightarrow \text{acetyl-CoA} + NADH_2 + CO_2 \qquad (8)$$

This enzyme requires thiamin pyrophosphate, coenzyme A and NAD, and is activated by phosphate (Carlsson *et al.*, 1985). Induction of the enzyme requires aerobic conditions and the presence of pyruvate in the medium (Carlsson, *et al.*, 1985). Aerobically, the ratio of fermentation products differs from that seen anaerobically in that ethanol increases, acetate decreases and formate is no longer produced (van Beelen *et al.*, 1986). The ethanol increase is linked to NAD regeneration since it is through this pathway that $NADH_2$ is oxidized. The third pathway, pyruvate oxidase has been

reported in S. sanguis and S. mitis (Carlsson and Kujala, 1984).

Oxygen is used by flavin nucleotides for NADH oxidation in the NADH oxidase system with different reactions occurring between strains within the species S. mutans (Thomas and Pera, 1983). For example, the NADH oxidase system of S. mutans GS5 produces water from oxygen (reaction 9),

$$2\ NADH_2 + O_2 \xrightarrow{\text{NADH oxidase}} 2\ NAD^+ + 2\ H_2O \qquad (9)$$

While in S. mutans Ingbritt, there is a two-step process involving the generation of superoxide radical (O^-) (reaction 10),

$$NADH_2 + O_2 \xrightarrow{\text{NADH oxidase}} NAD^+ + 2\ O^- + 2H^+ \qquad (10)$$

$$2\ O^- + 2H^+ \xrightarrow{\text{superoxide dismutase}} O_2 + H_2O_2 \qquad (11)$$

Most cells synthesize superoxide dismutase to remove the highly toxic superoxide radical (O^-) from the cell (reaction 11). Hydrogen peroxide is also toxic to cells yet studies with S. mutans Ingbritt demonstrated that continued exposure to oxygen did not result in the accumulation of hydrogen peroxide in the cells. It was either expelled from cells or rendered non-toxic by the activity of NADH peroxidase (reaction 12),

$$NADH + H^+ + H_2O_2 \xrightarrow{\text{NADH peroxidase}} NAD^+ + 2\ H_2O \qquad (12)$$

The above reaction requires active metabolism by the cell to provide a constant supply of reduced NADH to fuel the reaction.

Glycogen

Streptococci deal with the fluctuating carbohydrate levels by storing intracellular carbohydrate as glycogen, a polymer of glucose with $\alpha(1-6)$ linkages branching along a $\alpha(1-4)$ backbone. Oral streptococci are generally active glycogen synthesizers with increases of 37 and 50% in dry weight due to glycogen formation reported for S. mitis (Gibbons and Kapsimalis, 1963) and S. salivarius (Hamilton, 1968), respectively. S. mutans cells found in deep layers of plaque contain more glycogen granules than those isolated from the surface. This suggests a role for glycogen in energy storage in the organisms survival close to the tooth where carbohydrate restriction plays a role in the survival of the bacteria (Hamada and Slade, 1980).

Glycogen is formed from glucose-1-P by the action of ADP-glucose synthase (reaction 13) and α-1,4 glucan transferase (reaction 14) (Khandelwal et al., 1973; Hamilton, 1976):

$$\text{glucose-1-P} + \text{ATP} \xrightarrow{\text{ADP-glucose synthase}} \text{ADP-glucose} + \text{PPi} \qquad (13)$$

$$\text{ADP-glucose} + \text{glycogen}_n \xrightarrow{\alpha\text{-1,4 glucan transferase}} \text{ADP} + \text{glycogen}_{n+1} \qquad (14)$$

The key to regulation of glycogen formation is ADP-glucose synthase which is activated by FBP_2, present at high

concentrations when the bacteria are exposed to excess carbohydrate. This shunting of glucose into a storage form rather than glycolysis is a mechanism used by bacteria to prevent 'sugar killing' (Carlsson, 1986). Glucose from glycogen can re-enter glycolysis through the action of glycogen phosphorylase (reaction 15) and a debranching enzyme (Spearman et al., 1973) with the resultant glucose-1-P readily converted to glucose-6-P in the Embden-Meyerhoff-Parnas pathway (Huis in't Veld and Backer-Dirks, 1978).

$$\text{glycogen}_n + P \xrightarrow{\text{glycogen phosphorylase}} \text{glycogen}_{n-1} + \text{glucose-1-P} \qquad (15).$$

Glycogen phosphorylase is activated by PEP and AMP, both of which are increased in starved cells (Spearman et al., 1973; Carlsson, 1986).

Although most oral streptococci synthesize glycogen, differences in the rates of formation and degradation between oral streptococci have been reported (Hamilton, 1976). S. mutans and S. sanguis produced low levels of glycogen compared to some strains of S. salivarius, with the slower glycogen synthetic rates coupled to slower utilization rates.

Glycogen production and utilization by bacteria has been associated with caries activity in some studies, but not all. Berman and Gibbons (1966) surveyed plaque from healthy and carious sites in rats and humans. They reported that isolates from caries-prone subjects were more likely to produce glycogen than those taken from caries-inactive plaque

sample (60% versus 13% for the human isolates) and were more likely to maintain the ability after prolonged culture *in vitro*. In one study, however, strains of *S. mutans* unable to form large amounts of glycogen were cariogenic in animal models (Freedman *et al.*, 1976). Tanzer *et al.* (1976) reported opposite results with mutants defective in glycogen production. In this case, the mutant cells were impaired in cariogenic potential compared to wild-type *S. mutans* however. When rats were infected with one of the glycogen-defective mutants, colonization of the wild-type parent was delayed or diminished. Thus, pre-infection of the rats with the mutant conferred protection from caries. Glycogen synthesis did not impair colonization by the mutant, or place it at a competitive disadvantage when challenged by the glycogen-active wild-type strain.

Direct evidence linking glycogen metabolism with cariogenic activity comes from the enamel demineralization model developed by Zero and co-workers (1986a,b), consisting of an intra-oral appliance made of enamel blocks covered with bacteria. *S. mutans* were generated with and without glycogen, by growing cells with excess or limiting glucose, respectively. Both types of cells were shown to have comparable cell densities and glucose metabolizing ability. Following application of cells onto the appliance and its insertion into the mouth of volunteers, a large prolonged pH drop without the addition of any exogenous carbohydrate, was obtained with cells containing glycogen and this was

associated with demineralization. Carbohydrate rinses did not significantly modify this observation and no acidification or demineralization was seen with the other cells in the absence of sugar rinses.

More recently, S. mutans mutants defective in glycogen production were obtained by recombinational inactivation (Harris, 1992). The mutants produced little glycogen and were significantly less cariogenic in a gnotobiotic rat model however, the defective gene did not share homology with the known sequences for glycogen metabolic genes, suggesting that the mutation may be in a regulatory rather than structural gene of glycogen synthesis (Harris et al., 1992). Thus, the relationship between glycogen synthetic and degradative abilities and virulence of oral bacteria may be more complex than originally thought.

Extracellular polysaccharide

A diet high in sucrose will not only affect glycogen production, but also allow for the production of extracellular polysaccharide by oral bacteria. It is estimated that streptococci will convert between 3-5% of available sucrose into glucans or fructans while the rest is catabolized (Chassey, 1983). External sucrose can be simultaneously cleaved by the action of free and cell-associated fructosyltranferases and glucosyltransferases with the transfer of fructose or glucose to a growing

polysaccharide chain by the following reactions (Hamada and Slade, 1980):

$$\text{Sucrose} + \text{fructan}_n \xrightarrow{\text{fructosyltransferase}} \text{glucose} + \text{fructan}_{n+1} \qquad (16)$$

$$\text{Sucrose} + \text{glucan}_n \xrightarrow{\text{glucosyltransferase}} \text{fructose} + \text{glucan}_{n+1}. \qquad (17)$$

Glucans are divided into two types based on their solubility. The soluble glucan, also termed dextran is composed predominantly of glucose moieties linked in a $\alpha(1-6)$ backbone with $\alpha(1-3)$ and $\alpha(1-4)$ branching. Insoluble glucan or mutan has a predominantly $\alpha(1-3)$ linkage backbone with $\alpha(1-6)$ and $\alpha(1-4)$ branchings (Baird et al., 1973; Inoue and Smith, 1976). Fructans produced by S. mutans are predominantly $\beta(2-1)$-linked fructose molecules unlike the $\beta(2-6)$-linked fructans formed by other bacteria and have varying degrees of solubility (Baird et al., 1973; Newbrun, 1976).

Recent studies have identified three distinct glucosyltransferases in S. mutans. The glucosyltransferase B (*gtfB*) gene product breaks down sucrose to produce mainly insoluble glucan, some soluble glucan and fructose (Shiroza et al., 1987; Fukushima et al., 1992). The *gtfC* gene product produces free fructose and either soluble or insoluble glucan (Hanada and Kuramitsu, 1988). The *gftD* gene converts sucrose to soluble glucan and free fructose (Hanada and Kuramitsu, 1989). There is some evidence, based on sequence analysis, that the *gftC* gene arose from the *gftB* gene through recombination and mutation and it is located

adjacent to the *gftB* gene on the S. mutans chromosome (Ueda et al., 1988). Recently, a strain of S. mutans has been identified in which the *gftB* and *gftC* have recombined so that it contains only a single gene for the enzyme regulating insoluble glucan synthesis which appears to be a hybrid of the two genes normally found in S. mutans (Yamashita et al., 1992). In addition, considerable heterogeneity of the *gftB* gene exists within the species. This is believed to occur due to point mutations that do not appreciably alter the activity of the gene products (Chia et al., 1991). Fructan production results from the presence of a single gene, *ftf* coding for the fructosyltransferase (Shiroza and Kuramitsu, 1988).

Fructans and glucans contribute significantly to the oral environment in several ways. Soluble glucans and fructans can be degraded by plaque bacteria, and therefore, serve as extracellular carbohydrate storage (Schachtele et al., 1973). Dextranases, enzymes capable of degrading glucans, can be cell-associated or free, and are produced by many residents of the plaque including streptococci, Fusobacterium fusiforme, Actinomyces israelii, Bacteroides ochraceus and Bifidobacterium (Sund et al., 1987). In the case of S. mutans and A. israelii, their dextranases reduce glucans to oligosaccharides that they cannot metabolize but which may be used by other bacteria in the plaque community (Staat and Schactele, 1974, 1975). It is recognized that various dextranases are produced by the plaque microflora and their

joint action will convert glucans to free glucose. For example, the action of the dextran-glucosidase of S. mitis on soluble glucans formed by strains of S. mutans results in the formation of glucose which could become available to S. mutans and other bacteria present in plaque (Sund et al., 1987).

Schroeder and co-workers (1989) prepared a mutant of S. mutans defective in the *ftf* gene and, therefore, unable to produce fructans. The mutant adhered to rat tooth surfaces as well as the wild-type parent, although it was significantly less cariogenic. It was suggested that fructans may contribute to the virulence of S. mutans by providing a carbohydrate storage compound for the bacteria to consume during periods of low carbohydrate availability. S. mutans produces a fructan hydrolase that is induced by the presence of fructans and repressed by glucose (Walker et al., 1983). Because animals in the Schroeder experiments were fed ad libitum, fructan utilization may not have been a critical factor since the microflora in plaque may not have been sufficiently limited in carbohydrate.

Extracellular polysaccharides also act as a site for adherence of many bacteria. Glucans act as the receptors for adhesins present on mutans streptococci (Hamada and Slade, 1980; Tanzer et al., 1985). By producing glucans that serve for adhesion in the mouth, mutans streptococci can displace other bacteria that cannot bind to glucans. Some of the adhesins may be cell-associated glucosyltransferases that,

through the action of proteolytic enzymes in plaque, have lost their catalytic function while retaining glucan-binding ability (Mooser and Wong, 1988).

Munro and co-workers (1991) looked at the adhesion and cariogenicity of wild type S. mutans and mutants defective in various combinations of glucosyltranferase and fructosyltransferase genes. They used an in vitro model of adhesion that relied on the fact that S. mutans cannot adhere to glass unless grown with excess sugar, which then allows adherence through extracellular polysaccharides. The insoluble glucans seem to be critical for adherence to glass since inactivation of *gftB* or *gftC* abrogated adhesion to glass even in the presence of a functional *gftD* or *ftf* gene. When all glucosyltransferase and fructosyltransferase genes were inactivated, mutants were significantly impaired in cariogenicity and the addition of a functional *gtfD* gene did not significantly increase cariogenicity suggesting that, in the gnotobiotic rat model used, soluble glucans contributed little to virulence. Addition of a functional *ftf* gene increased cariogenicity slightly, but caries rates were still well below those seen in animals inoculated with the wild-type. One mutant defective in *gftB* and *gftC*, but otherwise normal, was intermediate in caries activity. The reduction in caries activity is doubtless related in part to interference with adherence and therefore, reduction in mutants' ability to colonize the rats, although, all the mutants examined in this study successfully infected the

rats. This confirms earlier observations, made both in animal and human studies, that glucans and glucosyltransferases are not an absolute requirement for S. mutans colonization or pathogenicity but certainly enhance both (Gibbons, 1983).

Didbin and Shellis (1988; Shellis and Didbin, 1988) suggest other roles for extracellular polysaccharides. Extracellular polysaccharides in plaque can displace bacteria, thereby reducing the microbial numbers. This is particularly evident in the deeper layers of plaque. Organisms in the presence of polysaccharide therefore, have reduced competition for available carbohydrate substrate and the acid damage to enamel may be greater. This increase in acid will occur, in part, because with the lower bacterial concentration, lactate will not accumulate to levels that inhibit further acid production by the plaque microflora. Finally, extracellular polysaccharides are less dense than bacteria, providing a larger extracellular volume and a medium which facilitates the diffusion of sugar substrates.

Many of the conclusions of Didbin and Shellis (1988; Shellis and Didbin, 1988) which are derived from computer modeling, have been confirmed in in vitro studies looking at S. mutans grown with and without sucrose (van Houte et al., 1989). S. mutans grown without sucrose on a solid phase produced negligible extracellular polysaccharides and had a higher cell density than cells grown with sucrose that were widely spaced by extracellular polysaccharides. Again,

bacterial cells with extracellular polysaccharides responded to a glucose challenge with a greater pH fall and more sustained acidity compared to cells without polysaccharide. There was no difference in ability of cells to produce acid from sugar, but the ability to deal with acid was very different because the extracellular polysaccharide assumed a greater volume of the biomass, and unlike bacteria, did not contribute to buffering. The increased extracellular polysaccharide content of the biomass was also associated with greater porosity.

Acidurance

For successful colonization of the oral cavity, S. mutans must be able totolerate the low pH environment often found in some habitats on the tooth surface. Many cytosolic bacterial enzymes have a narrow pH range for activity and cease functioning at low pH even when isolated from bacteria grown at low pH (Padan et al., 1981). As reviewed by Padan (1984), aciduricity does not lie in cytosolic functions occurring at low pH, but in the ability of cells to maintain the intracellular pH close to neutrality despite an acidic environment. The membrane-bound H^+/ATPase plays a central role in maintaining pH homeostasis in bacterial cells.

Abrams and Jensen (1984) and Kinoshita et al. (1984) have reported increased levels of H^+/ATPase in cells of S. faecalis grown at low pH, compared to cells grown at or above neutral pH. Kinoshita et al. reported that when cells of S.

faecalis were grown in the presence of the ionophore gramicidin, the pH range of growth was restricted to values between pH 6.5 and pH 8.8 compared to values between pH 4.5 and pH 8.8 for control cells, reflecting the loss of cytoplasmic pH regulation in gramicidin-treated cells.

Bender et al. (1986) examined membrane damage, assessed by magnesium release from cells, following the exposure to acid of four oral bacteria; S. salivarius , S. sanguis ,S. mutans and Lactobacillus casei. The streptococci exhibited membrane damage when exposed to pH 4.0, while L. casei was more aciduric, withstanding acid exposure to pH 3.0. The membrane proton permeability of the different bacteria was assessed by acid-pulsing the cells and measuring the time required for the pH to stabilize, reflecting equilibration of protons across the membrane. The minimal proton permeability of S. mutans occurred at pH 5.0, while the minimum for less aciduric streptococci was at higher pH values. Proton permeability was increased by treatment of the cells with the H^+/ATPase-inhibitor N,N'-dicyclohexylcarbodiimide (DCCD) indicating that the perceived proton barrier represented a balance between proton influx down a gradient and energy-requiring efflux of protons mediated by the H^+/ATPase. The pH optima of the H^+/ATPases assayed in the bacteria corresponded to their aciduricity, with values of 7.5, 7.0, 6.0, and 5.0 reported for S. sanguis, S. salivarius, S. mutans and L. casei, respectively.

Recent studies with enteric bacteria, such as Salmonella typhimurium and Escherichia coli, have indicated that external pH can modulate cell function at the genetic level. For example, while cells of Salm. typhimurium grown at neutral pH were able to survive several hours at an external pH of 4.0, they were rapidly killed by lowering the pH to 3.3. If the culture was shifted to pH 5.8 and the cells allowed to adapt for one generation, survival rates following severe acid shock increased 100 to 1000-fold (Foster and Hall, 1990). This phenomenon, termed the 'acid tolerance response', allows cells to maintain higher internal pH than that found in unadapted cells exposed to low pH. Foster and Hall (1990) showed that bacterial cells have inducible proteins (acid-shock proteins) which were expressed when cells were exposed to pH below 4.0. If cells were exposed to medium above pH 4.0, a similar set of proteins that were constituitive conferred some protection against acid. During the adaptation phase when the cells were incubated at pH 5.8, the synthesis of 18 polypeptides was shown to change, 12 increasing while 6 decreased when compared to levels found in unadapted cells. Some 49 proteins, all distinct from the 12 already mentioned, were synthesized in pH 3.3 acid-shocked versus unchallenged cells (Foster and Hall, 1991). It is interesting that the latter cells required an 'adaptive phase' for induction and de novo protein synthesis for survival at pH 3.3, and did not require these proteins for cell survival at pH 4.3 (Foster, 1991). Foster suggested

that this system of acid tolerance represented the response of an organism to an environment that acidifies slowly.

An 'acid tolerance response' has been reported in S. mutans (Belli and Marquis, 1991). The ability of cells grown in continuous culture at pH 7.0, 6.0 and 5.0 to adapt to an acidic environment was assessed by comparing the minimum pH produced by harvested cells exposed to excess glucose with the H^+/ATPase activity of the same cells and their ability to withstand potentially lethal acidification. As expected, the cells grown at pH 5.0 produced the lowest pH values in response to glucose challenge, had the highest H^+/ATPase activity and were better able to survive exposure to pH 3.5. Cells grown at pH 6.0 were intermediate and cells grown at pH 7.0 were least able to adapt to the acid environment. However, the differences in minimum pH produced in response to glucose challenge were very small with reported values of pH 3.14 versus 3.26 for S. mutans IB1600 and pH 3.74 versus 3.38 for S. mutans GS-5 grown at pH 7.0 and pH 5.0, respectively. Adaptation occurred very rapidly, usually within one generation, while de-adaptation required several generations.

From the above studies, the precise mechanism whereby external pH can regulate gene transcription is not revealed. However, lysine decarboxylase and other enzymes including arginine decarboxylase have increased activity in cells grown at low pH. Watson and co-workers (1992) examined the *cad A* gene from E. coli, which codes for lysine decarboxylase, an

inducible enzyme that produces the amine cadavarine from lysine which is then excreted and acts to reduce the acidity of bacterial products in an acidic environment (Sabo et al., 1974). The gene for the enzyme and its flanking regions were cloned, and the promoter responsible for the pH-regulated expression of *cadA* was shown to be located upstream from the gene. A second gene, *cadB* was identified between the promoter and the *cadA* gene. Another gene, *cadC*, whose expression was required for pH-induced expression of the lysine decarboxylase, was located upstream from the promoter. These studies indicate that complex interactions govern cellular pH regulation and hint at the possible mechanisms that may be occurring in other bacteria, such as the oral streptococci, that are subjected periodically to an acidic environment.

Protonmotive Force

The cytoplasmic membrane of cells acts as a barrier, or rather a gate, discriminating between diverse molecules in the environment, allowing passage of some into the cell while excluding others. The cells of most living organisms maintain concentrations of ions that differ greatly from those of their surroundings. For example, in streptococci, internal concentrations of potassium are 500 mM, or higher, even when the potassium concentration outside the cell is as low as 50 μM, creating a concentration gradient of 10^4 (Harold and Kakinuma, 1985). On the other hand, while

bacterial growth media commonly contain 100-200 mM sodium ions, the internal concentration rarely exceeds 1 mM. In part, these gradients result from the need to maintain a suitable ionic milieu within the cell for metabolism in what may be a hostile environment. Energy input is required to maintain these and other gradients, but cells have evolved several mechanisms to conserve this energy by coupling gradients to various cell functions including flagellar movement in motile bacteria (Armitage, 1992), and the transport of various solutes including sugars and amino acids (Konings et al., 1987).

Unlike catabolism, energy generation involving gradients across the membrane presupposes that in chemical reactions, substrates and products enter and leave reactions along defined pathways in specific orientations (Mitchell, 1972). The first evidence for this came from observations with aerobic organisms by Mitchell (1962) indicating that enzymes involved in the transfer of electrons to molecular oxygen during respiration, were strictly oriented in membranes. Over the years, he developed the chemiosmotic theory which stated that the transfer of electrons would be accompanied by the net expulsion of protons out of the cell leading to the formation of an electrochemical proton gradient or protonmotive force (PMF) across the membrane. Furthermore, the potential energy in this gradient was coupled to ATP generation through the H^+/ATPase (Mitchell, 1972). This defines one feature of energy-transducing membranes: that is

protein assemblies (pumps) that allow for the extrusion of protons from cells (Nicholls, 1982). The second feature required is a link to the H^+/ATPase/H^+/ATP synthetase that uses the generated proton-gradient to form ATP (Harold, 1977; Nicholls, 1982). The enzyme can be involved in both ATP hydrolysis and ATP formation, however, for simplicity, the term H^+/ATPase will be used throughout the thesis for the enzymes catalyzing either reaction.

Combinations of proton pumps can be viewed as a proton circuit which can be compared to an electrical circuit transferring electrons. For an electrical circuit to perform work, electron flow must have a limited number of pathways across the electrical potential, otherwise electron flow would be too low to have sufficient energy to perform work. For the proton circuit to work, the cell membrane must be impermeable to protons so that the proton flow is controlled and restricted through specific membrane ports or pumps. Membranes must possess four properties in order for the chemiosmotic theory to apply to proton flux coupled to ATP generation:

1. Respiratory chains or alternative mechanisms must translocate protons out of the cell.
2. Must contain a reversible proton-translocating ATPase.
3. Must be impermeable to protons.

4. Should possess specific exchange carriers to allow osmotic stability and entry of metabolites in the presence of a high membrane potential.

Proton electrochemical potential ($\Delta\mu_{H+}$)

The proton electrochemical potential is a special case of an ion electrochemical potential difference. The free energy (ΔG) of an ion electrochemical potential difference is the sum of the two forces acting in the ion gradient. One force is related to the concentration of the ion and represents the free energy change occurring when 1 mole of solute is transferred from a concentration $[X]^1$ to a concentration $[X]^2$ in the absence of an electrical potential. It is represented by equation 18:

$$\Delta G = 2.303\ RT \log \frac{[X]^2}{[X]^1} \qquad (18)$$

The second force is applied when one mole of an ion, X^{m+}, is transferred down an electrical potential difference in the absence of a concentration gradient and is expressed as:

$$\Delta G = -mF\ \Delta\psi \qquad (19)$$

where $\Delta\psi$ is the electrical potential difference and can be viewed as the difference in total charges on all the ions between the starting and end point of the ion transfer. F is Faraday's constant and has a value of 23,062 cal/volt·mol. In most cases, the two forces will be operating simultaneously, so the net ΔG will be the sum of the two

constituent equations when an ion X^{m+} is moving down an electrical potential from concentration $[X]^1$ to a concentration $[X]^2$ or:

$$\Delta G = - m F \Delta\psi + 2.303 RT \log \frac{[X^{m+}]^2}{[X^{m+}]^1} \quad (20)$$

As already stated, the situation is analogous to electricity so ΔG is commonly expressed as the ion electrochemical gradient $\mu_X{}^{m+}$, and expressed in units of electrical potential, volts:

$$\Delta\mu_X{}^{m+} = m \Delta\psi - 2.303 \frac{RT}{F} \log \frac{[X^{m+}]^2}{[X^{m+}]^1} \quad (21)$$

To describe the proton gradient:

$$\Delta\mu_{H^+} = \Delta\psi - 2.303 \frac{RT}{F} \log \frac{[H^+]^2}{[H^+]^1} \quad (22)$$

The log $[H^+]^2/[H^+]^1$ term is equivalent to the pH difference, ΔpH, between the two sides of the membrane so the equation becomes:

$$\Delta\mu_{H^+} = \Delta\psi - 2.303 \frac{RT}{F} \Delta pH \quad (23)$$

and at 37°C:

$$\Delta\mu_{H^+} = \Delta\psi - 59 \Delta pH \quad (24)$$

Equilibrium occurs when $\Delta G = 0 = \Delta\mu_{H^+}$. If this is applied to the general ion equation, we obtain:

$$\Delta\psi = 2.303 \frac{RT}{F} \log \frac{[X^{m+}]^2}{[X^{m+}]^1} \quad (25)$$

These last two points provide the theoretical framework for the measurement of $\Delta\psi$ and ΔpH in order to determine $\Delta\mu_{H^+}$ experimentally. From this, it is apparent that an ion potential difference may exist in the absence of a concentration gradient, or conversely, may be achieved without equalizing the ion concentration across the membrane and it shows that the disruption of one does not necessitate complete disruption of the other.

Experimentally, $\Delta\psi$ and ΔpH can be measured in bacteria with indicator molecules that possess certain qualities: (a) they must be transported by a single, simple mechanism and once inside the cell, must be metabolically inert, (b) the intracellular and extracellular concentrations must be readily and accurately measured, and (c) the indicator must be able to reach equilibrium without disturbing other gradients in the process.

With most bacteria, such as streptococci, the membrane potential ($\Delta\psi$) is measured with a cation such as methyltriphenylphosphonium iodide moving down an electrochemical gradient into the negatively-charged cell interior (Keevil and Hamilton, 1984). To measure the pH gradient (ΔpH), an electroneutral molecule is used, because it will be unaffected by $\Delta\psi$ and reaches equilibrium when the concentration on either side of the membrane is the same. Weak acids and bases can permeate the membrane in uncharged form and their dissociation inside the cell is dependent on the internal pH and the dissociation constant (K_a) for the

molecule. The interior of most bacterial cells is usually alkaline so that weak acids such as salicylate are used to measure ΔpH. Detailed descriptions of these techniques have been reviewed by Rottenberg (1979) and Kashket (1985).

The membrane resistance to ion transfer can be lowered by ionophores which act by shielding the charge of the ion from the membrane bilayer. Ionophores fall into two types, each with distinct modes of action and corresponding properties. Mobile carriers are ionophores that pick up an ion, form a cage around it and deposit it on the other side of the membrane then diffuse back through the membrane to pick up another ion. Typically, they demonstrate selectivity between different ions, can transport as many as 1000 ions per second and, because they diffuse through membranes, are sensitive to membrane fluidity (Nicholls, 1982). The second class, called channel-formers, extend through the membrane forming charge-masking channels and are usually poorly selective between different ions and, since diffusion back and forth is not required, they transport up to 10^7 ions per second.

Ionophores can be further divided according to their ability to transfer protons and other charged ions, or both simultaneously. Valinomycin and gramicidin transfer ions except protons. For example, valinomycin is a mobile carrier that mediates the electrical transport of Cs^+, Rb^+, K^+ or NH_4^+; protons and Na^+ ions are too small to interact with the molecule. Gramicidin D usually refers to a group of related hydrophobic linear polypeptides that form channels in

membranes that allow free passage of any monovalent cations (Wallace, 1990). Gramicidin S refers to a cyclic decapeptide that forms an ion carrier. Nigericin is a mobile carrier that transfers protons with no net transfer of charge because protons are exchanged for cations such as K^+. The addition of nigericin to cells often results in the dissipation of a ΔpH provided H^+ and K^+ gradients exist. The ionophore A23187 has a similar function except that it is specific for divalent cations, exchanging 2 protons for a calcium or magnesium ion and does not alter monovalent ion gradients. Compounds such as carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) are called uncouplers because they catalyze the net transport of protons effectively removing proton resistance of the membrane resulting in a short circuit of protonmotive force.

Generation of protonmotive force in streptococci

Streptococci lack a respiratory chain but are able to generate proton gradients, either through the expulsion of acidic end products in a process called end-product efflux, or via the membrane-bound H^+/ATPase. The result is the net efflux of protons from the cell creating a proton gradient across the membrane with the concentration of protons outside the cell being greater than that inside the cell (Padan, 1984, Michels et al., 1979).

As mentioned previously, the H^+/ATPase is viewed as a reversible proton pump that catalyses the synthesis or

hydrolysis of ATP depending on the direction of proton movement. H^+/ATPases have been identified in all Streptococcus species examined to date (Maloney and Wilson, 1975; Leingruber et al., 1981; Khan and Berg, 1983, Otto et al., 1984; Sutton and Marquis, 1987) and are believed to be present in all bacteria (Abrams and Smith, 1974; Downie et al., 1979; Futai and Kanazawa, 1983). Bacterial H^+/ATPases share a common structure consisting of the integral membrane component, F_0, and the catalytic F_1 portion. The latter is readily solubilized from the membrane by mild washing procedures (Abrams and Smith, 1974). H^+/ATPases are multioligomeric enzymes with the F_0 portion comprised of three different units (a, b and c) and the F_1 consisting of five different subunits ($\alpha, \beta, \gamma, \delta$ and ε). The stoichiometry of the different units is not known for most ATPases and may differ among different species, as will the size and composition of the subunits (Munoz, 1982; Futai and Kanazawa, 1983). The ε and δ units, previously called nectin (Abrams and Smith, 1974), mediate binding of F_1 to the F_0 portion. The α unit has also been implicated in the attachment of the F_1 to the F_0 in Streptococcus faecalis (Munoz, 1982), while the ATPase catalytic function is associated with α and β subunits of the F_1 component (Munoz, 1982, Futai and Kanazawa, 1983). The F_0 portion acts as a proton port with proton expulsion during ATP hydrolysis and ATP generation coupled to the uptake of protons into the cell (Abrams and Smith, 1974; Munoz, 1982).

The study of H^+/ATPases was greatly facilitated by the finding that the membrane-bound enzyme was inhibited by DCCD (Abrams and Smith, 1974). DCCD has no effect on the catalytic activity of solubilized F_1 although activity is lost when the F_1 is reattached to DCCD-treated membranes. This suggested that DCCD acts by binding to the F_0 portion of the H^+/ATPase which has been substantiated by the finding that DCCD-resistant mutants of S. faecalis were unable to bind DCCD due to alterations in the F_0 (Abrams and Smith, 1974). Other mutants of S. faecalis, defective in energy coupling due to increased proton permeability, could be restored to many functions by treatment with DCCD (Leingruber et al., 1981). Their mutation had occurred in a gene coding for one of the F_0 subunits and produced a leaky proton port lacking coupling to the ATPase activity effectively short-circuiting the membrane. DCCD corrected the defect by blocking the faulty open F_0 port. Thus, with wild-type cells, DCCD acts by blocking proton transport and uncouples the proton pump from the ATPase activity.

One of the main tenets of the chemiosmotic theory is the coupling of ATP synthesis via the H^+/ATPase to the protonmotive force. Maloney and Wilson explored the relationship between protonmotive force (that is $\Delta\psi$ and ΔpH) and ATP synthesis in S. lactis (Maloney and Wilson, 1975; Maloney, 1977). A positive electrochemical gradient, interior negative, was generated in bacteria by treating the cells with the ionophore valinomycin resulting in the efflux

of K^+ ions, while pH gradients were generated by placing the cells in buffers at pH 5.0 or 6.0 Under either of these conditions, a transient rise in the intracellular ATP level was observed. The accumulation of ATP could be halted by the the addition of DCCD indicating that the elevated levels of ATP resulted from ATP synthesis and not an impairment of hydrolysis under these conditions. The increase in ATP was sensitive to proton permeability of the membrane and was removed by cell treatment with FCCP. The addition of inhibitors of substrate level phosphorylation did not impair the observed increase in ATP, indicating that the source of the ATP was not cellular catabolism. It was found that a threshold protonmotive force of 215 mV was required before the increase in ATP was observed. With greater values, ATP synthesis and increased proton influx was observed in cells with a fully functional H^+/ATPase, independent of the primary component of the protonmotive force, $\Delta\psi$ or ΔpH (Maloney, 1977). Maloney concluded that the H^+/ATPase was responsible for the ATP synthesis and its activity was directly coupled to proton influx (Maloney, 1982). The low level of proton influx observed when protonmotive force was less than 175 mV was related to slight leakiness of the membrane and reflected some ion movement in response to the imposed protonmotive force (Maloney, 1982).

When describing ATP synthesis in terms of proton influx alone, it may not be obvious how this relates to the electrochemical gradient. It must be recalled that the

driving force on a proton is the sum of its concentration gradient and the electrochemical gradient ($\Delta\psi$) composed of net charge differences across the membrane. When the actual external concentration of protons is low, a high membrane potential will still move protons into a negatively-charged cell interior. Thus, the protons described in membrane reactions are subject to additional forces compared to their counterparts in solution. The stoichiometry of proton/ATP synthesis was calculated, by relating the free energy for ATP synthesis with the free energy required to translocate a proton, to be $2H^+$/ATP synthesized (Maloney, 1982). Later, calculations were refined to take into account any proton efflux accompanying the proton influx and a value of $3H^+$/ATP synthesized was reported (Maloney, 1983). These values are within the range reported by Khan and Berg (1983) for Streptococcus V4051. Otto et al. (1984) found that the H^+/ATP ratio varied from 2 in resting cells to values of 4 or more in actively growing cells of S. cremoris. They explained their finding by saying that the change in free energy for ATP formation increased in actively growing cells as the reaction was displaced from thermodynamic equilibrium. In his experiments, Maloney (1983) attempted to keep conditions close to thermodynamic equilibrium. The precise mechanism by which the H^+/ATPase is able to transform the energy in the electrochemical gradient to chemical energy in ATP is not understood. Models have been proposed that suggest conformational changes (Nicholls, 1982).

While Maloney demonstrated the ATP-generating properties of the H^+/ATPase by the uptake of protons, the enzyme also functions in reverse by expelling protons in response to acid challenges at the expense of ATP. Such ATP hydrolysis is energetically costly and to obviate this, streptococci conserve energy in a process called end-product efflux that moves protons out of the cell in association with the expulsion of acid end-products (Michels et al., 1979). The stoichiometry of end-product efflux can be seen in the following example which assumes glucose metabolism results only in lactate formation (equation 25):

$$\text{glucose} + 2\ ADP_{in} + 2\ P_{in} \longrightarrow 2\ \text{lactate}_{in} + 2\ H^+_{in} + 2\ ATP_{in} + 2H_2O \qquad (25)$$

H^+/lactate excretion can be expressed as:

$$\text{lactate}_{in} + nH^+_{in} \longrightarrow \text{lactate}_{out} + nH^+_{out} \qquad (26)$$

where n is the number of protons translocated out of the cell in symport with lactate. Combining equations 25 and 26, the process becomes:

$$\text{glucose} + 2\ ADP_{in} + 2\ P_{in} + 2(n-1)H^+_{in} \longrightarrow \text{lactate}_{out} + 2\ nH^+_{out} + 2\ ATP_{in} + 2H_2O \qquad (27)$$

The value of n determines the size of the protonmotive force that will be generated during lactate efflux (ten Brink and Konings, 1986) . If n is equal to 1, electroneutral symport will take place with no $\Delta\psi$ and a minimal ΔpH being generated.

At n values greater than 1, electrical symport occurs with both $\Delta\psi$ and ΔpH being generated, consequently, the dependence of the cell on ATP hydrolysis for proton efflux and PMF generation is reduced. The key to end-product efflux will be the number of protons transported out of the cell in symport with lactate. The n value or stoichiometry between protons and lactate decreases with an increase of the lactate gradient and/or pH gradient. It is reasonable to expect less energy to be produced as $\Delta\mu_{lactate}$ and ΔpH increase since as thermodynamic equilibrium is approached ΔG decreases. Values of less than one have been observed that would not result in net expulsion of protons generated during glycolysis. Thus, the H^+/ATPase is still required to expel protons.

ten Brink and Konings (1986) have calculated the energy conserved in ATP units when end-product efflux is occurring and have predicted savings from 20-100% depending on the fermentative pathway used by the organism. As mentioned previously, streptococci usually metabolize carbohydrate by either homolactic or mixed acid fermentation, and when lactate is the product of fermentation, a 50% energy gain can be calculated. For mixed-acid production, a 67% energy gain is predicted over that resulting from substrate level phosphorylation alone (ten Brink and Konings, 1986).

Experimental evidence for a carrier-mediated, end-product efflux has come from studies with both S. cremoris (Otto et al., 1982) and S. faecalis (ten Brink and Konings, 1986). In both cases, externally applied $\Delta\psi$ induced lactate influx into

cells. On the other hand, the transfer of lactate-loaded cells to lactate-free buffer generated a protonmotive force to drive uptake of leucine and other PMF-coupled transport systems in S. cremoris. DCCD (ten Brink and Konings, 1986) had only a slight inhibitory effect demonstrating that generation of protonmotive force was not dependent solely on the H^+/ATPase. As anticipated, increasing membrane permeability to protons abolished the leucine uptake.

Transport of carbohydrates

The first step in the cariogenic process initiated by S. mutans is the transport of carbohydrate into the cell. While sugars provide the carbon and energy source for biosynthesis and cell maintenance, the end-products of metabolism are mainly acids that act as 'toxins' in the teeth. As stated earlier, carbohydrates require priming before they are catabolized through glycolysis. In S. mutans, the first priming step can be one of two types: (a) the coupling of transport and phosphorylation in a group translocation process known as the phosphotransferase system (PTS) with the phosphate provided by PEP, and (b) a permease transport reaction with phosphorylation by an ATP-dependent kinase subsequent to transport. The second step usually occurs at the phosphofructokinase step producing FBP_2 at the expense of ATP.

Phosphoenolpyruvate phosphotransferase system

The phosphoenolpyruvate (PEP) phosphotransferase system (PTS) is used by Gram-positive and Gram-negative bacteria for the transport and phosphorylation of sugars (Meadow et al., 1990). The PTS was first described in 1964 by Kundig and co-workers in a paper in which the authors state that "the biological significance of the new phosphotransferase system is not clear and fruitful speculation must await further experimental results". The importance of the PTS has surpassed these authors' expectations and it is now recognized as an essential pathway for carbohydrate metabolism in most saccharolytic pathogens. Not only does it serve as a transport system, but the PTS also contributes to the regulation of other cell functions including non-PTS sugar uptake and chemotaxis towards sugars (Grübl et al., 1990; Meadow et al., 1990).

The PTS consists of two soluble general proteins, Enzyme I (EI) and the heat-stable protein (HPr), which are required for the transport of all PTS-sugars (Postma and Lengeler, 1985). EI mediates the transfer of a phosphate from PEP to a histidine residue in HPr, which in turn transfers the phosphate to a sugar-specific, membrane-bound Enzyme II (EII) that catalyzes phosphorylation of the incoming sugar (Figure 4). In some cases, a second sugar-specific component, EIII permanently or transiently associates with EII, to mediate the phosphoryl transfer from HPr to the specific EII (Meadow et al., 1990).

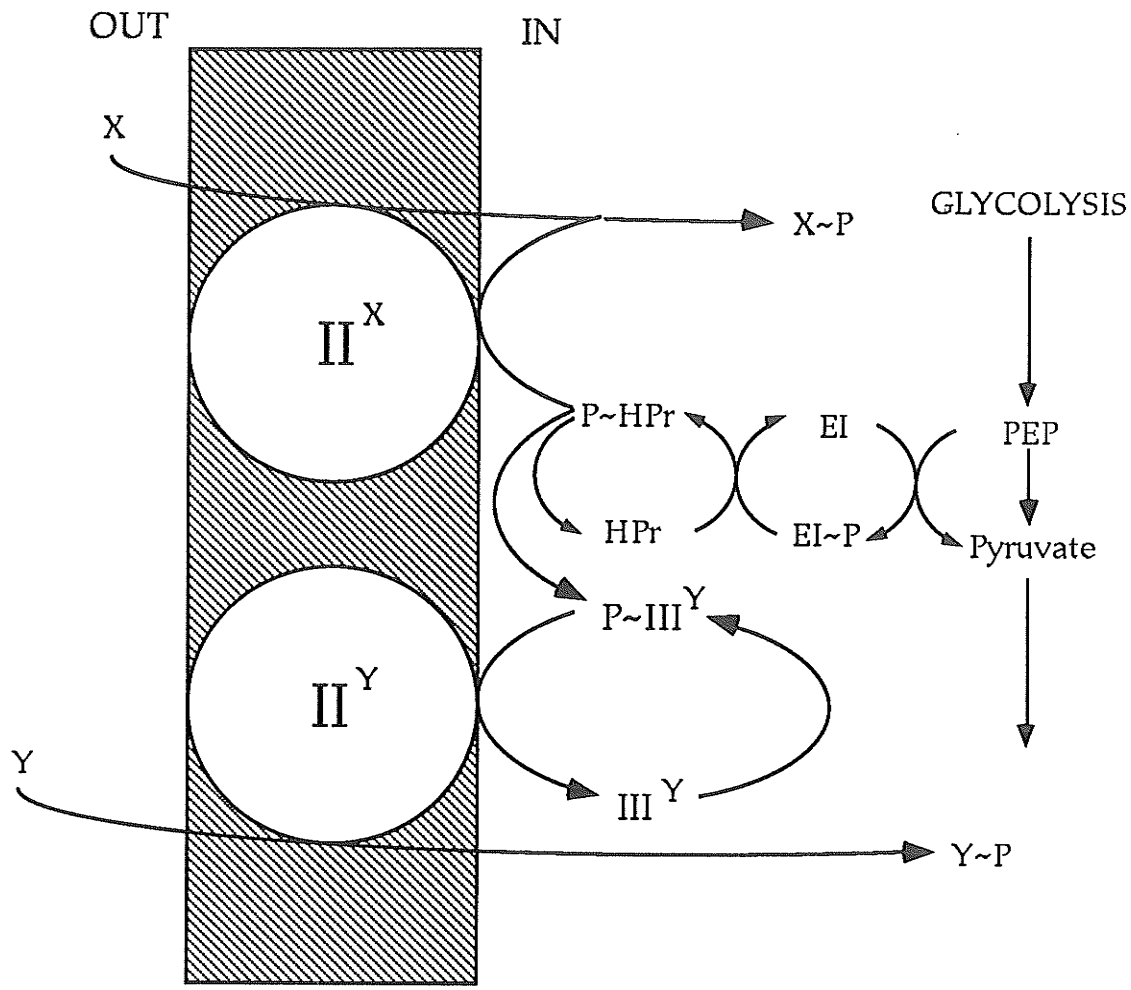

Figure 4. General model of the phosphoenolpyruvate phosphotransferase system. X and Y are sugars and sugar alcohols.

Components of the PTS

Enzyme I EI is common to all bacteria containing a functional PTS and, as far as is known, with the exception of the EI isolated from Mycoplasma capricolum, consists of identical monomers that self-assemble to form the functional dimeric EI (Postma and Lengeler, 1985). Both subunits may be autophosphorylated by PEP with a requirement for Mg^{+2}, Mn^{+2} or some divalent cation (Meadow et al., 1990). Because of the ready reversibility of the reaction, it has been suggested that regulation at the level of EI may be affected by the pyruvate/PEP ratio, however, a potentially more powerful regulation of EI may involve the dissociation/association of its constituent monomers.

Vadeboncoeur and co-workers (1983) have shown that antibodies raised to the EI of S. salivarius cross-react with the EI of S. mutans, but not with the enzyme from E. coli, Staph. aureus, or S. faecalis. It was somewhat surprising that the antibodies did not recognize the S. faecalis EI as the proteins from S. faecalis and S. salivarius have very similar amino acid content with a high degree of homology surrounding the active site (Reizer et al., 1989a).

Heat Stable Protein HPr has been isolated from a variety of bacteria, including E. coli, Salm. typhimurium and S. salivarius and shown to be a very small protein of 10,000 daltons or less (Meadow et al., 1990). Unlike the HPr's isolated from Gram-negative bacteria, those from Gram-

positive bacteria contain cysteine and tyrosine residues. Regardless of their source, all HPrs have similar active sites and are phosphorylated by EI at the histidine-15 (Meadow et al., 1990).

Enzymes II and EIII There appears to be little similarity in amino acid structure between the EIII: isolated from various bacteria. This is not surprising as they all differ in their contribution to sugar phosphorylation and translocation. In the transfer of phosphate from the HPr~P to the incoming sugar, there are two intermediate phosphorylation steps. Both occur on a translocating unit which may consist of an EII alone, an EII-EIII membrane-bound complex, or membrane-bound EII that is transiently associated with a soluble EIII. To avoid the confusion arising from the wide range of names given to the sugar-specific PTS components, Saier and Reizer (1992) proposed a new nomenclature for the various enzymes that was related to their constituent protein domains and their function. The vast majority of permease protein complexes, regardless of their precise structures, consist of three functional domains: (a) a hydrophilic domain, resembling many of the EIII proteins, that contains the first phosphorylation site, usually on a histidine residue, (b) a second hydrophilic site that contains the second phosphorylation site, which for the majority of PTS permeases is on a cysteine residue, and, (c) a hydrophobic transmembrane domain which binds and transports the sugar,

These they termed the A,B and C domains, respectively (Saier and Reizer, 1992). EIII protein can contain one (A) or both (AB) of the hydrophilic domains and, in some cases, there is no EIII and all the domains (ABC) reside on the EII molecule. Despite the requisite domains in the PTS permeases, they share little homology in protein sequences.

Weegel et al. (1991a) recently cloned a segment containing the histidine phosphorylation site of the mannitol-translocating EII or EII^{mtl} of E. coli. The segment was able to substitute for the $EIII^{mtl}$ of Staph. carnosus and partially restore PTS activity in extracts lacking the native $EIII^{mtl}$. In reciprocal experiments, $EIII^{mtl}$ of Staph. carnosus provided the same function in phosphorylation assays containing substitutions in the histidine site of the E. coli EII.

While there are differences in primary sequences between the EII and EIII isolated from various bacteria for the transport of different sugars, the suggestion has been made that the PTS permeases (EII with any necessary EIII) share many features and they arose from a common ancestor (Saier et al., 1988; Lengeler, 1990). Common features of EII and EII-EIII complexes include: (a) similar size of around 650 amino acids, (b) secondary structures that include a hydrophobic portion of about 370 amino acids and a hydrophilic region of 250-305 amino acids, (c) a propensity to have hydrophilic and hydrophobic amino acids arranged in clusters, and (d) 250 strictly hydrophobic amino acids in the first half of the molecule, arranged in up to 12 potential transmembrane

helices that appear to be separated by extramembrane loops. These loops are made up of hydrophilic and neutral amino acids with an amphipathic helix that serves as a membrane anchor (Lengeler, 1990).

Lolkema and Robillard (1990) recently demonstrated that the predominant active form of EII in membranes is dimeric although the EII monomer has limited transport and phosphorylation properties. This was confirmed by studies involving constructs of EII^{mtl} of E. coli that had substitutions at either the histidine or cysteine phosphorylation site. Substitutions at either site completely disrupted phosphotransferase activity although the EII carrying an alternate amino acid residue at the cysteine site retained some phosphorylation activity (Weeghel et al., 1991b). When the substituted EII were combined, heterodimers formed that allowed phosphoryl transfer between the intact histidine phosphorylation site on one EII and the intact EII cysteine site on the other. Based on these and associated studies, a model was presented that suggested that the phosphate donated by PEP was transferred from the HPr~P to the histidine residue on EII or EIII protein, then to the cysteine residue, and finally transferred during translocation onto the incoming sugar (Lolkema et al., 1992).

Many of the individual EII enzymes transport not one but a related group of carbohydrates, with the different sugars displaying a hiearchy in EII affinity (Reizer et al., 1989a). This results in the preferential uptake of a specific sugar

based on its concentration and affinity of EII for that sugar.

Shortly after the initial discovery of the PTS, two models were advanced to explain its mechanism, (1) vectorial phosphorylation and (2) the 'phosphorylating' model where the PTS acts as a 'trap' after the sugar enters the cell through its corresponding permease (Roseman, 1969). In vectorial phosphorylation, phosphate transfer is mandatory to the sugar translocation process and the EII functions only when all the PTS components are intact. In the second model, the transport and phosphorylation are discrete steps in which the EII 'permeases' should mediate the facilitated diffusion of the appropriate sugar even in the absence of the HPr and EI. Studies on a PTS-defective mutant were used to identify the appropriate model. An EI mutant of Salm. typhimurium was isolated that contained no measurable EI but normal levels of HPr and somewhat elevated EII (Simoni et al., 1967). It was determined that the defect in the mutant arose from a single point mutation and not a disruption of several genes. The mutant was unable to grow on glucose and several other carbohydrates, including both known PTS-sugars and non-PTS sugars. The mutant could grow as well as the wild type when provided with glucose-P, which enters the cell through a specific permease, indicating that the defect involved the conversion of glucose to glucose-P and not the subsequent metabolism of glucose phosphate. If the vectorial phosphorylation model applied, then both transport and

phosphorylation would be blocked. If the phosphorylating model were valid for the PTS, then transport of glucose would occur at rates comparable to the wild type, and while PTS-mediated phosphorylation would be blocked, glucose entering the cell could be phosphorylated by glucokinase, found at normal levels in the mutant (Fraenkel et al., 1964). The EI mutant of Salm. typhimurium was unable to transport several PTS sugars and the glucose analogue, α-methyl D-glucopyranoside (mGlc) at a significant rate, confirming the vectorial phosphorylation model (Roseman, 1969). Further confirmation of this model came from studies conducted by Kaback (1968) using membrane vesicles of E. coli. Vesicles are advantageous for use in transport studies because cytoplasmic components for substrate metabolism are removed, allowing exclusive study of transport phenomena. The vesicles contained an intact PTS and with the addition of PEP accumulated sugars as their phosphate esters. Vesicles were loaded with C^{14}-glucose and then exposed to external H^3-glucose and PEP. If phosphorylation was not tightly coupled to transport then C^{14}-glucose-P would be rapidly formed and H^3-glucose would appear in an unaltered form in the vesicles. If PTS-phosphorylation was dependent on translocation, then the C^{14}-glucose would be unaltered but H^3-glucose-P would increase in the vesicles. The latter was found to occur demonstrating that the EII did not transport substrate in the absence of phosphorylation.

Gachelin (1970) disputed these findings and reported facilitated diffusion of α-methyl D-glucopyranoside (mGlc) by the EII^{glc} of E. coli in the absence of phosphorylation. However, in his studies, galactose was repeatedly shown to compete strongly with the mGlc transport and as galactose is not a substrate of the EII^{glc}, while glucose is a substrate of the galactose permease, it is likely that the observed diffusion was through the galactose permease and not via the EII^{glc}.

The vectorial phosphorylation model for the PTS, and an important corollary of the model, that is, that EII does not transport its substrates in the absence of phosphorylation, was supported by a variety of experimental evidence (Roseman, 1969; Postma and Roseman, 1976; Postma and Stock, 1980). However, a Salm. typhimurium mutant that transported glucose in the absence of phosphorylation and apparently contained an uncoupled EII^{glc} was isolated from a bacterial stock containing a *pst HI* deletion affecting both HPr and EI (Postma, 1981). The mutant was isolated by screening revertants that had regained the ability to grow on glucose. There was, howeverm a 1000-fold decrease in glucose affinity when compared to Salm. typhimurium with an intact PTS. The mutation that allowed growth on glucose occurred in the EII^{glc}, since further specific mutations in the EII^{glc} abolished growth on glucose and also restoration of EI and HPr genes allowed the cell to grow on other PTS sugars with the exception of glucose indicating the EII had not reverted

to the wild-type EII. The altered EII^{glc} lost all ability to phosphorylate sugars even when provided with HPr, EI and PEP. Ruijter *et al.* (1991) confirmed that glucose transport by the EII^{glc} of the mutant required no energy and confirmed that it occurred via facilitated diffusion, albeit with low affinity for glucose. The mutant originally isolated by Postma (1981) was grown in continuous culture in decreasing concentrations of glucose to select for mutants with a higher affinity for glucose (Ruijter *et al.*, 1990). Initially, this scheme was successful for selecting strains possessing EII^{glc} with increasing affinity for glucose, but continuing selection pressure resulted in the isolation of high affinity strains due to mutations occurring in the gene for the galactose permease that were unrelated to EII.

Ruijter *et al.* (1992) created a strain of *E. coli* with defective EII^{glc} due to substitutions near the cysteine residue that, as previously mentioned, is the second site of phosphate transfer during the PTS-mediated translocation of sugars. The defective EII^{glc} transported glucose via facilitated diffusion. However, unlike the mutants already described, they were also able to carry out PTS phosphorylation of sugars without transport. While these studies do demonstrate that it is possible to separate the translocation and phosphorylation functions of the PTS, the mutants possessing uncoupled systems would be at a disadvantage when competing with their wild type counterparts containing intact PTS due to their reduced substrate

affinity. Reports of facilitated diffusion by EII are rare and when detected, the transport is not physiologically relevant (Elferink et al., 1990). However, these studies provide information on the mechanisms underlying the exquisite coupling of transport and phosphorylation seen with the PTS.

Regulation of the PEP-phosphotransferase system

The PTS is the focus of intense study in part because it is the primary system for the transport of carbohydrates in many bacteria and essential for their growth (Meadow et al., 1990). Associated studies have examined the regulation of the system and how it acts to direct other cell functions including the transport of non-PTS substrates. A connection between the PTS and metabolism of non-PTS sugars is demonstrated by the inability of PTS-defective mutants to grow on non-PTS substrates (Roseman, 1969; Saier, 1989). An early observation now explained in part by PTS regulation is the diauxie effect first described by Monod (1942). The diauxie effect occurs when bacteria challenged with two different sugars, degrade one preferentially, before catabolizing the second, usually after a brief stationary phase. Monod (1942) divided sugar substrates into two classes: the A substrates that impede the transport of the B substrates. In enteric bacteria, A substrates include glucose, mannitol and N-acetylglucosamine, while B substrates include glucitol, fructose, maltose and lactose. The

division of substrates into class A and class B is not rigid. In some cases, a class B can interfere with transport of an alternate class B substrate. The diauxie effect was shown to occur because the A substrates could decrease the activity of the class B transport systems through two mechanisms called catabolite repression and inducer exclusion, whereby the transport of substrates that serve as inducers to associated operons is inhibited (Postma and Lengeler, 1985).

One well-characterized example of catabolite repression concerns the diauxie effect where glucose is metabolized before lactose. In E. coli, the genes for β-galactoside transport and metabolism are located on the lac operon (Beckwith, 1970). The operon consists of a promoter and operator region preceding three structural genes *lac Z*, *lac Y* and *lac A* coding for β-galactosidase, β-galactoside transport protein and β-galactoside transacetylase respectively. β-galactosidase cleaves lactose into glucose and galactose, while the transacetylase acetylates non-metabolizable β-galactoside analogues promoting their extrusion from the cell. The promoter sequence binds RNA polymerase and initiates transcription. The operator, associated with the promoter, can bind regulatory molecules that repress transcription by blocking RNA polymerase binding to the promoter. Another stuctural gene, the *R* gene, is located just upstream from the lac operon promoter and this codes for the lactose repressor, a protein that can bind the

lac operator to block transcription (Gilbert and Müller-Hill, 1970).

When exposed to a β-galactoside, some of the substrate enters the cell and interacts with the lactose repressor to impede its binding to the operator, allowing RNA polymerase binding of the promoter and transcription of the *lac* operon. The original extracellular concentration of the inducer must be high to gain access to the cell, but once the operon is fully induced, the cell is able to concentrate the inducer even as the external concentration declines.

Experimental findings suggested that another level of regulation also occurs. For example, with glucose present, full induction of the operon was not seen, even when inducers were present (Magasanik, 1970). Moreover, mutants with defective repressors unable to bind the lac promoter, still had depressed levels of β-galactosidase when grown with glucose. A similar effect was produced in other ways, such as limiting nitrogen or phosphate in culture, but all the conditions resulted in cells whose catabolic functions exceeded anabolic functions, hence, this type of repression was called 'catabolite repression' (Magasanik, 1970).

The mediator of this effect was identified as adenosine-3',5'-phosphate or cyclic adenosine monophosphate (cAMP) (Pastan and Perlman, 1970). In 1965, Makman and Sutherland showed that the addition of glucose to E. coli cultures caused the rapid depletion of cAMP in cells. Ullman and Monod (1968) demonstrated that the addition of cAMP to

cultures of E. coli growing on glucose could reverse most of the observed repression of the lac operon. cAMP binds a protein called the cAMP-binding receptor protein, or CRP, and the resulting complex in turn interacts with the *lac* promoter and RNA polymerase to promote transcription of the *lac* operon (Pastan and Adhya, 1976). The cAMP-CRP complex induces the transcription of many genes such as those of the arabinose and melibiose operons and represses others, such as a major porin and genes involved in capsular polysaccharide synthesis. The complex also regulates *cya* that codes for adenylate cyclase, the enzyme responsible for cAMP production and *crp*, the gene coding for CAP (Ullmann and Danchin, 1983; Botsford and Harman, 1992).

Saier (1989) proposes a model whereby the PTS can directly regulate catabolite repression through adenylate cyclase interaction with EIII protein. The activity of adenylate cyclase is enhanced by the binding of phosphorylated-III^{glc} (Saier, 1989). Thus, when the cell has ample energy and no PTS sugar is available for transport, EIII will be phosphorylated and interact with adenylate cyclase to synthesize cyclic AMP, which in turn, promotes transcription of the *lac* operon. Conversely, in the presence of PTS-sugars, the EIII protein will not be phosphorylated, adenylate cyclase will be inactivated causing cAMP levels to decline and non-PTS transport will be inhibited. During the lag phase following exhaustion of the preferred sugar, cAMP

levels increase and induction of the enzymes for the metabolism of the second sugar occurs.

Unlike catabolite repression which results in up to 80% repression of the lac operon and persists for the duration of glucose metabolism, a second type of repression, called 'transient repression', is observed during diauxie growth and completely represses operon expression, but only lasts for one half to one generation of bacterial growth (Magasanik, 1970). Transient repression can be relieved by the addition of cAMP at five times the concentration required to relieve catabolite repression. However, it has been suggested that the mechanism is distinct from the cAMP-CRP mediated repression and that cAMP may interact with a second as yet unidentified mediator (Ullmann and Danchin, 1983)

cAMP occurs in diverse bacteria and its known regulatory function is mediated through CRP. However, it is responsible for more than catabolite repression, such as regulation of the cell cycle in Saccharomyces cerevisiae, and anaerobic metabolism in Salm. typhimurium and likely transient repression by mechanisms not yet identified (Botsford and Harmen, 1992).

Inducer exclusion also contributes to the diauxie effect. In enteric bacteria, many non-PTS carbohydrate permeases including the maltose, melibiose and lactose permeases, and glycerol kinase normally present in an active form are rendered either inactive, or less active, by the binding of free EIII (Saier, 1989). In E. coli, it was shown that free

EIII, unlike the phosphorylated form, bound the lac permease (Nelson et al., 1983). This binding inactivates lactose transport via the permease in studies with purified lac permease reconstituted into proteoliposomes (Newman and Wilson, 1980). Thus, in the presence of PTS sugars, EIII protein will block transport of β-galactoside, resulting in an active lac repressor that can bind the operator blocking transcription of the *lac* operon.

A diauxie effect is seen in bacteria exposed to two PTS sugars using distinct EII. E. coli will transport α-methyl-glucoside before hexitols (Lengeler and Steinberger, 1978). When two or more EII permeases catalyzing the phosphotransfer of distinct sugars are present, the EIIs will compete for the HPr~P and it has been proposed that the EI-catalyzed transfer from HPr to EII is the rate-limiting step in PTS-transport (Postma and Lengeler, 1985).

Competition also results when bacteria are exposed to two sugars that are transported by a common EII. For example, the EII^{Man} of S. pyogenes will transport glucose, 2-deoxyglucose, mannose and glucosamine with rates and affinities which decrease in the order given (Reizer et al., 1989b). Thus, the uptake of glucosamine is inhibited if cells are simultaneously exposed to five-fold excess of glucose or 2-deoxyglucose .

The uptake of sugars via the PTS results in the appearance in the cell of the phosphorylated form of the sugar transported. As discussed earlier, sugar-killing could

result if this is not controlled. In addition to regulation already discussed, Gram-positive organisms have three mechanisms to prevent the accumulation of the sugar phosphate in the cell: (1) inducer exclusion, (2) exchange of intracellular sugar phosphate with external phosphate by means of antiport, and (3) exchange of the sugar of the intracellular sugar-phosphate with extracellular sugar via PTS-mediated transphosphorylation reaction (Reizer et al., 1989a).

The first mechanism was demonstrated by Reizer and Panos (1980) with S. pyogenes. Cells incubated with thiomethylgalactoside (TMG) and β-isopropylthiogalactoside (IPTG) transported these non-metabolizable lactose analogues into the cell via the lactose-PTS. The intracellular pool of phosphorylated analogues was stable and was not affected by the addition of a wide range of non-metabolizable analogues. However, the addition of glucose, mannose or glucosamine resulted in the rapid cleavage of the accumulated sugar-phosphate and the expulsion of the free analogue in a process known as 'inducer expulsion' (Reizer et al., 1989b). The two steps in the expulsion process, dephosphorylation and transport, could be separated. The presence of high concentrations of TMG or IPTG outside the cell inhibited transport, but not the internal dephosphorylation of accumulated phosphorylated analogues. On the other hand, the addition of arsenate curtailed dephosphorylation, but did not impede transport of free analogue out of the cell (Reizer et

al., 1983). EII^{Lac} was shown to mediate the expulsion of the free analogue since EII^{Lac}-mutants did not show the glucose-mediated expulsion and specific competitive inhibitors of the EII^{Lac} also curtailed expulsion. Dephosphorylation is an energy-requiring process since metabolizable sugars promote activity while energy inhibitors, particularly those affecting ATP formation, abolish dephosphorylation (Saier, 1989) and one enzyme with this activity has been described (Thompson and Chassy, 1983).

These findings indicate that the intact EII can efficiently mediate the efflux of free sugar while operating efficiently to bring in sugars during group translocation. Not only will this protect against build-up of potentially toxic sugar phosphates in the cell, but also allows the cell to discriminate between carbohydrate sources. Sugars, such as lactose, often require induction by the sugar-phosphate of other enzymes associated with their metabolism. Inducer expulsion, stimulated by glucose and other carbohydrates, will inhibit this induction and cells will preferentially use glucose.

In some strains of S. lactis, the presence of an electroneutral phosphate:sugar 6-phosphate antiport has been reported (Ambudkar and Maloney, 1984; Maloney et al., 1984). The antiport may compensate for the inability of the EII of S. lactis to expel β-galactoside, although currently, the relevance of the system is not clear. The third mechanism involves the exchange and phosphorylation of an external

sugar with the dephosphorylation of an internal sugar-phosphate via the EII has been reported (Reizer et al., 1989a). This mechanism has been demonstrated with the EII^{mtl} of E. coli (Lolkema and Robillard, 1990) and may allow for the expulsion of nonmetabolizable sugars transported by the PTS without the energy requirement of inducer expulsion. However, the importance of this mechanism and whether this is a general property of EII enzymes is not known.

One mechanism of PTS regulation that so far is restricted to Gram-positive bacteria concerns a second phosphorylation on HPr (Reizer et al., 1985). In addition to a PEP-phosphorylation site at histidine-15, HPr can be phosphorylated by ATP at serine-46 and this acts to regulate the phosphotransfer by the PTS (Reizer et al., 1989b). When HPr is phosphorylated at the serine, EI is unable to catalyze the histidine phosphorylation thereby inhibiting the transport function of the PTS. The inhibition of the PTS can be partly lifted by the interaction of the HPr(Ser-P) with EIII proteins. In S. faecalis, HPr(Ser-P) is phosphorylated at the same rate as free HPr in the presence of the EIII protein for gluconate, but at a much slower rate in its absence. In Staph. aureus, phosphorylation of HPr(Ser-P) by PEP was 100 times faster when III^{lac} was present, but this was still 50 times slower than PEP phosphorylation of the unmodified HPr (Reizer et al., 1989a). It is believed that the addition of a negative charge on the HPr at the serine

site impairs its subsequent interaction with EI and EIII reduces this interference (Reizer et al., 1989b).

A specific kinase that catalyses the phosphorylation of HPr at the serine site, HPr(Ser) kinase, has been isolated from S. pyogenes, S. faecalis and B. subtilis. It is membrane-associated and can act on the HPr from any Gram-positive bacteria, including S. mutans, but not those of Gram-negative bacteria (Reizer et al., 1989a). In addition, HPr(His-P) cannot serve as a substrate. The kinase is strongly inhibited by phosphate and is activated by fructose-1,6,-bisphosphate. Thus, when cells need energy and phosphate levels are high, PEP accumulates in the cell and can be used to phosphorylate HPr for PTS transport. The HPr(Ser) kinase will be inhibited by phosphate and the histidine-15 site will be available for phosphorylation by PEP. Conversely, when the cells are exposed to excess sugar, FBP_2 and ATP will both accumulate activating the HPr(Ser) kinases that in turn act to slow the entry of sugars by the PTS.

Recently, it has been demonstrated in E. coli and Salm. typhimurium, that acetate kinase and ATP (or GTP) can replace PEP as the phosphoryl donor for EI (Fox et al., 1986). Such a mechanism occurs in S. faecalis where an ATP-dependent kinase that phosphorylates dihydroxyacetone and glycerol can reversibly exchange a phosphate with HPr at the histidine residue (Deutscher and Sauerwald, 1986). Fox et al. (1990) have suggested that these pathways may provide links between

metabolism and transport, and in the case of S. faecalis, the glycerol and HPr(His-P) may compete for the kinase allowing unphosphorylated glycerol to accumulate in the cell resulting in inhibition of glycerol uptake.

In Gram-negative cells, protonmotive force has been shown to inhibit the EII permeases by reducing the affinity of the enzyme for its sugars and promoting the efflux of the free sugar (Postma and Lengeler, 1985). To date, Brochothrix thermosphacta is the only Gram-positive organism showing PMF-related inhibition of the PTS (Singh et al., 1985), thus the extent of this regulation is not known.

Sugar transport in Streptococcus mutans

The first step in the cariogenic process initiated by S. mutans is the transport of sugar. This organism uses carbohydrates as a carbon and energy source and transports several sugars by the PTS including glucose, mannose, lactose, sucrose, mannitol and sorbitol (Thompson, 1987). Glucose transport in S. mutans is complex with multiple transport systems and strain differences reported (Hamilton, 1987). Schachtele and Mayo (1973) were the first to report the presence of a glucose-PTS in S. mutans and most strains have two glucose PTS with distinct EII, one with affinity for mannose, glucose and 2-deoxyglucose called the EII^{Man} and the other with affinity for glucose, α-methylglucoside and 2-deoxyglucose called the EII^{glc} (Vadeboncoeur, 1984; Néron

and Vadeboncoeur, 1987). One notable exception is S. mutans GS5 which is reported to possess EII^{glc}, but not EII^{man} (Liberman and Bleiweis, 1984b). A soluble III^{man} protein is also required for the mannose PTS (Néron and Vadeboncoeur, 1987).

Some oral streptococci exhibit diauxie growth when challenged with glucose and lactose. Unlike enteric bacteria, however, oral streptococci transport lactose via the PTS (Hamilton and Lo, 1978) with the exception of S. mutans HS6 that possesses a lac-permease:β-galactosidase system (Calmes and Brown, 1979). In S. mutans, lactose is metabolized via the tagatose 6-phosphate pathway to triose phosphates of the glycolytic pathway (Hamilton and Lebtag, 1979). The enzymes of both the tagatose pathway and the lactose PTS are repressed by growth on glucose and induced by growth on lactose (Hamilton and Lebtag, 1979). Hamilton and Lo (1978) demonstrated transient repression of the lactose PTS and associated metabolic enzymes with the addition of cAMP to cells grown in low concentrations of the permeabilizing agent toluene. Permanent catabolite repression was not affected significantly by this treatment. In enteric bacteria, lower concentrations of exogenous cAMP than required to overcome transient repression can suffice, suggesting some differences in transcriptional control between E. coli and S. mutans.

Catabolite repression was mediated through EII^{man} since mutants defective in this protein lost the diauxie effect.

S. mutans GS5 has a single PTS system for glucose transport and mutants defective in the glucose PTS, were devoid of catabolite repression confirming the importance of the EII complex (Liberman and Bleiweis, 1984a). Regulatory mechanisms of carbohydrate transport may differ between strains of S. mutans as EII proteins that are inducible in one strain may be constitutive in another. For example, strains GS5-2 and NCTC 10449 of S. mutans displayed typical diauxie when exposed to glucose and lactose, while S. mutans ATCC 27352 failed to display diauxie by the absence of a lag phase, although it used glucose before lactose (Vadeboncoeur and Trahan, 1983). Conversely, S. mutans 6715 used lactose before glucose and, when grown first on lactose, the EIIs specific for glucose, mannose and fructose were repressed.

Brown and Wittenberger (1973) showed that in S. mutans, enzymes required for metabolism of mannitol and sorbitol, namely mannitol-1-P dehydrogenase or sorbitol-6-P dehydrogenase, were found following growth on the sugar-alcohol. Moreover, glucose addition to cultures grown on mannitol or sorbitol resulted in the repression of the inducible enzymes required for the preparation of the sugar alcohols for the glycolytic pathway. In this example of carbohydrate transport regulation, two mechanisms are operating: competition by distinct EII for HPr~P and inducer exclusion. Dill and Seno (1983) demonstrated that inducer exclusion was responsible for the rapid repression of the hexitol systems. These workers showed that 2-deoxyglucose

was as efficient at causing repression as glucose, fructose and sucrose. Therefore, hexose transport by the PTS without subsequent metabolism was sufficient to cause repression. The EII of the other sugars successfully competed for HPr~P such that the hexitols were no longer transported into the cell to induce their respective genes resulting in rapid loss of hexitol transport systems in the presence of assorted hexoses.

The importance of sugar competition for a common EII is demonstrated by the susceptibility of S. mutans to xylitol. Xylitol is transported by the EII^{fru} and accumulates inside the cell as xylitol-P, which cannot be further metabolized (Gauthier et al., 1984). When S. mutans was grown on glucose, mannose, and lactose, xylitol had a bacteriostatic effect on cell growth, but cells grown on fructose or sucrose were not susceptible to xylitol inhibition. Exogenous fructose effectively competed with xylitol for the EII^{fru} excluding xylitol from the cells. The sucrose inhibition of xylitol transport is believed to result from extracellular cleavage of sucrose to glucose and fructose with the latter preventing xylitol entry into the cell.

S. mutans has at least four different mechanisms for the utilization of sucrose. These include: (1) the hydrolysis of sucrose to glucose and fructose by invertase, (2) the transport of sucrose via an inducible, sucrose-PTS followed by its hydrolysis by the constitutive enzyme sucrose hydrolase to glucose-6-P and fructose, (3) the degradation of

sucrose by fructosyltransferases resulting in formation of free glucose and fructans, and (4) the degradation of sucrose by glucosyltransferases resulting in formation of free fructose and glucans. The regulation of the sucrose-PTS is not well-understood although it is repressed by growth in high concentrations of sucrose.

Many of the studies examining the metabolism and carbohydrate transport of S. mutans have used the technique of continuous culture which allows for the cultivation and collection of bacteria under strictly defined growth conditions in a culture device called the chemostat (Tempest, 1970). By limiting an essential nutrient in the medium, the growth rate becomes proportional to the dilution rate of the culture allowing for the examination of the physiology of bacteria grown at different growth rates (Herbert, 1958). A range of factors, approximating environmental stresses encountered in the mouth can be manipulated in continuous culture, in particular, limiting nutrient levels and low growth rates. These conditions are not readily achieved using batch culture techniques. Under continuous culture, S. mutans grown at low versus high dilution rates, or limited versus excess carbohydrate supply, differ in products of fermentation (Carlsson and Griffith, 1974; Ellwood and Hamilton, 1982), aciduricity (Hamilton and Bowden, 1982) and carbohydrate transport systems (Hamilton et al., 1979).

In continuous culture studies, the glucose-PTS in S. mutans Ingbritt repressed by a variety of conditions including

growth at low pH (Ellwood and Hamilton, 1982), at high growth rates and with excess glucose (Ellwood et al., 1979; Hamilton et al., 1979) and following transition to growth on sucrose (Ellwood and Hamilton, 1982). Glucose uptake in the chemostat and glycolytic rates of washed cells exceeded that which could be accounted for by the repressed glucose-PTS suggesting the presence of an alternative non-PTS glucose transport system (Hamilton, 1986). More recent research indicated that the repression of the glucose-PTS is mediated through the sugar-specific EII components of the system (Vadeboncoeur et al., 1987, 1988, 1991; Hamilton et al., 1989). In the earlier work, the highest glucose-PTS activity in S. mutans Ingbritt was detected in permeabilized cell assays with cells grown in continuous culture with a glucose limitation at neutral pH and a dilution rate (D)= 0.1 h^{-1} (Ellwood et al., 1979). Membranes isolated from cells grown with conditions also displayed the highest EII activity when tested with mannose and glucose indicating both systems were fully induced (Vadeboncoeur et al., 1987). The activities of the two EII were decreased by one third in cells grown at a D= 1.0 h^{-1}, and 45% and 54% by decreasing the pH to 5.5 at a D= 0.1 h^{-1}. In cells grown at pH 5.5 with a D= 0.4 h^{-1}, EII^{glc} and EII^{man} activities decreased 24- and 27-fold respectively (Vadeboncoeur et al., 1987) and were undetectable in cells grown at pH 5.0 (Vadeboncoeur et al., 1991). The levels of the soluble, common components of the PTS, HPr and EI, were resistant to changes in growth

conditions and were repressed only up to 4-fold in cells grown on excess glucose at pH 5.0 and with increases in growth rate (Vadeboncoeur et al., 1987; Hamilton et al., 1989).

Similar results have been reported for a fresh isolate of S. mutans (Rodrigue et al., 1988), but somewhat different results were reported for S. mutans DR0001 (Vadeboncoeur et al., 1987) and will be discussed below. The mechanisms of repression and inhibition of the PTS are not known, but as the degrees of repression of the individual components differ, a single, coordinated mechanism is not indicated. Repression cannot be due to free glucose since significant repression of both EII^{man} and EII^{glc} were detected in the presence and absence of glucose in the growth medium by Hamilton et al., 1989. These authors suggested that part of the regulation may be mediated through sugar-phosphate accumulation in the cell which could be examined by testing the effect of various non-metabolizable glucose analogues transported by the PTS on synthesis and activity of EII enzymes. By growing permeabilized cells of S. mutans in the presence of cAMP, as previously described by Hamilton and Lo (1978), and assaying the various PTS components, it may be possible to delineate the role, if any, of cAMP in regulation of the PTS components. cAMP usually affects enzyme activity at the genetic level. However, Lodge and Jacobson (1988) have demonstrated that part of the increase in PTS activity associated with a shift from glucose-excess to glucose-

limited conditions is seen even when protein and RNA synthesis are blocked. They reported that stimulation of PTS activity correlated with the loss of a phosphate on the HPr. Thus, as with other Gram-positive bacteria, the HPr of S. mutans has two sites of phosphorylation, one involved in phosphoryl transfer to EI, while the other likely serves a regulatory role (Reizer et al., 1989a,b).

Unlike other systems, very few mutants of S. mutans defective in some aspect of glucose transport have been described. One mutant that has proved valuable is a PTS-defective mutant, S. mutans DR0001/6, that possesses approximately 15% of the glucose-PTS activity of its wild-type parent, DR0001, in permeabilized cell assays (Hamilton and St. Martin, 1982). Recently, it has been shown that the transport of glucose, mannose and 2-deoxyglucose by S. mutans DR0001 is controlled by a common EII or separate EIIs under co-ordinate regulation (Vadeboncoeur et al., 1987). Unlike S. mutans Ingbritt, strain DR0001 showed almost 2-fold stimulated EII activity for glucose in cells grown at pH 5.5. The EII activity of the mutant was 12% for glucose and 7% for mannose of the respective EII activities of the wild type parent and HPr activity was comparable (Vadeboncoeur et al., 1987).

In continuous culture, the mutant DR0001/6 exhibited glucose uptake and glycolytic rates comparable to those of the wild-type parent, suggesting the presence of a second, non-PTS system of glucose transport (Hamilton and St. Martin,

1982). Supporting the hypothesis were kinetic experiments of glucose uptake which demonstrated that the wild type strain possessed two uptake systems with K_m values of 6-8 μM and 57-125 μM, while the mutant possessed only one system with a K_m of 72-130 μMm The nature of the non-PTS system was studied by looking at the effect of inhibitors and ionophores on glucose-related activities. Glycolysis of the mutant following glucose challenge was more susceptible to various ionophores, such as the protonionophore, carbonylcyanide-m-chlorophenylhydrazone (CCCP), than the wild-type parent. This suggested that the second transport system was coupled to the gradient energy of the protonmotive force. The PMF-associated transport model was supported by subsequent studies showing that agents which perturb the protonmotive force inhibited glycolytic activity or glucose transport (Hamilton and St. Martin, 1982; Keevil et al., 1984; Hamilton, 1987). In addition, DCCD, which inhibits the H^+/ATPase known to generate protonmotive force in streptococci, inhibited glucose uptake and the glycolytic ability of S. mutans (Hamilton, 1984). Protonmotive force is also generated by lactate efflux, and it was also shown that high concentrations of lactate in the external medium can inhibit the growth of the PTS-defective mutant S. mutans DR0001/6 (Hamilton and St. Martin, 1982). One problem with these studies is that control cells with a fully induced, competent glucose PTS often showed some sensitivity to the various agents, albeit less than S. mutans DR0001/6 or cells

grown under conditions known to repress the glucose PTS. While these studies provided circumstantial evidence for protonmotive force-linked transport, more direct evidence was required.

Keevil et al. (1986) reported the transport of 6-deoxyglucose by S. mutans Ingbritt in response to protonmotive force. 6-deoxyglucose is a valuable tool for studying glucose transport because it is a non-metabolizable glucose analogue that cannot be transported by the PTS (Schachtele and Mayo, 1973). In their system, transient uptake of 6-deoxyglucose was reported that peaked in 4-fold accumulation of the substrate, however, since their data was obtained with heat-treated cells without corresponding protonmotive force measurements, these results are questionable.

In the non-PTS model linking glucose transport to protonmotive force, glucose in an unaltered state would enter the cell, possibly in symport with a proton, as is the case for the lac permease of E. coli (Kaback, 1983), where it would be phosphorylated by glucokinase. Porter et al. (1980) identified a glucokinase in S. mutans that transfers the terminal phosphate from ATP to glucose, was specific for glucose and had no activity when tested with other hexoses. Glucokinase is under some genetic control, being repressed two-fold in cells grown in continuous culture with limiting glucose, conditions that are optimal for the glucose-PTS (Hamilton, 1984). The Km for ATP of glucokinase was

unchanged in different growth conditions, however, its Km for glucose increased two-fold in conditions favouring the glucose-PTS system, but decreased in conditions known to repress the glucose-PTS indicating a somewhat higher affinity for glucose (Hamilton, 1984).

Research Aims

The alternative glucose transport system could be of great importance in defining the ecology and pathogenicity of S. mutans. Existing evidence indicating repression of the glucose-PTS at low pH and high extracellular concentrations of glucose and sucrose, suggests it would be favoured under cariogenic conditions. It is the goal of this thesis to identify and characterize the non-PTS glucose transport in S. mutans. The specific aims are:

1. To examine the effect of various ionophores on components of the protonmotive force, glycolytic rates and glucose uptake at a range of pH in cells grown in PTS-repressed and induced conditions,
2. To determine the activity of the H^+/ATPase in cells grown under different conditions and the sensitivity of glycolysis and glucose uptake in these cells to agents that interfere with the H^+/ATPase,
3. To measure uptake of glucose and glucose analogues in the presence of artificially-generated protonmotive force of known strength,
4. To determine the preferential phosphate donor to glucose under a variety of glucose PTS-stimulated and repressed conditions, and,
5. To examine glucose transport in membrane vesicles derived from S. mutans as these will be devoid of PTS activity to the absence of HPr and EI.

Chapter 2

Effect of Ionophores and Inhibitors on Glucose Transport and Metabolism by Streptococcus mutans Ingbritt

Introduction

Streptococcus mutans is a major etiological agent of dental caries, catabolizing dietary carbohydrates to acid end-products that, once excreted, can contribute to the demineralization of the tooth enamel (Hamada and Slade, 1980; Van Houte, 1986). Early studies on sugar transport by S. mutans have indicated that the organism possesses at least two distinct systems for glucose uptake (Hamilton, 1984, 1987). The principal transport system for glucose and a variety of sugars, including mannose, fructose, sucrose, lactose and maltose, is the phosphoenolpyruvate (PEP)-phosphotransferase system (PTS) (Vadeboncoeur, 1984). In the PTS, high-energy phosphate from PEP is transferred via the general proteins HPr and EI, through the sugar-specific, membrane-bound carriers, EII, onto the incoming carbohydrate (Meadows et al., 1990). In some cases, the latter phosphoryl transfer requires the participation of a sugar-specific Enzyme EIII, or simply EIII.

Continuous culture studies have shown that the glucose-PTS is repressed under a variety of conditions including growth at low pH (Hamilton and Ellwood, 1978), at high growth rates

and with excess glucose (Ellwood et al., 1979; Hamilton et al., 1979), and following transition to growth on sucrose (Ellwood and Hamilton, 1982). More recently, it has been demonstrated that most of the observed repression is mediated through the sugar-specific, membrane-bound EII component of the PTS and not the general proteins (Vadeboncoeur et al., 1987, 1991; Hamilton et al., 1989). Although the PTS was repressed, the calculated glucose uptake in the chemostat and the glycolytic rates were not inhibited to a comparable degree suggesting that during PTS repression, S. mutans employed an alternative, non-PTS glucose transport system (Ellwood et al., 1979).

Further evidence for this hypothesis came from studies with the PTS-defective mutant S. mutans DR0001/6. This mutant exhibited approximately 10% of the EII^{glc} activity of the parent (Vadeboncoeur et al., 1987) and yet had glycolytic and growth rates comparable to those of the wild-type parent (Hamilton and St. Martin, 1982). Kinetic studies with S. mutans DR0001/6 and its parent S. mutans DR0001 also indicated two glucose transport systems (Hamilton and St. Martin, 1982). The wild type parent was found to possess two kinetically distinct glucose transport systems, a high affinity system with a K_m of 6.7-8.0 μM and a low affinity system with a K_m of 57-125 μM, while the PTS-defective mutant possessed only the low affinity system.

The nature of the second system in S. mutans DR0001/6 was, in part, defined by looking at the effect of inhibitors and

ionophores on glucose-related cell activities. For example, low concentrations of the proton ionophore, carbonylcyanide-m-chlorophenylhydrazone (CCCP), inhibited glycolytic activity more strongly in the PTS-defective mutant than in the wild-type parent (Hamilton and St. Martin, 1982). This suggested that the second transport system was coupled to the gradient energy of protonmotive force (PMF), which is the sum of the transmembrane pH (ΔpH) and electrochemical ($\Delta\psi$) gradients (Mitchell, 1966). Subsequent studies supported this model by demonstrating that other ionophores which perturb ΔpH or $\Delta\psi$ inhibited glycolytic activity and total glucose transport (Keevil et al., 1984; Hamilton, 1987).

Further support for the PMF-associated transport model was provided by studies with agents that interfere with the generation of protonmotive force. For example, streptococci are known to generate PMF through proton extrusion via the proton-translocating, membrane-associated ATPase (H^+/ATPase) (Harold, 1977) and lactate efflux (Michels et al., 1979; Otto et al., 1980). The H^+/ATPase blocking agent, N,N'-dicyclohexylcarbodiimide (DCCD), inhibited glucose uptake and glycolytic activity by S. mutans strains (Keevil et al., 1984), while the presence of high concentrations of lactate in the external medium inhibited the growth and glycolytic activity of the PTS-defective mutant, S. mutans DR0001/6 (Hamilton and St. Martin, 1982; Keevil et al., 1984; Hamilton, 1987).

Keevil et al. (1986) used the non-PTS glucose analogue, 6-deoxyglucose (6-DG), to test for the presence of the protonmotive force-coupled transport system in studies with S. mutans Ingbritt. The uptake of 6-DG was shown to be inhibited by several ionophores that depleted one or both components of the PMF and the addition of lactate and DCCD completely abolished 6-DG transport.

The purpose of this study was to examine more closely the effect of various agents known to impair protonmotive force on glucose transport and metabolism on S. mutans Ingbritt. Cells were grown in continuous culture under four sets of conditions, one that was optimal for the glucose-PTS and three which result in PTS repression. By examining transport and glycolysis separately it should be possible to distinguish between transport-specific effects and generalized effects associated with ionophore activity.

Materials and Methods

Bacterial strain

The S. mutans strain Ingbritt used in this study was kindly supplied by J. Sandham, University of Toronto, Canada. Each chemostat experiment was started from a lyophilized culture whose identity was confirmed by Gram-staining and a battery of biochemical tests (Shklair and Keene, 1976; Hamada and Slade, 1980; Coykendall and Gustafson, 1986).

Growth conditions

Cultures were grown either in a LH 500 series EIII chemostat (L.H. Engineering, Stoke Poges, Buckinghamshire, UK) with a working volume of 670 ml or a New Brunswick chemostat (model C30; New Brunswick Scientific Co., Inc., Edison, N.J.) with a working capacity of 375 ml as described previously (Hamilton and Bowden, 1982). The growth medium was the semi-defined medium of Bowden et al. (1976) and the pH in the chemostat was maintained at 7.0 or 5.5 with the automatic addition of 2M KOH and the gas phase was 5% CO_2 in nitrogen. Four growth conditions were employed: (A) 5 mM glucose at a dilution rate (D) of 0.1 h^{-1}, pH= 7.0; (B) 50 mM glucose at D=0.1 h^{-1}, pH= 7.0; (C) 50 mM glucose at D=1.0 h^{-1}, pH= 7.0 and (D) 50 mM glucose at D=0.1 h^{-1}, pH= 5.5. Glucose was limiting in all cases (i.e., free glucose <250 μM) and cells were devoid of endogenous glycolytic activity.

Routine chemostat maintenance, including dry weight measurements and daily pH determinations, was performed as described previously (Hamilton et al., 1979). The purity of chemostat cultures was monitored periodically by streaking on blood agar plates which were then incubated aerobically and anaerobically at 37°C. Steady state was considered to have been achieved when at least 10 culture volumes had passed through the chemostat.

Phosphorylation of glucose by ATP or PEP

Cells of S. mutans Ingbritt were harvested from the chemostat overflow over 3-16 hours into a container cooled to 0°C, centrifuged at 10,000xg for 10 minutes, washed twice and resuspended at 10 mg/ml in 10 mM potassium phosphate buffer (pH 7.0) containing 14 mM 2-mercaptoethanol and 5 mM $MgCl_2$. Cells were permeabilized (decryptified) with toluene as described by Hamilton et al. (1989) and assayed in the following reaction mixture (final concentrations): 50 mM potassium phosphate buffer (pH 7.0), 5 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 10 mM NaF, 2 mM C^{14}-glucose (225 dpm/nmol), 2mM ATP or PEP and 250 µg (dry weight) cells in a 500 µl volume. Controls contained no phosphoryl donor. Following 15 minutes of incubation at 37°C, the phosphorylated product was precipitated with 30 mM BaBr in 90% ethanol (Gachelin, 1970), collected on filters and the filters counted in Aquasol.

Determination of intracellular glucose and glucose-6-phosphate concentrations

Triplicate 35 ml samples of steady state chemostat cultures were rapidly drawn directly from the chemostat into a flask containing perchloric acid (1 M final concentration) using a vacuum. The cell-perchlorate mixture was incubated at room temperature with gentle mixing for 30 minutes to complete the extraction of the cell contents. The samples were then neutralized to pH 7.6 with the addition of 10M KOH and stored

at -70° overnight. The following day, samples were thawed, centrifuged at 8000g for 10 minutes to remove debris and the supernatant collected for assays which were performed within 48 hours of chemostat sampling. Glucose-6-phosphate was measured with a modification of the glucose-6-P dehydrogenase assay of Bergmeyer (1974). Samples were combined with (final concentration) 200 mM Triethanolamine buffer (pH 7.5), 8.0 mM $MgCl_2$, and 0.8 mM $NADP^+$ in a final volume of 1 ml and assayed in a UV/visible spectrophotometer (Hewlett Packard model 8452A Diode Array Spectrophotometer, Hewlett Packard Co., U.S.A.). Glucose-6-phosphate dehydrogenase (5 units) was added to initiate the assay and the conversion of glucose-6-P to 6-phosphogluconolactone, monitored by the reduction of $NADP^+$ at 340 nm. The reaction went to completion within 3 minutes at which time known quantities of glucose-6-phosphate were added to samples to serve as internal standards and a second absorbance reading was made.

Glucose was assayed using the glucose oxidase-peroxidase coupled reaction of Kingsley and Getchell (1960). Samples were combined with (final concentration): 400 mM Tris-HCl (pH 7.0), 0.01% o-dianisidine, 0.1 mg peroxidase and 0.67 mg/ml glucose oxidase (final concentrations). Following incubation at 37° for 60 minutes, 400 µl of 37.4% sulfuric acid was added and the tubes allowed to cool to room temperature. Absorbance was then read at 530 nm. A duplicate set of tubes was included in each assay to which known amounts of glucose were added to act as an internal standard.

Glucose uptake

Glucose uptake by S. mutans Ingbritt was determined with intact cells grown under all four conditions (A, B, C and D) and incubated with C^{14}-glucose over the pH range 5.0 to 8.5. Prior to the assay, cells were washed twice in 10 mM potassium/sodium phosphate (K/NaP) buffer (pH 7.0) containing 14 mM 2-mercaptoethanol and 5 mM $MgCl_2$. The reaction mixture contained (final concentration): 50 mM K/NaP buffer (pH 5 to 8.5), 5 mM C^{14}-glucose (0.1 C/mole) and 1.0 mg (dry weight) cells in a final volume of 1.0 ml. Following a pre-incubation period of 10 minutes at 37°C, the reaction was initiated by the addition of the labelled glucose and the cellular accumulation of C^{14}-glucose was measured at 1 minute by filtering the reaction mixture through a 0.45 µm membrane filter (Type HA, Millipore Corporation, Bedford, MA). The filters were washed twice with 1 ml of pre-warmed assay buffer of the same pH and the cellular radioactivity on the filter was determined by counting in Aquasol. The following ionophores and and inhibitors were tested for their effect on glucose transport: 50 µM valinomycin, 50 µM nigericin, 50 µM gramicidin S, 20 µM carbonylcyanide-*m*-chlorophenylhydrazone (CCCP), 40 µM tetrachlorosalicylanilide (TCS), 100 µM DCCD or 2 mM sodium fluoride (NaF). These compounds were added to the reaction mixture at the beginning of the pre-incubation period and since the ionophores were prepared in ethanol, controls containing the appropriate level of ethanol were included in all experiments. The ethanolic control values

for all experiments ranged from 0.89 to 1.16 of the controls values. Various agents were also tested for their effect on cells actively transporting glucose by their addition at the same concentrations 0.8 minutes following C^{14}-glucose addition. These assays were performed with 5 ml of Condition B cells incubated at 1.0 mg/ml in K/NaP buffer (pH 6.0) with 5 mM C^{14}-glucose. Samples (0.5 ml) were taken at 0.25 minute intervals over a 2 minute period, filtered, washed and counted as described above.

Glucose uptake was also measured with Condition B cells following pre-treatment with 2-deoxyglucose to rundown the cellular PEP pool. Washed cells were incubated with 25 mM 2-deoxyglucose and incubated at 37° for 30 minutes. Previous experiments had demonstrated that 2-deoxyglucose transport was abolished after 5 minute incubation at this concentration.

Measurement of glycolytic activity

The glycolytic activity of Condition B and D cells was assayed as described previously (Hamilton and St. Martin, 1982). Briefly, cells were harvested and suspended at 1.0 mg/ml in K/NaP buffer in a pH stat at a final volume of 5.0 ml. The suspension was constantly mixed under a stream of nitrogen gas and the endpoint pH was maintained at pH 5.5 or 7.5 by the addition of 0.1 M NaOH from a Radiometer Autoburette (model ABU 1a, Radiometer, London, Ontario, Canada). The glycolytic rate was determined after the

addition of glucose to a final concentration of 5 mM. The inhibiting effect of various agents was tested by their addition to the glycolyzing mixtures once a linear rate of acid production had been established. Glycolytic activity was defined as nanomoles metabolic acid neutralized per milligram (dry weight) of cells per minute.

Measurements of ΔpH and Δψ

The protonmotive force (Δp) was determined by measuring the transmembrane pH gradient (ΔpH) and the electrochemical gradient (Δψ) according to the equation: $\Delta p = \Delta\psi\ 59 - \Delta pH$ (Hamilton, 1990). The transmembrane pH gradient was measured using ^{14}C-salicylate (20 μM final concentration, 1200 dpm/nmol) and the Δψ was measured with ^{14}C-methyltriphenlyphosphonium iodide (40 μM final concentration, 600 dpm/nmol) by published methods (Keevil and Hamilton, 1984; Keevil et al., 1984; Hamilton, 1990). Cells from Condition B (50 mM glucose at D=0.1 h^{-1}, pH= 7.0) were washed and resuspended at 1 mg/ml in K/NaP buffer (pH 5.5 or 7.5) containing either ^{14}C-salicylate or ^{14}C-methyltriphenlyphosphonium iodide. Duplicate samples were removed at 2 and 5 min after the addition of glucose (20 mM final concentration) and the cells separated from the aqueous phase by centrifugation through the silicone oil (1:1 Dow Corning fluid 550 and 556) as described previously (Hamilton, 1990). Corrections for non-specific binding of probes were

made by subtracting the label in cells treated for 5 minutes with 5% butanol and 50 μM gramicidin S ($\Delta\psi$) or with butanol alone (ΔpH). To determine the effect of ionophores and inhibitors, cells were pre-treated for 5 minutes with the agents at the same concentrations as described above and then assayed.

The intracellular and extracellular water content of cells was determined as previously described (Kashket and Barker, 1977; Keevil and Hamilton, 1984). Briefly, S. mutans Ingbritt was incubated in K/NaP buffer with either ^3H_2O (2000 dpm/ml final volume)(total aqueous space), or ^{3}H-polyethylene glycol (1500 dpm/ml final volume)(extracellular water volume). Following a 10 minute incubation, the cell pellets were collected by silicone centrifugation and counted. Cellular volumes were not significantly affected by the external pH. The intracellular water volume for S. mutans Ingbritt under experimental conditions employed was 2.49 ± 0.06 μl/mg (dry weight) at pH 7.5 and 2.52 ± 0.05 μl/mg (dry weight) at pH 5.5, while the extracellular volumes were 0.33 ± 0.03 and 0.36 ± 0.02 μl/mg (dry weight), respectively.

Chemicals

Aquasol, radioactive salicylate, methyltriphenylphosphonium iodide and PEG were purchased from New England Nuclear (Montreal, Quebec, Canada) and tritiated water was obtained from Amersham (Oakville, Ontario, Canada). Radioactive

glucose was purchased from ICN Radiochemicals (Irvine, CA.,U.S.A).

Results

PEP and ATP-dependent phosphorylation of glucose

Glucose transport by S. mutans Ingbritt was characterized in cells grown in continuous culture under conditions optimal for the glucose-PTS(Condition A), and three conditions known to repress the glucose-PTS (Conditions B, C and D)(Table 4). PEP is the phosphoryl donor for the PTS resulting in the intracellular formation of glucose-6-P. Non-PTS active transport, on the other hand, would result in the appearance of the glucose inside the cell which would be phosphorylated by ATP in a glucokinase reaction. We tested for ATP and PEP-dependent phosphorylation activity in decryptified cells grown under the four sets of conditions. As shown in Table 4, PEP-dependent phosphorylation of glucose by cells grown under Condition A was almost 5-fold higher than that by ATP, confirming that the glucose-PTS is the primary mode of glucose transport in these cells. Cells growing with a 10-fold higher glucose concentration in Condition B exhibited a 2-fold increase in PEP phosphorylation, however, ATP phosphorylation was 4-fold higher and this resulted in a decrease in the PEP/ATP ratio to 1.85. This is consistent with the fact that although the PTS is repressed to levels one third of those measured in condition A, it is still

active (Hamilton et al., 1989). Increasing the dilution (growth) rate of the B cells to 1.0 h^{-1}(Condition C), which is near the μ_{max} of S. mutans Ingbritt (Hamilton, 1986), stressed the cells resulting in a decline in the total phosphorylating activity, but ATP was the donor of choice as exhibited by the PEP/ATP ratio of 0.59. When cells were grown with 50 mM glucose at pH 5.5, D= 0.1 h^{-1}(Condition D), the overall phosphorylating activity was low compared to similar cells at pH 7.0 (Condition B), but ATP-phosphorylating activity was 3-fold greater than that with PEP with a resulting PEP/ATP ratio of 0.33. In addition, Condition B cells, pretreated with 2-deoxyglucose to reduce the intracellular 'PEP pool' and cripple the PTS exhibited a PEP:ATP ratio of 0.38 indicating that ATP became the preferred phosphoryl donor. These results indicate that PTS and non-PTS glucose uptake systems can be distinguished by assaying decryptified cells in the presence of ATP or PEP and supports earlier observations on the repression of the glucose-PTS obtained by other methods (Table 4).

Intracellular concentrations of glucose and glucose-6-phosphate

As an extension of the above study, the actual intracellular concentrations of free glucose and glucose-6-phosphate were determined in S. mutans Ingbritt

Table 4

PEP and ATP phosphorylation of glucose by decryptified cells of Streptococcus mutans Ingbritt grown in continuous culture under conditions resulting in optimal (A) and repressed (B, C, D) PTS activity.

Cells	Glucose Concn	D (h^{-1})	Growth pH	EII activity[a]		Phosphoryl Donor[b]		Ratio PEP/ATP
				glucose	mannose	PEP	ATP	
A	5	0.1	7.0	319[c]	350[c]	74.4	16.4	4.54
B	50	0.1	7.0	96[d]	102[d]	110.1	59.4	1.85
B[e]						36.5	94.9	0.38
C	50	1.0	7.0	29[f]	19[f]	13.7	23.2	0.59
D	50	0.1	5.5.	6[c]	9[c]	6.9	20.7	0.33

a Nmoles per milligram of membrane protein per minute in the presence of excess HPr, EI and III.

b Nmoles per milligram dry weight of cells per minute.

c Adapted from Vadeboncoeur et al., 1987.

d Adapted from Hamilton et al., 1989.

e B cells treated with 2-deoxyglucose as per Table 8.

f Adapted from Vadeboncoeur et al., 1991.

grown in Conditions A, B and D to test if the concentration of free glucose inside the cells reflected the activity of the non-PTS glucose transport system. With increasing repression of the glucose PTS (Condition A to D), the free glucose concentration inside the cells increased 100-fold and the ratio of glucose:glucose-6-phosphate increased over 30-fold (Table 5). It is interesting to note that the concentration of intracellular glucose-6-phosphate was similar (1-2 fold) to the concentration of glucose in the medium while the intracellular glucose varied from 0.5 fold(Condition A) to almost 10-fold (Condition D) compared to the medium glucose.

Effect of pH on total glucose uptake

The uptake of C^{14}-glucose by steady state cells of S. mutans Ingbritt under Conditions A, B, C and D was tested at pH values from 5.0 to 8.5. This assay measures total transport activity and does not distinguish between the PTS and any non-PTS system. Cells from Condition A, possessing optimal PTS activity, exhibited the highest transport activity of the four cell types with a maximum ranging from pH 7.0 to 8.5 (Figure 5). The rates observed at pH 7.0 were twice those of cells from Condition B (164 vs 84 nmole/mg per min), 5-fold more active than cells from Condition C (164 vs 30 nmole/mg per min), and 8-fold more active than cells from Condition D (164 vs 20 nmole/mg per min). In addition, transport

Table 5

Intracellular concentrations of glucose and glucose-6-phosphate and extracellular concentrations of glucose in Streptococcus mutans Ingbritt grown in continuous culture

Growth Condition	Medium Glucose	Intracellular concentration[a]		G/G6P ratio	Culture Glucose[a]	G_{in}/G_{out} ratio
		Glucose (G)	Glucose-6-P (G6P)			
A[b]	5	4.5 ± 2.1	17.5 ± 5.5	0.25	0.003 ± 0.002	1500
B	50	122.9 ± 56.1	99.6 ± 20.5	1.23	0.019 ± 0.014	6470
D	50	419.3 ± 216.4	51.0 ± 15.9	8.22	0.254 ± 0.100	1600

a Millimolar ± standard deviation.
b Medium glucose concentration = 10 mM.

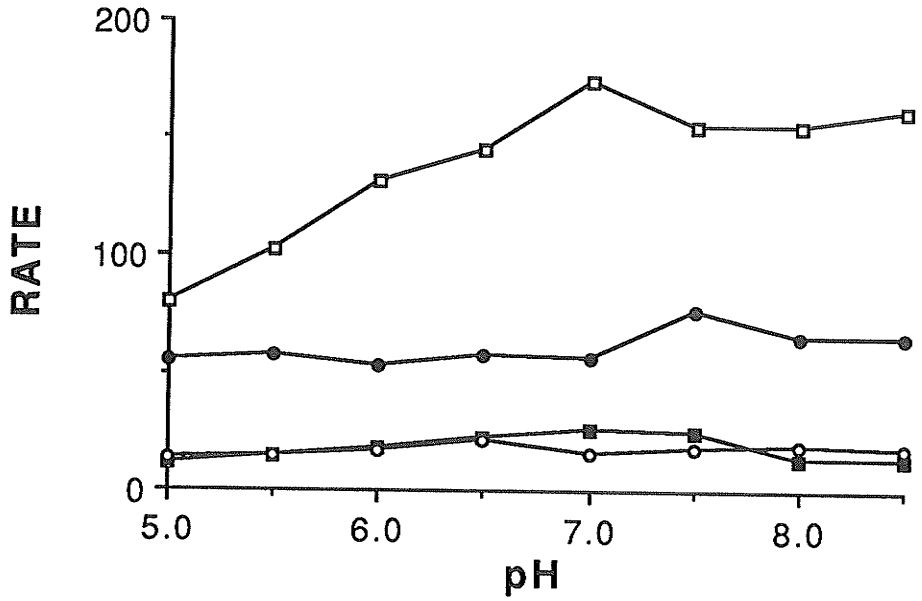

Figure 5. Rate of glucose uptake in washed, intact cells of Streptococcus mutans Ingbritt grown in continuous culture under four different growth conditions; Condition A (□) 10 mM glucose at D= $0.1h^{-1}$, pH=7.0; Condition B (●) 50 mM glucose at D= $0.1h^{-1}$, pH=7.0; Condition C (■) 10 mM glucose at D= $1.0h^{-1}$, pH=7.0; Condition D (○) 50 mM glucose at D= $0.1h^{-1}$, pH=5.5. Rate= nmol per mg (dry weight) cells per min.

activity by A cells was much more sensitive to pH values lower than 7.0 compared to B, C and D cells.

Effect of ionophores and inhibitors on glucose uptake

Since the cells grown under Conditions B, C, and D exhibited lower PTS activity (Table 4), we were interested in the effect of various ionophores and inhibitors on C^{14}-glucose uptake to determine if there was increased sensitivity to ionophores and agents known to disrupt the protonmotive force in these cells. Washed cells from all four growth conditions were pre-incubated with 50 µM valinomycin, nigericin, gramicidin or 20 µM CCCP, 40 µM TCS, 100 µM DCCD or 2 mM NaF and assayed over the pH range 5.0 to 8.5 with C^{14}-glucose.

As seen in Figure 6, gramicidin S, nigericin and valinomycin were the most inhibitory with their effects decreasing with the changes in growth conditions from A to D. For example, glucose uptake with A, B, C and D cells was consistently inhibited by 50 µM gramicidin S at all pH values, although, a small amount of uptake activity (e.g., 4.6-7.5 nanomoles/mg per minute) was apparent under all conditions. Consequently, as the total uptake activity decreased in transitions from A to B to C to D (Fig. 5), the activity with gramicidin relative to the control value increased, e.g. from 0.04 (SD ± 0.02) for A cells to 0.25 (SD ± 0.05) for C cells grown near μ_{max}. As expected, the H^+/K^+ exchanger nigericin was very inhibitory at pH 5.0 and 5.5 for

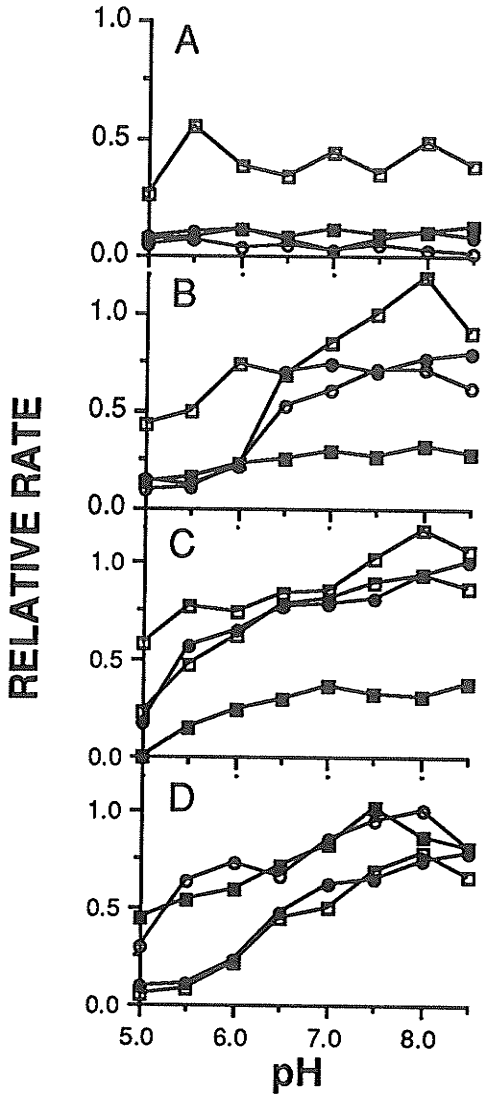

Figure 6. Effect of ionophores, gramicidin S (A), valinomycin (B), nigericin (C) and TCS (D), on glucose transport in washed, intact cells of Streptococcus mutans Ingbritt grown in continuous culture under optimal for the glucose-PTS, Condition A (□) and three conditions resulting in PTS repression, Condition B (●), Condition C (■) and Condition D (O). Relative rate = glucose transport activity of treated cells/glucose transport activity of untreated cells.

the A and B cells (e.g., relative activity, 0.09-0.15), but less so for the C and D cells (relative activity, 0.52-0.54). The inhibitory effect diminished with increasing pH such that above pH 7.5, the relative activity ranged from 0.70 to 1.04. The profile for the K^+ ionophore valinomycin was similar, however, less overall inhibition was observed. Not surprisingly these results indicate that ionophores known to dissipate H^+ and K^+ gradients are particularly effective at acidic pH values, but are less effective under more alkaline conditions where the pH gradient is low (Hamilton, 1984; 1990). Of particular interest was the observation that the uptake rate by the cells has an influence on the effect of the ionophores such that cells with the lower uptake rates (C and D cells) were less affected than cells exhibiting rapid rates (A cells).

In addition to the above agents, the protonionophores, CCCP and TCS, were tested and shown to inhibit glucose uptake more strongly at low pH (data not shown). CCCP was less inhibitory than TCS with maximum inhibition (53%) observed with Condition A cells at pH 5.0 and decreased to 10% inhibition at pH 7.0. TCS-treated cells grown in condition A and B exhibited similar patterns of glucose transport susceptibility with a maximum of 94% and 91% inhibition of glucose uptake at pH 5.0 with a steady decline in inhibition to values 65% of control at pH 7.0 and above (Figure 6).

The effect of the H^+/ATPase inhibitor DCCD on glucose uptake was also examined. Condition A cells were the most

affected with B, C and D cells being relatively immune to its effect. However, maximal inhibition for A cells was only 38% at pH 5.0 and decreased as the assay pH increased (Figure 7).

Fluoride is known to affect bacterial cell physiology in several ways including acidification of the cytoplasm, inhibition of enolase and H^+/ATPase (Hamilton, 1990). At pH 5.0 and 5.5, glucose uptake in A and B cells was inhibited 96% and 92%, respectively, and the inhibition was only partially alleviated to 40% and 65% as the pH rose (Figure 7). Inhibition by fluoride was independent of pH for C cells with glucose uptake 40% of control values at all pH values tested. Glucose uptake in D cells was inhibited 50% at pH 5.0 and 5.5, but resistant to the effects of fluoride above pH 7.0.

Effect of ionophore addition to actively metabolizing cells

In the previous experiment, the inhibiting agents were added 5 minutes prior to C^{14}-glucose addition. To test the effect on actively metabolizing cells, glucose uptake was measured at pH 6.0 with Condition B cells and gramicidin, nigericin and valinomycin added 0.8 min after the C^{14}-glucose addition. As shown in Figure 8, valinomycin had no detectable effect on the uptake rate, while the addition of nigericin had the immediate effect of inhibiting the further uptake of glucose. The addition of gramicidin was dramatic as glucose uptake was not only immediately curtailed, but the

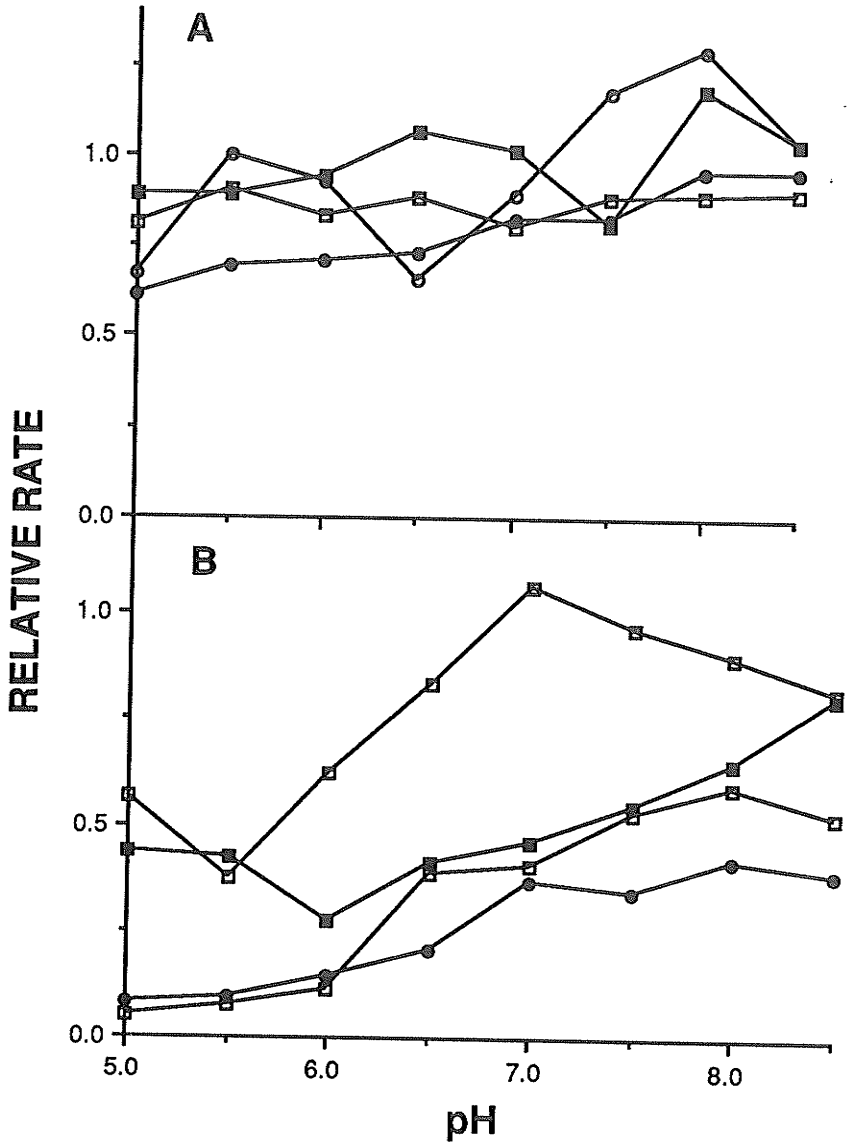

Figure 7. Effect of DCCD (A) and fluoride (B) on glucose transport in washed, intact cells of Streptococcus mutans Ingbritt grown in continuous culture conditions optimal for the glucose-PTS, Condition A (□) and three conditions resulting in PTS repression, Condition B (●), Condition C (■) and Condition D (O). Relative rate = glucose transport activity of inhibitor cells/glucose transport activity of untreated cells.

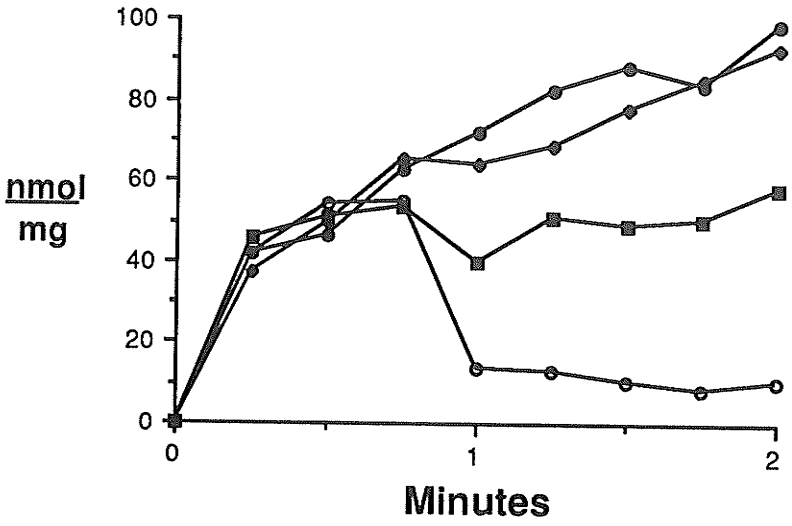

Figure 8. Effect of ionophores on glucose transport at pH 6.0 by cells of Streptococcus mutans Ingbritt grown in continuous culture under Condition B (50 mM glucose at D= $0.1h^{-1}$, pH=7.0) incubated with 5 mM glucose. Additions were made 0.8 minutes after glucose addition as follows: ethanol (●), nigericin (■), valinomycin (◆) and gramicidin S (○).

previously accumulated radioactivity was rapidly lost from cells. The addition of DCCD resulted in reduced transport of glucose, while TCS treatment stopped further uptake (data not shown).

Sensitivity of glycolysis to ionophores

Cells grown under Condition B and D were examined to determine how closely glycolysis mirrored glucose uptake with respect to the sensitivity to the various ionophores and agents known to disrupt protonmotive force. Cells in these two conditions were chosen because both possessed repressed PTS systems, however, B cells were sensitive to the various agents, while D cells were relatively resistant. The glycolytic rates for cells grown in Condition B were 423 ± 36 (mean ± SEM)(nmoles/mg (dry weight)) cells per minutes at pH 7.5 and 265 ± 36 (nmoles/mg (dry weight) cells per minutes at pH 5.5 while the values for Condition D cells were 123 ± 4 and 60 ± 4 at pH 7.5 and 5.5, respectively. Gramicidin, which was the most effective at disrupting glucose uptake at all pH values tested, was equally effective at disrupting glycolysis in cells from both conditions regardless of the assay pH (Table 6). Glycolysis by cells grown at pH 5.5 was more sensitive to the various agents than cells grown at pH 7.0. Furthermore, cells grown at both pH values were more sensitive to agents when incubated at pH 5.5 than pH 7.0, with no detectable glycolytic activity at pH 5.5 with

Table 6

Relative glycolytic rates of washed, intact cells of Streptococcus mutans Ingbritt grown in continuous culture at pH 7.0 and pH 5.5 (50 mM glucose, D= $0.1h^{-1}$) incubated anaerobically with 20 mM glucose and various ionophores and inhibitors (in a pH stat).

	Assayed at pH 7.0		Assayed at pH 5.5	
Agent	Growth 7.0	Growth 5.5	Growth 7.0	Growth 5.5
Ethanol	0.92[a]	0.84	0.89	1.00
Gramicidin	0	0.08	0	0
Nigericin	0.77	0.70	0	0
Valinomycin	0.92	0.77	0	0
TCS	0.92	0.77	0	0
CCCP	1.00	0.94	0.21	0
DCCD	0.92	0.78	0	0
NaF	1.00	0.78	0	0

a. Value of treated cells/value of untreated cells.

gramicidin, nigericin, TCS and fluoride. With the exception of gramicidin, cells grown at pH 7.0 were only mildly inhibited when assayed at pH 7.0 although strong inhibition of glycolysis was seen when the cells were assayed at pH 5.5.

Effect of ionophores on the generation of protonmotive force

With the background information on the effect of the various agents on glucose transport and metabolism, we undertook to examine their effect on protonmotive force, or more specifically, on the pH and electrical gradients (ΔpH and $\Delta\psi$). For this, cells were grown in Condition B at pH 7.0, washed and incubated with glucose (5 mM) in a pH stat with the endpoint at either pH 7.0 or 5.5. As expected, the ΔpH contribution at pH 5.5 was much larger than that detected in cells incubated at neutral pH, while $\Delta\psi$ was the major component of protonmotive force at pH 7.0 (Table 7). Ethanol treatment alone reduced pH and electrical gradients in both cells and reduced protonmotive force by 40% and 20% at pH 5.5 and 7.0, respectively. As expected, gramicidin S dissipated protonmotive force at both assay pH with only a small pH gradient remaining at pH 5.5. Nigericin was almost as effective at reducing significantlym both components. Valinomycin had no effect at pH 7.0 and actually increased protonmotive force values over that found in ethanol-treated cells to levels found in untreated cells, while at pH 5.5, both pH and electrical gradients were sharply reduced so Δp

Table 7

Effect of ionophores and inhibitors on protonmotive force (ΔpH and $\Delta\psi$) generated by washed cells of Streptococcus mutans Ingbritt, grown in continuous culture with 50 mM glucose ($D=0.1h^{-1}$) at pH 7.0 (Condition B) and incubated anaerobically in a pH stat at pH 5.5 or 7.0 with 5 mM glucose.

	EP 7.0			EP 5.5		
Agent	$z\Delta pH$	$\Delta\psi$	Δp[a]	$z\Delta pH$	$\Delta\psi$	Δp
Control	-6[b]	47	53	-37	24	61
Ethanol	-1	30	31	-27	22	49
Gramicidin	0	0	0	-5	0	5
Nigericin	0	18	18	-7	2	9
Valinomycin	-16	42	58	-5	5	10
TCS	0	42	42	-7	14	21
CCCP	-11	25	36	-14	0	14
DCCD	-7	34	41	-5	25	30
NaF	-10	46	56	-3	0	3

[a] Δp= protonmotive force calculated from $\Delta p = \Delta\psi - z\Delta pH$.
[b] millivolts.

was reduced 84% from control levels. TCS had no effect on the electrical gradient, but abolished the pH gradient at neutral pH, while both gradients were sharply reduced at pH 5.5. With CCCP, the proton gradient was maintained at pH 7.0, while at pH 5.5, the pH gradient was reduced (37 to 14 mV) and the electrical gradient was completely abolished. The addition of DCCD at pH 7.0 had little effect on either gradient, while at pH 5.5, the electrical gradient was intact but the pH gradient was reduced by 86%. The addition of fluoride at neutral pH had very little effect on protonmotive force, however, both gradients were essentially abolished at pH 5.5.

Effect of 2-deoxyglucose pre-treatment of cells

To ascertain whether non-PTS transport could be detected in cells with significant PTS activity, cells were treated with 2-deoxyglucose to deplete the 'PEP pool' in order to cripple the PTS system (Thompson and Chassy, 1982). For this, glucose transport was examined with cells of S. mutans Ingbritt grown in Condition B, unwashed or washed in buffer. These latter cells were treated either to deplete endogenous energy reserves or the 'PEP pool'. As shown in Table 8, the first three treatments did not alter glucose uptake significantly, while 2-deoxyglucose treatment reduced uptake sharply to levels comparable to those seen in PTS-repressed Condition D cells (Figure 9). The 2-deoxyglucose-treated

Table 8

Effect of pre-treatment on glucose transport by cells of Streptococcus mutans Ingbritt grown in continuous culture under Condition B

Treatment	Glucose Uptake
No washing	30.4[a]
Washed (2X)	
- No further treatment	35.2
- Depleted of energy reserves[b]	27.2
- Depleted of the 'PEP pool'[c]	14.8

[a] Nanomoles per mg (dry weight) cells per min.

[b] Incubation at 37° at pH 7.0 for 30 min.

[c] Incubation with 25 mM 2-deoxyglucose for 30 min and subsequently washed to remove 2-deoxyglucose.

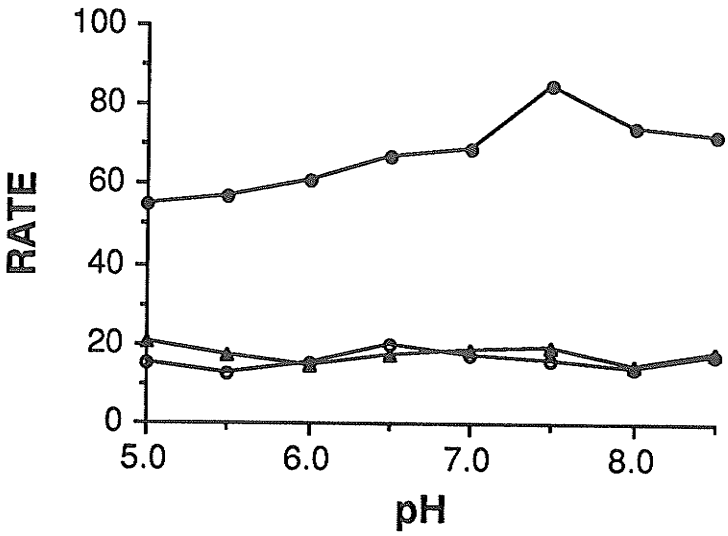

Figure 9. Effect of 2-deoxyglucose treatment on rate of glucose uptake by intact, washed cells of S. mutans Ingbritt grown in continuous culture under Condition B and D. Condition B cells untreated (●) and treated with 2-deoxyglucose (▲). Condition D cells (○). Rate = nmol per mg (dry weight) cells per min.

cells were further characterized by treatment with inhibitors and ionophores to determine the sensitivity of the remaining non-PTS glucose transport to perturbation of protonmotive force. As shown in Figure 10.A., glucose transport in the 2-deoxyglucose-treated cells was much more sensitive to DCCD than either untreated B or D cells, but showed less susceptibility than untreated B cells to fluoride (Figure 10.B.).

Discussion

Growth rate-dependent repression of the glucose PTS in S. mutans Ingbritt was reported as early as 1973 (Hunter et al.) and, since that time, several other factors that result in repression have been identified. Early studies relied on the use of the decryptified cell assay to demonstrate glucose-PTS repression associated with increasing growth rate (Ellwood et al., 1979), sucrose as the carbon source (Ellwood and Hamilton, 1982), decreasing growth pH (Hamilton and Ellwood, 1978) and growth in excess glucose (Ellwood et al., 1979). More recently, these results have been confirmed with chemostat-grown S. mutans Ingbritt by looking directly at the activity of PTS components in cells. It was been found that the previously observed PTS repression is mediated through the membrane-bound, sugar-specific EII^{Glc} and EII^{Man} (Vadeboncoeur et al., 1987, 1991; Hamilton et al., 1989), both of which are capable of transporting and phosphorylating

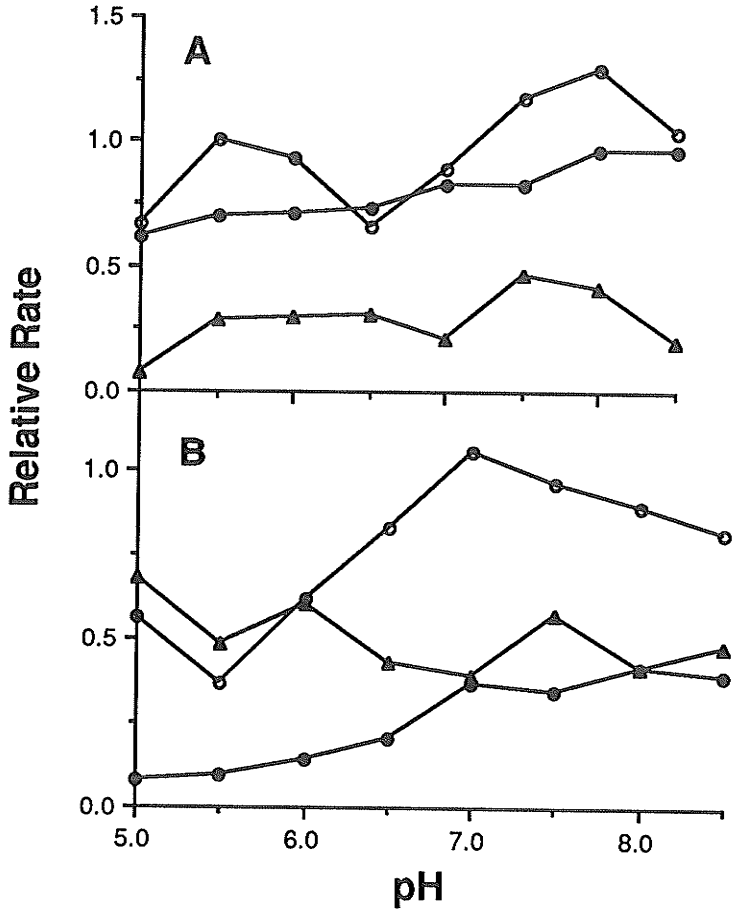

Figure 10. Effect of the inhibitors, DCCD (A) and fluoride (B) on glucose transport by Streptococcus mutans Ingbritt grown in continuous culture under Conditions B and D. Condition B cells untreated (●) and treated with 2-deoxyglucose (▲). Condition D cells (O). Relative rate = glucose transport activity of inhibitor cells/glucose transport of untreated cells.

glucose (Vadeboncoeur, 1984). Furthermore, these later studies indicated that the levels of the general, cytosolic proteins of the PTS, EI and HPr, were largely unaffected by the growth conditions resulting in the repression of the PTS. Studies with other Gram-positive bacteria have shown that PTS activity can also be down-regulated when a serine residue on the HPr, distinct from the histidine involved in phosphotransfer to carbohydrates, is phosphorylated (Reizer et al., 1989a). Lodge and Jacobson (1988) have reported the loss of a covalently-bound phosphate on HPr associated with shifting S. mutans GS5 from growth in excess glucose to glucose-limited conditions. Thus, it appears S. mutans employs more than one strategy to restrict PTS activity.

The presence of non-PTS glucose transport has been indicated for some time by continuous culture studies demonstrating that observed glucose-PTS activity is not commensurate with glucose utilization in S. mutans under PTS-repressed conditions (Ellwood et al., 1979; Hamilton et al., 1979; Hamilton and St. Martin, 1982). Unlike the PTS, where PEP serves as the phosphoryl donor in the group translocation process, glucose transport via an active, non-PTS transport process could use ATP and glucokinase to generate glucose-6-phosphate (Hamilton, 1987). To test this theory, we measured glucose-6-phosphate produced by permeabilized cells in the presence of excess ATP or PEP. We selected growth conditions for S. mutans Ingbritt that produced cells with high glucose-PTS activity (Table 4, Condition A), one with intermediate

activity due to increased medium glucose (Condition B) and two conditions where the glucose-PTS was strongly repressed by a high growth rate (Condition C) or by low pH (Condition D). The ratio of glucose phosphate produced by decryptified cells with excess PEP versus ATP decreased as glucose-PTS activity decreased from Condition A to D (Table 4) indicating that ATP became the preferred phosphoryl donor as the PTS was increasingly repressed.

The phosphorylation activity measured in Condition B cells was much higher than that reported individually for any of the cell types tested undoubtedly reflecting in part, the higher glucose concentration in the medium. Glucokinase has been reported to increase two-fold as cells are switched from PTS-optimal to PTS-repressed conditions with accompanying changes in the affinity of the enzyme for glucose (Hamilton, 1984). Thus, the high phosphorylation activity may be due to a particularly active glucokinase in these cells or to an increased synthesis of the enzyme. The high activity seen in the presence of PEP is more difficult to explain. However, it must be recalled that, unlike glucose phosphorylation which is the product of a single enzyme, PEP phosphorylation involves the participation of several components, HPr, EI, EII and, in some cases EIII, all of which are subject to regulation (Reizer et al., 1989). Thus, the individual activity of these components will contribute to the final PEP phosphorylation activity and it is conceivable that the components of the PTS may adapt to the increased glucose in

the medium to stimulate the glycolytic pathway (Marsh and Keevil, 1986).

The intracellular concentration of glucose also increased with PTS repression (Table 5) which would be expected if the cells turned to an active glucose transport system that translocated glucose in an unmodified form. If the PTS were the sole mechanism of transport for glucose in S. mutans, it would be expected that the intracellular concentration of glucose would be low, as via the PTS, glucose enters the cells as glucose-6-phosphate. This is the case in Condition A cells which have high PTS activity and transport glucose predominantly through the PTS (Table 4). With these cells, the intracellular glucose-6-phosphate concentration was four times the internal glucose concentration and three times the medium glucose concentration. In Condition B cells, which have intermediate PTS activity due to substrate-dependent repression, glucose and glucose-6-phosphate are present at comparable concentrations and twice the medium glucose concentration. With Condition D cells possessing the lowest PTS activity, glucose-6-phosphate is present at the same concentration as glucose in the medium and intracellular glucose is present at 10 times the medium concentration. The most striking feature of this experiment is the increasing intracellular glucose concentration seen with increasing PTS repression, which can be best explained by the presence of a second non-PTS process transporting glucose into the cell without chemical modification. The non-PTS system is an

active transport mechanism because the intracellular glucose concentration in the highly PTS-repressed Condition D cells was 10 times the medium concentration entering the chemostat. In fact, since the Condition D cultures were essentially 'glucose-limited' with a glucose concentration in the spent culture at 0.25 mM, the intracellular glucose concentration was actually more than 1600-fold higher than that in the culture medium.

The observed rates of glucose transport measured over a pH range from pH 5.0 to 8.5 differed between cells grown under the 4 conditions (Figure 5). Cells with the highest PTS activity (Condition A) exhibited the highest rates of glucose transport (175 nmol/mg per min at pH 7.0), while cells with one third the glucose-EII PTS (Condition B) of PTS optimal cells had glucose uptake rates reduced to one third (57 nmol/mg per min at pH 7.0) and cells grown in PTS-repressed conditions had the lowest rates (Condition C and D). Cells with high PTS activity were also very sensitive to acid pH with the rate of glucose transport inhibited 50% at pH 5.0 (Figure 5). When grown at pH 5.0, S. mutans is devoid of any PTS activity (Vadeboncoeur et al., 1991), and as several mechanisms may be used to restrict PTS activity including ones that do not rely on growth adaptation or even de novo protein synthesis (Lodge and Jacobson, 1988, Reizer et al., 1989a), the observed inhibition is not unexpected. Cells with intermediate PTS activity were largely unaffected by the assay pH. Glucose transport in cells that relied on

alternative glucose transport, as indicated by the PEP:ATP ratios of 0.59 and 0.33 were strongly inhibited by shifts in assay pH away from neutrality (Figure 5). For example, transport was reduced 60% at pH 5.0 and 50% at pH 8.5 for Condition C cells and 40% at pH 5.0 and 15% at pH 8.5 for cells from Condition D. These results alone are ambiguous when applied to the question of one versus two glucose transport systems in S. mutans. It could be argued that the reduced glucose transport seen in Condition C and D cells is due solely to the remaining, albeit repressed, activity of the PTS and that the sensitivity to extreme pH reflects the reduced energy available for pH homeostasis. Alternatively, it could be suggested, as previously reported by Ellwood and Hamilton (1978), that the very low PTS activity seen in these cells is not sufficient to account for the observed glucose uptake. This latter argument is bolstered by the increasing use of ATP as a phosphate donor to glucose in permeabilized cells grown in Conditions C and D. In addition, the sensitivity of glucose transport to pH in Condition D cells was unexpected because they were grown at acidic pH and previous data (Hamilton and Buckley, 1991) indicate that the cells have adapted to growth at low pH. If the sensitivity was not due to pH homeostasis, then a case can be made for the vulnerability of the non-PTS transport system to acidic conditions.

Hamilton and St. Martin (1982) proposed a protonmotive force-coupled model of non-PTS glucose transport based on the

sensitivity of PTS-repressed cells to ionophores and other protonmotive force-disruptive agents. For the most part, these and subsequent studies did not examine glucose transport, but actually measured inhibitory effects on glycolytic rates which reflect both transport and metabolism of the glucose (Hamilton and St. Martin, 1982; Keevil et al., 1984; Hamilton, 1987). Without actual measurements of intracellular glucose accumulation, it would have been premature to interpret their results as demonstrating ionophore vulnerability of the first transport step, rather than any of the subsequent steps. In particular, the last step of glycolysis, which involves the excretion of acid end-products now believed to occur via a protonmotive force-linked process, called end-product efflux, could be affected by the ionophore treatment (Michels et al., 1979; ten Brink and Konings, 1986). For these reasons, further study on the effect of ionophores was required. By abolishing or reducing transmembrane gradients required for transport, metabolism could be limited by the lack of a primary substrate. Alternatively, a direct metabolic effect could indirectly impair glucose uptake regardless of the transport system by limiting PEP or ATP and increasing intracellular glucose concentrations forcing transport against a smaller glucose gradient. In acidic conditions, limitations on the protonmotive force would lead to acidification of the cytosol and the latter scenario might result. At higher pH values, cytosolic events are not reliant on ion gradients per se as

long as suitable ion environments are maintained (Kobayashi and Unemoto, 1980; Thibodeau and Marquis, 1983; Dashper and Reynolds, 1992), therefore, ionophore sensitivity of glucose uptake at pH 6.5 and above is likely due to disruption of gradients needed for transport and not due to interference with metabolism.

In this study, gramicidin S, a cyclic decapeptide, was shown to reduce glucose transport to a common low level in all cells regardless of assay pH or growth conditions (Figure 6). This fact can become somewhat obscured when reporting relative rates because control uptake rates between cells differ. Glycolysis was also abruptly curtailed by this agent (Table 6) and its ionophore properties were demonstrated by the dissipation of both $\Delta\psi$ and ΔpH in cells tested at pH 7.0 and 5.5 (Table 7) confirming earlier findings (Hamilton, 1987). The addition of gramicidin S to cells actively transporting glucose (Figure 7) resulted in the rapid expulsion of accumulated radiolabel to low levels comparable to those seen in cells pre-treated with gramicidin S (Figure 4). The precise mechanism of action of this compound has not been elucidated. However, these results suggest that gramicidin S interferes with both transport and metabolism by compromising the integrity of the cytoplasmic membrane. Gramicidin S causes the release of inorganic phosphate (Katchalski *et al.*, 1956), the loss of nucleotides from *S. pyogenes* and the lysis of protoplasts of *Micrococcus lysodeikticus* (Izumiya *et al.*, 1979). Katsu *et al.* (1986,

1988) demonstrated that gramicidin S treatment also resulted in similar increases in permeability of E. coli membranes, red blood cells and liposomes in a concentration-depedent fashion. These observations and the current results are consistent with disruption of membrane permeability. Contrary findings were reported by Dashper and Reynolds (1990) when they found that glycolysis was completely inhibited at pH 5.0, but the gramicidin effect was negligible in cells assayed at pH 7.0. However, they used gramicidin D, a linear polypeptide that generates channels in membranes making them permeable to protons and other monovalent ions (Wallace, 1990) rather than the gramicidin S used here.

Nigericin strongly inhibited glucose transport in cells with high glucose PTS activity grown in Conditions A and B with inhibition increasing at low pH such that transport was reduced by 96% and 86%, respectively (Figure 6). Inhibition was less for low PTS cells (Condition C and D), however, as less active transport was occurring in these cells, the activity following nigericin treatment was comparable in cells grown in Conditions A, B, C and D. The sensitivity of glucose uptake to nigericin was confirmed by the immediate cessation of glucose uptake following nigericin addition to cells at pH 6.0 (Figure 8). At pH levels above 6.5, nigericin inhibition was around 30% for all cells becoming, negligible in cells with the least PTS activity (Condition D) above pH 7.5. The pattern of inhibition of glycolytic rates was similar to that of glucose transport with complete

inhibition of glycolysis at pH 5.5 and less than one third reduction at pH 7.0 regardless of the cell growth conditions (Table 6).

Nigericin is an ionophore that results in the electroneutral exchange of ions across the membrane, exchanging a K^+ for a H^+ (Nicholls, 1982). The overall effect is usually dissipation of the ΔpH with concomitant reduction of the $\Delta\psi$, as is seen at pH 7.0 (Table 7). When assayed at pH 5.5, both gradients were completely dissipated, as could be predicted, since the greater extracellular proton concentration in the acidic environment would result in greater exchange and, therefore, more K^+ efflux. At neutral pH, the reduction in glucose transport was accompanied by a reduction in glycolytic activity. As stated previously, disruption of gradients would not effect metabolism significantly at pH 7.0, therefore, the results suggest that the primary effect of nigericin is on transport.

Unlike nigericin, valinomycin inhibited glycolysis more than glucose uptake at pH 5.5 (66% and 100% inhibition of glycolysis <u>versus</u> 43% and 23% inhibition of glucose uptake) for Condition B and D cells. At neutral pH, both uptake and glycolysis were only slightly affected. This, coupled with the fact that valinomycin did not instantly effect glucose uptake in time course experiments (Figure 8), suggested that the immediate effect of valinomycin is on metabolism and not transport. Valinomycin is an ionophore that catalyzes the transport of monovalent ions with the exception of protons

(Nicholls, 1982) collapsing the electrochemical gradient ($\Delta\psi$) of cells while leaving the pH gradient intact. Valinomycin has little effect on gradients in cells assayed at pH 7.0, as previously reported (Hamilton, 1991), although, both gradients were reduced significantly at pH 5.5. Our results indicate greater sensitivity of glycolysis than transport to valinomycin suggesting the primary effect of valinomycin is on glucose metabolism and not transport.

TCS inhibited glucose uptake in a pH-dependent fashion with maximal inhibition at pH 5.0. In time-course experiments, glucose uptake was immediately stopped following addition of TCS (data not shown) suggesting that direct repression of transport was occurring. Following TCS addition, glycolysis was completely inhibited at acidic pH and mildly affected at neutral pH (Table 6). TCS sharply reduced ΔpH at both pH 7.0 and 5.5 and also reduced $\Delta\psi$ 40% at pH 5.5. Comparable inhibition of glycolysis and glucose transport is seen following treatment with TCS, making it difficult to assign a primary target for TCS effects and also to apply these results to a protonmotive force-driven model of glucose transport. The lack of effect at neutral and higher pH does not argue for or against this model since under these conditions the pH gradient is only a minor component of the protonmotive force.

Inhibition by CCCP mirrored that of TCS for glycolysis (Table 6) but was less extreme for glucose transport (data not shown). CCCP had little effect on ion gradients at

neutral pH, but reduced ΔpH 62% and completely dissipated $\Delta\psi$ at pH 5.5. The limited effect of CCCP on glucose uptake, glycolysis and pH gradients compared to earlier results (Hamilton and St. Martin, 1982), may be a reflection of the CCCP concentration. However, like TCS, it is impossible to determine whether CCCP treatment affects glucose transport or metabolism.

Previously, the sensitivity of glycolysis in S. mutans to DCCD has been used as evidence for protonmotive force-driven glucose transport (Hamilton and St. Martin, 1982). In addition, Keevil et al. (1986) have demonstrated DCCD inhibition of the transport of 6-deoxyglucose, a glucose analogue that is not transported by the PTS. In this study, the effect of DCCD on glucose transport was limited, with inhibition never exceeding 40% and often much less, if any inhibition occurred at all (Figure 7). DCCD specifically interacts with the H^+/ATPase which is active in pH regulation and energy conservation (Jones, 1986; Booth, 1987,). Previously, we have reported an increase in H^+/ATPase activity in cells grown at low pH (Hamilton and Buckley, 1991), thus, it was reasonable to expect cells grown at pH 5.5 in Condition D to demonstrate the greatest sensitivity to this agent. However, this was not the case as cells grown at pH 7.0 showed comparable DCCD sensitivity of glucose transport (Figure 7). Glycolytic activity in cells grown at pH 5.5 (Condition D) was very sensitive to this compound with 47% and 79% inhibition when assayed at pH 7.0 and 5.5,

respectively. Condition B cells, containing significant PTS activity, were unaffected at neutral pH but inhibited 63% at pH 5.5 (Table 6). This is similar to the increased DCCD sensitivity in the PTS-defective mutant S. mutans DR0001/6 compared to the wild-type parent (Hamilton and St. Martin, 1982). In both cases, the sensitivity of the glycolytic pathway in cells grown at neutral pH to DCCD, but assayed at acid pH, likely reflects the acidification of the cell interior since the cells were not adapted to this pH. The effect on the condition D cells, which were adapted to growth at low pH (Hamilton, 1991), is not likely due solely to internal acidification since cells assayed at neutral pH were also severely inhibited. Hamilton and St. Martin (1982) suggested that the cells with little PTS were reliant on H^+/ATPase for protonmotive force generation to drive non-PTS glucose transport, however, the minimal inhibition of glucose transport seen in this study following DCCD treatment at neutral pH, does not support this proposal.

Fluoride inhibition of glycolysis by S. mutans DR0001/6 has been used to suggest an energy requirement for non-PTS transport (Hamilton, 1987). Fluoride mediates a myriad effects on cells (reviewed by Bowden and Hamilton, 1988; Hamilton, 1990) including inhibition of enolase thereby blocking glycolysis, reducing the formation of PEP and inhibiting the PTS. Acidification of the cell interior occurs as fluoride readily translocates across the cytoplasmic membrane at acid pH as HF, which then dissociates

liberating protons inside the cell (Whitford et al., 1977; Kashket and Kashket, 1985). The F^- released in this process inhibits H^+/ATPase which is essential for maintenance of pH gradients (Marquis,1977, Sutton et al., 1987). Electrical potentials may also be affected as fluoride treatment has been shown to lead to expulsion of K^+ and inorganic phosphate (Luoma, 1980). The 'fluoride-pH effect' denotes the pH dependence of fluoride uptake whereby with increasing external pH, less fluoride accumulates in cells and fewer fluoride-mediated effects are observed.

Glucose uptake in cells from all four conditions was strongly inhibited by fluoride at pH below 6.5 (Figure 7). Above pH 6.5, inhibition declined but was maintained at 40-60% in all the cells grown at pH 7.0 (Conditions A, B and C) but declined to less than 20% in Condition D cells. Fluoride abolished protonmotive force and glycolysis at pH 5.5 but had little effect on cells at neutral pH (Tables 5 and 6). Because glycolysis in not inhibited at neutral pH, the inhibition in glucose transport seen in PTS active cells cannot be attributed to depletion in PEP stores but appears to be more direct. The unidentified target must be very sensitive as the low levels of fluoride that accumulate in the cells at and above neutral pH reduced transport by over 50%. Condition C and Condition D cells had the least glucose PTS activity, were most likely to have non-PTS activity and were very sensitive to fluoride even at pH above 6.5.

Cells grown under Condition B and possessing one third the PTS activity of cells grown under optimal PTS-conditions (Table 1), were treated with 2-deoxyglucose to remove PTS activity by depleting the 'PEP pool' (Thompson and Chassy, 1982). Following 2-deoxyglucose treatment, ATP became the phosphoryl donor of choice for glucose in permeabilized cells, unlike untreated cells where PEP was the dominant donor (Table 4). In addition, glucose transport was much lower than that of untreated B cells and was comparable to cells grown in condition D, which had very low levels of glucose-PTS activity (Tables 4 and 7, Figure 9).

Unlike any of the other cells tested here, 2-deoxyglucose-treated cells were very sensitive to DCCD with 93% inhibition of glucose transport at pH 5.0 declining to 54% at pH 7.5 (Figure 10). Along with PEP depletion, it has been reported that ATP levels are reduced by 60% in S. lactis following 2-deoxyglucose treatment (Thompson and Chassy, 1983). The combination of reduced ATP content and DCCD-mediated inhibition of the H^+/ATPase may interfere with protonmotive force generation required for transport, or the H^+/ATPase may be playing a secondary, as yet, undefined role whose requirement is enhanced in these cells by the energy depletion resulting from 2-deoxyglucose-treatment.

From pH 5.0 to 6.5, the actual rates of glucose transport were indistinguishable between Condition B cells treated or untreated with 2-deoxyglucose and then fluoride. Above pH 6.5 following fluoride addition, glucose uptake in untreated

Condition B cells recovered to higher levels than those found in 2-deoxyglucose-treated Condition B cells although relative rates were comparable (Figure 9). This suggests that fluoride has a similar effect on cells whether treated with 2-deoxyglucose or not. However, as the increased sensitivity to DCCD indicates a prominent role for the H^+/ATPase in glucose transport in these cells, fluoride inhibition may also be acting through this enzyme.

The studies reported here support the presence of non-PTS transport in S. mutans as first indicated by Hamilton and Ellwood (1978). Although not characterized, non-PTS transport would differ from PTS transport in that unmodified glucose presumably enters the cell whereupon it is phosphorylated by ATP via glucokinase. We further propose that this system is constitutive based on the observation that cells with growth-repressed PTS (Conditions C and D) have comparable activity to 2-deoxyglucose-treated cells although they do not respond in the same manner to various inhibitors, particularly DCCD.

Hamilton and St. Martin (1982) presented a model for non-PTS transport in S. mutans coupled to protonmotive force based on the sensitivity of glucose transport or metabolism to agents known to perturb protonmotive force generation. In the present activity study, comparisons were made of ionophore sensitivity of glucose uptake and metabolism between cells with various levels of PTS activity. We found that valinomycin sensitivity did not support a protonmotive

force-associated model of transport since its primary effect is on metabolism and not transport. The effects of gramicidin S are difficult to interpret without knowing the extent of membrane disruption following its addition. The use of the remaining agents may support the participation of protonmotive force in glucose transport. Some caution must be exercised since these agents clearly affect metabolism also, so it is difficult to discriminate between direct transport effects or secondary effects associated with metabolism. The fluoride and DCCD sensitivity could indicate a role of the H^+/ATPase in the process, however, whether it is energy conservation or protonmotive force generation or related to pH homeostasis in the cell cannot be answered from this study. The situation is further complicated by the fact that the glucose-PTS is also sensitive to many of the compounds used in this study. It is well documented that protonmotive force modulates the PTS in E. coli (Postma and Lengeler, 1985) and the Gram-positive organism Brochothrix thermosphacta (Singh et al., 1985). Ultimately, the only way to prove conclusively the PMF-associated model is to demonstrate glucose uptake in response to artificially-generated protonmotive force of known magnitude.

Chapter 3

Search for Protonmotive Force-Coupled Glucose Transport in *Streptococcus mutans* Ingbritt

Introduction

Previous attempts to identify the protonmotive force-coupled model of glucose transport in *Streptococcus mutans* have relied on the sensitivity of glucose transport and metabolism to various ionophores and inhibitors of the protonmotive force (PMF). The difficulty in interpreting data obtained from these types of studies is described in the last Chapter and centers on the inability to assign the primary inhibitory effect of agents to transport or metabolism since disruption of one will often affect the other. This problem is not new. However, it is recognized that there are two types of experiments which furnish direct proof of PMF-coupled transport systems.

West (1970) was the first to demonstrate direct coupling of the PMF with transport looking at lactose transport in *E. coli*. Cells were treated with glycolytic inhibitors to exclude proton extrusion with metabolic end-products and respiration was prevented by maintaining stringently anaerobic conditions. The permeant anion thiocyanate was added to inhibit formation of a membrane potential, positive inside, associated with proton influx. When the cells were placed in a weakly buffered medium with a lactose analogue

β-galactoside, the pH of the medium increased concurrently with transport. The alkalization was not observed in mutants deficient in the carrier protein or in wild-type E. coli with a chemically-inactivated lactose carrier. Thus, proton-lactose symport was demonstrated by the alkanization of the medium concurrent with transport of substrate.

Not long after this, Kashket and Wilson (1972) used a different approach to demonstrate protonmotive force-coupling of galactoside transport in Streptococcus lactis (Lactococcus lactis). They induced an electrochemical gradient in cells by first loading them with potassium, then adding the ionophore valinomycin which induced the exit of potassium ions creating a large transmembrane electrical gradient ($\Delta\psi$) (interior negative). Accumulation of galactosides was associated with the electrochemical gradient as well as the acidification of the cell interior indicating proton influx. In a subsequent experiment, galactoside accumulation was shown to be related to imposed pH gradients by resuspending cells in buffers at various pH values (Kashket and Wilson, 1973). A 22-fold concentration of galactoside was observed when cells were exposed to buffer of pH 6.0, while very little influx of galactoside was detected at pH 8.0. Galactoside accumulation was proportional to the size of the applied protonmotive force whether ΔpH or $\Delta\psi$ was the major contributing component (Kashket and Wilson, 1973).

In 1979, Ellwood and co-workers (1979) performed a variation of the West experiment on S. mutans Ingbritt.

Cells grown under PTS repressive conditions (pH= 7.0, D= 0.4 h^{-1} with excess glucose) were harvested and resuspended in either saline or a high potassium, lightly buffered solution. The pH was then recorded in cells immediately following the addition of a range of sugars including glucose or 2-deoxyglucose. No alkalization of the external medium was detected indicating no proton symport with sugar transport. The results of this preliminary experiment was not conclusive for several reasons. Firstly, although the PTS in S. mutans was repressed under these growth conditions, it was still active, albeit at a relatively low level, and was capable of transporting glucose and 2-deoxyglucose (Hamilton et al., 1991). If the PTS were the dominant system, then no alkanization would be expected and any transport by the PTS would reduce the potential proton influx. Secondly, although all endogenous glycolytic activity was exhausted prior to the sugar addition, no metabolic inhibitors were included in their experiments. Thus, it was possible that the rapid catabolism of glucose to acid end-products would mask the potential alkalization of the medium. Thirdly, the concentration of cells used in this experiment might not have been sufficient to produce a detectable change in the external pH. In similar experiments conducted by Kashket and Wilson (1973) using twice the bacterial concentration used by Ellwood and co-workers, an increase of approximately 0.03 pH units was detected following exposure to thiomethyl-β-galactoside in S. lactis.

We did not attempt to repeat the experiment as described above but decided to look at glucose transport in response to imposed protonmotive force. By manipulation of the electrochemical and pH gradients, transport activity can be clearly demonstrated and raised to greater levels than may be seen in standard culture conditions. In addition, extensive use was made of the glucose analogue, 6-deoxyglucose, since it is not transported by the PTS (Schachtele and Mayo, 1973). Therefore, treatment to inhibit the PTS was unnecessary.

Materials and Methods

Bacterial strain and growth conditions

Cells of S. mutans Ingbritt were grown in continuous culture in Condition B (50 mM glucose at D=0.1 h^{-1}, pH= 7.0) as described in Chapter 2.

H^3-6-deoxyglucose uptake in response to artificially-generated gradients

pH gradient (ΔpH): The uptake of 6-deoxyglucose by S. mutans Ingbritt was determined in response to artificially-generated pH or electrochemical gradients. The pH gradients were generated by two different techniques, acid-pulsing or chemical dilution (CD). To generate a large, transient ΔpH through acid pulsing, a modification of the technique of Kashket and Wilson (1973) was used. Cells were washed and incubated anaerobically at 37 °C in 150 mM KCl and 5 mM $MgCl_2$

at a cell concentration of 20 mg/ml. Cells were diluted 1:5 in a pH stat and small aliquots of 100 mM KOH + 50 mM KCl were added until the pH was stable at pH 8.5 for at least 5 minutes with the titration monitored on a recorder (CanLab, model 255 MM) attached to the pH stat. When the pH was stable, H^3-6-deoxyglucose (1.0 mM final concentration, 750 dpm/nmol) was added and an acid pulse, sufficient to drop the pH by approximately 4 pH units, was delivered as a single aliquot of 100 mM HCl + 50 mM KCl.

At one minute, duplicate 500 µl samples were removed and the cells separated from the aqueous phase by centrifugation through a 50:50 silicone oil mixture of Dow Corning fluids 550 and 556 (Dow Corning Corp., Midland, Michigan). The aqueous and oil layers were removed and the tubes cut just above the cell pellet and this portion added to scintillation vials. A 200 µl aliquot of 100 mM NaOH was added to the vials to ensure complete solubilization of the pellets. This amount of base did not interfere with radioactive determinations. Aquasol (5 ml) was added and the radioactivity measured in a liquid scintillation counter. Aliquots (50 µl) were removed from the remaining reaction mixture for determination of the total radioactivity.

The counts derived in this process represented the radioactivity associated with the pellet, including 6-deoxyglucose inside the cells and that trapped in the extracellular space and which did not represent transport. The internal radioactivity was calculated by subtracting the

label in the external water volume of the sample from the total radioactivity in the cell pellet. The external water volume was determined as described in Chapter 2. These values were converted to nmoles of internal 6-deoxyglucose per microliter using the specific activity of our isotope and the internal water volume of the samples. The ratio of the intracellular versus extracellular 6-deoxyglucose concentration was calculated by dividing the internal concentration by the concentration of 6-deoxyglucose in the cell mixture.

The uptake of 6-deoxyglucose was also measured in response to a pH gradient generated by a chemical dilution method which creates a H^+/acetate diffusion potential (Konings et al., 1986, Poolman et al., 1987). Cells were harvested from the chemostat overflow, washed twice in 100 mM K/acetate, 20 mM K/PIPES (pH 6.0) containing 5 mM $MgCl_2$ (KA buffer), resuspended at 10 mg/ml in the buffer containing 2 µM valinomycin and equilibrated on ice for 2 hours to enhance ΔpH formation (Konings et al., 1986). Cells were washed and resuspended at 50 mg/ml in KA buffer and then diluted 1:50 into one of three buffers containing 1 mM H^3-6-deoxyglucose: (1) 120 mM K/PIPES buffer, 5 mM $MgCl_2$ (pH 6.0) (KP buffer) for maximum ΔpH generation and two controls: (2) KP buffer containing 2 µM valinomycin and 2 µM nigericin to interfere with pH gradient formation, and (3) KA buffer. At three minutes, 500 µl samples were removed and the cells separated from the aqueous phase by centrifugation through a a 35:65

(vol/vol) ratio of fluid 550 to fluid 556 for conditions 1 and 2, and a 50:50 (vol/vol) mixture for condition 3 samples. Aliquots (50 µl) were removed and directly counted to determine the total radioactivity of the cell mixtures and the intracellular concentration of H^3-6-deoxyglucose was determined as described above.

Electrical ($\Delta\psi$) gradient: the uptake of 6-deoxyglucose was determined during the imposition of an artificial $\Delta\psi$ generated by the chemical dilution method. For this, chemostat cells were washed in 120 mM KP buffer and resuspended at 10 mg/ml in the same buffer containing 2 µM valinomycin and equilibrated on ice for 2 hours. Following the incubation, the cells were washed and resuspended in KP buffer at 50 mg/ml and diluted (1:50) into the appropriate buffer containing 1 mM H^3-6-deoxyglucose: (1) 120 mM Na/PIPES buffer containing 5 mM $MgCl_2$ (pH 6.0) (NP buffer) for maximal $\Delta\psi$ generation, and two controls: (2) NP buffer with 2 µM gramicidin to stop $\Delta\psi$ formation, and (3) KP buffer. At 1 minute, duplicate 500 µl samples were removed and the cells separated from the aqueous phase by centrifugation through a a 35:65 (vol/vol) ratio of fluid 550 to fluid 556 and the pellets treated as described for the acid-pulse samples. Aliquots were removed and directly counted to determine the total radioactivity of the cell mixtures. The experiment was repeated 5 times and ratio of internal:external 6-deoxyglucose was calculated as described above.

Determination of ΔpH and $\Delta\psi$.

When both the acid pulse technique and chemical dilution technique were employed to test for PMF coupled sugar transport, the actual ΔpH and $\Delta\psi$ generated were measured under the same conditions as those of the uptake assays to confirm that gradients were actually generated with the two methods. The gradients were determined as described in Chapter 2. Correction for non-specific binding of the probes was made by subtracting the label in cells treated for 5 minutes with 5% butanol and 50 μM gramicidin ($\Delta\psi$), or with butanol alone (ΔpH).

The intracellular and extracellular water content of cells was determined as previously described in Chapter 2. The intracellular water volume for S. mutans Ingbritt under experimental conditions employed was 2.13 ± 0.21 μl/mg (dry weight) and the extracellular volume was 0.51 ± 0.11 μl/mg (dry weight).

Counterflow

To test for the presence of a glucose-specific carrier protein in S. mutans Ingbritt, counterflow experiments were conducted using 6-deoxyglucose, 2-deoxyglucose and glucose. Cells were collected from the chemostat overflow for 16 hours, washed twice in 50 mM Na/K phosphate (pH 7.0) containing 5 mM $MgCl_2$, 10 mM mercaptoethanol and 10 mM sodium fluoride (PMMF buffer), and incubated for 1 hour at 37° with 25 mM 2-deoxyglucose to deplete the intracellular 'PEP

pools'. Cells were subsequently washed twice, resuspended at 12 mg/ml and aliquots (1 ml) were removed incubated for 1 hour at 37° with 20 mM 6-deoxyglucose, 2-deoxyglucose or glucose, respectively, to 'load' cells. Following incubation, the mixture was centrifuged, the supernatant removed and the inner surfaces of each tube carefully dried. The cell pellets were rapidly resuspended in 6 ml of PMMF buffer containing either 1 mM H^3-6-deoxyglucose (750 dpm/nmol) for 6-deoxyglucose-loaded cells, 1 mM C^{14}-2-deoxyglucose (360 dpm/nmol) for 2-deoxyglucose-loaded cells, and 1 mM C^{14}-glucose (230 dpm/nmol) for glucose-loaded cells. Samples (1 ml) were removed periodically and rapidly filtered through 0.45 μM filters (Type HA, Millipore Corporation, Bedford, MA) and filters washed twice with 1 ml of pre-warmed buffer. The cellular radioactivity was determined by counting the filters in Aquasol. The concentration of the labelled sugar in the extracellular water space after filter washing was considered negligible. Controls consisted of cells treated as above without the 2-deoxyglucose pretreatment and the unlabelled sugar loading step. Results are expressed as the average of three separate runs of loaded cells and the average of two control experiments.

Kinetics of glucose uptake

To determine the kinetics of glucose transport into cells, 2-deoxyglucose pre-treated cells were washed and resuspended in PMMF buffer at 20 mg/ml and 300 µl diluted into tubes containing 2.7 ml of PMMF buffer and increasing concentrations of C^{14}-glucose (0.5 to 50 mM). Samples (500 µl) were removed at 0.2 minute intervals for the first minute and filtered as described above to determine the rate of glucose transport.

Chemicals

C^{14}-glucose was supplied by ICN Radiochemicals, Irvine, CA, and H^3-6-deoxyglucose and C^{14}-2-deoxyglucose were purchased from NEN Research Products, Montreal, Canada. All other chemicals were reagent grade.

Results

pH gradient generation and 6-deoxyglucose transport in response to ΔpH

The definitive demonstration of protonmotive force-coupled transport in S. mutans would be glucose transport in response to an imposed pH or electrochemical gradient, or both. Two different techniques were used to generate a pH gradient, the acid pulse and 'chemical dilution' techniques. With the acid pulse technique, we first determined the time course of pH gradient formation (Figure 11). Following an acid pulse of

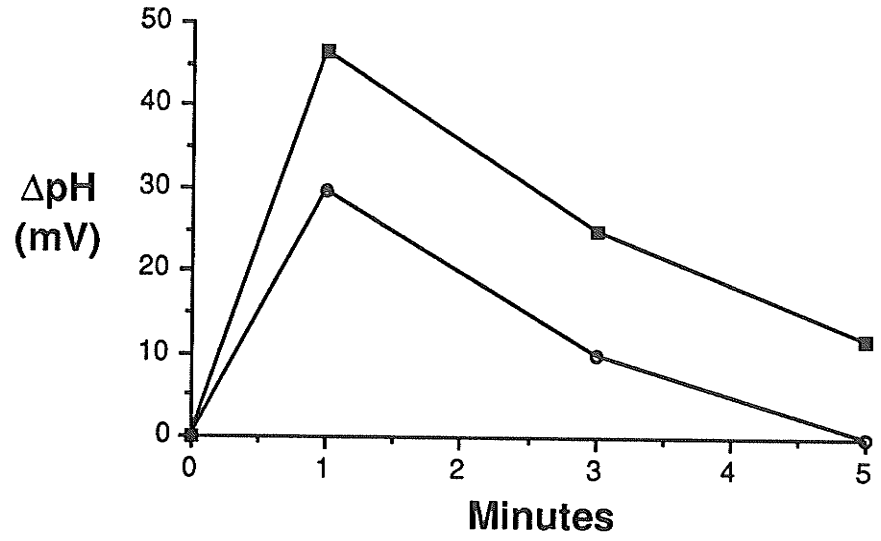

Figure 11. Time course of ΔpH formation in washed cells of Streptococcus mutans Ingbritt receiving acid pulses of 4.5 pH units (■) and 3.3 pH units (O).

4.5 pH units, a transient maximal pH gradient of 47 mV was detected at 1 minute which dissipated to 12 mV at 5 minutes. When a small acid pulse of 3.3 pH units was added, a peak of 30 mV occurred at 1 minute which completely dissipated by 5 minutes. In these experiments, the ionic environment was strictly maintained by the use of buffers consisting of combinations of protons and potassium ions equalling 150 mM, therefore, the electrochemical gradient would be constant regardless of the pH of the cells.

Figure 12 indicates the pH gradient generated in one minute following acid pulses of different sizes. No gradient was detected until the acid pulse exceeded 2.5 pH units, thus gradients were only formed when the acid pulse reduced the external pH below 6.5. With greater acid pulses, there was a linear relationship (r^2= 0.88) between the size of the acid pulse and the resulting pH gradient with a maximum gradient of 50 mV obtained with an acid pulse of 4.8 units.

Once it had been established that actual pH gradients could be generated, the uptake of 6-deoxyglucose was studied. Our results are reported as the ratio of intracellular versus extracellular 6-deoxyglucose concentration where a ratio of 1.0 represents equilibration of the analogue across the plasma membrane and a ratio greater than 1.0 would indicate active transport. Figure 13 plots the results of a variety of experiments and demonstrates the absence of a relationship between the size of the acid pulse and uptake of 6-deoxyglucose. The internal/external ratios of

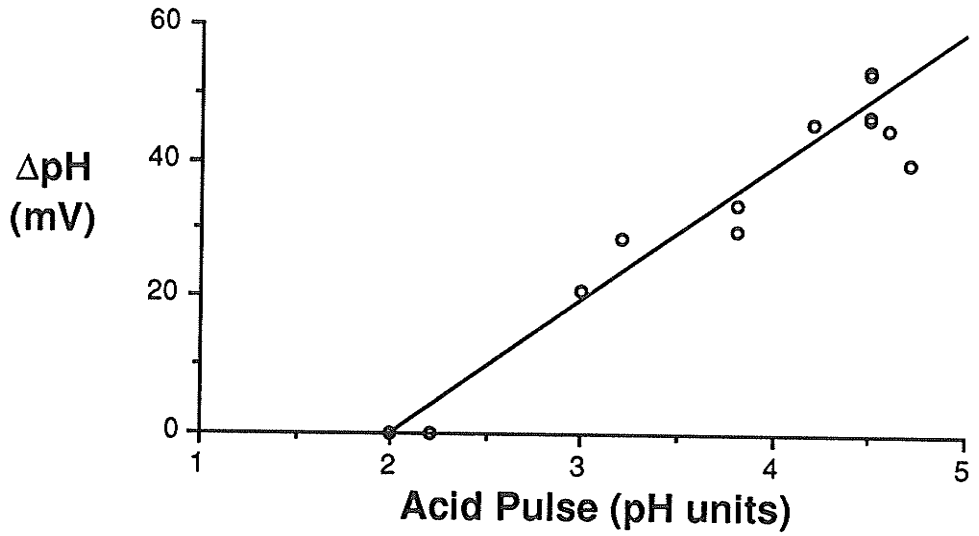

Figure 12. Relationship between the size of the acid pulse and the pH gradient generated by Streptococcus mutans Ingbritt.

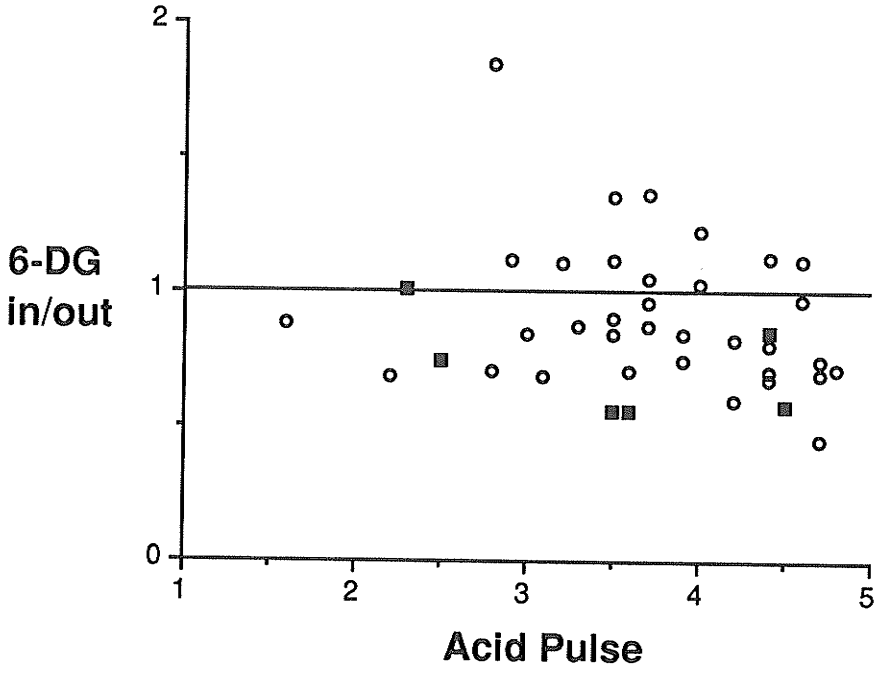

Figure 13. Relationship between the inside/outside concentration of 6-deoxyglucose and the acid pulse received by Streptococcus mutans Ingbritt. Untreated cells (O) and cells treated with the ionophores nigericin and gramicidin S (■).

6-deoxyglucose were clustered near 1.0 indicating equilibration of the analogue across the cell membrane. In cells treated with gramicidin and nigericin to permeabilize the membrane and inhibit gradient formation, 6-deoxyglucose ratios were comparable to untreated cells.

We were concerned that the exposure of the cells to extreme shifts in external pH with the acid pulse technique could possibly damage a non-PTS glucose transport system. As a consequence, the 'chemical dilution' technique was also employed to generate pH gradients. Cells were first loaded with acetate and then rapidly diluted into acetate-free buffer, generating a transient ΔpH (inside alkaline) since the acetate will exit the cell in the protonated form. When 6-deoxyglucose was included in the acetate-free diluting buffer, thereby creating a maximum ΔpH, no uptake of the analogue was detected but equilibration of 6-deoxyglucose across the membrane was evident by in/out ratios near 1.0 (Table 9). The controls, which included cells treated with the ionophores valinomycin and nigericin to interfere with ΔpH formation (Table 9, line 2) and those diluted into acetate buffer (chemical control)(Table 9, line 3), did not significantly deviate from the equilibration value of 1.0. The results agree with those we obtained from the acid pulse experiments and indicate no coupling between the pH gradient and non-PTS transport.

Table 9

Uptake of 6-deoxyglucose by Streptococcus mutans Ingbritt in response to imposed pH (ΔpH) and electrical ($\Delta\psi$) gradients generated by the chemical dilution technique.

		Protonmotive force (mV)			6-deoxyglucose uptake
		$-z\Delta pH$	$\Delta\psi$	Δp	
	ΔpH generated				
1	maximum ΔpH	26	0	26	1.08 ± 0.27[a]
2	val/nig control	0	0	0	1.21 ± 0.19
3	chemical control	0	3	3	0.86 ± 0.17
	$\Delta\psi$ generated				
4	maximum $\Delta\psi$	0	70	70	0.79 ± 0.14
5	gram control	0	0	0	0.86 ± 0.12
6	chemical control	0	0	0	0.81 ± 0.17

[a] Ratio of 6-deoxyglucose inside cells versus that outside the cells ± standard deviation.

6-deoxyglucose transport in response to $\Delta\psi$

The second component of the protonmotive force is the electrochemical gradient. If symport occurred between another cation and glucose, the ΔpH experiments would not have detected it. For this reason, we used a variation of the 'chemical dilution' technique to impose an electrochemical gradient across the cell membrane. Cells were pre-incubated in a high potassium buffer that resulted in potassium-loading of cells. Subsequently, they were diluted into potassium-free, high sodium buffer that resulted in the rapid movement of potassium out of the cells, however, as sodium was unable to traverse the plasma membrane, a transient electrochemical gradient (inside negative) was formed. As the proton content of the buffers was the same, no pH gradient would accompany the electrochemical gradient.

The time course of electrochemical gradient formation using this technique showed a maximal $\Delta\psi$ of 68 mV detected at one minute which rapidly decreased to a stable value of 30 mV at 5 minutes (Figure 14). When H^3-6-deoxyglucose was included in the diluting buffers, the in/out ratio of 6-deoxyglucose was 0.79 ± 0.14 and this value was not significantly different from the gramicidin control (eg. 0.86 ± 0.17)(Table 9, line 5) and the chemical control (0.81 ± 0.17)(Table 9, line 6). All these ratios approached 1.0 denoting equilibration rather than uptake, these results indicate that

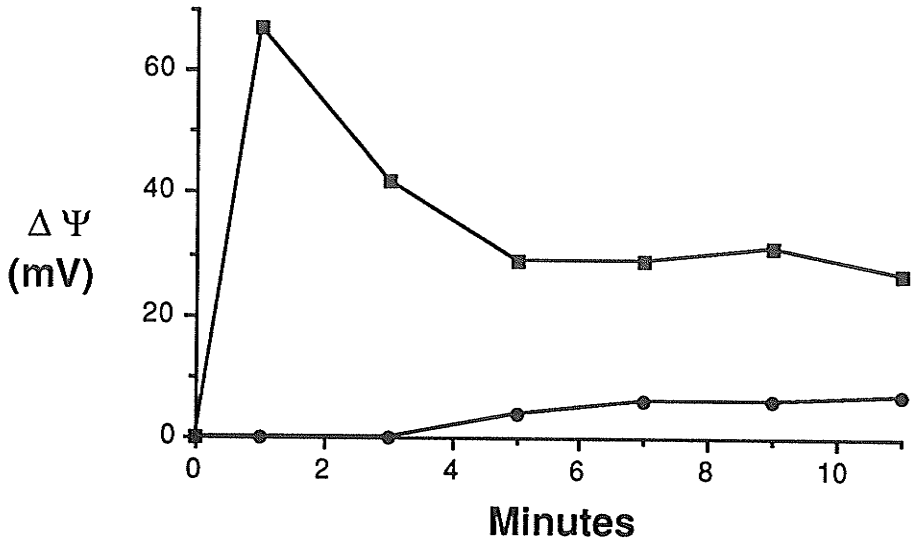

Figure 14. Time course of $\Delta\Psi$ formation in Streptococcus mutans Ingbritt using the chemical dilution technique. Cells diluted into NP buffer (■) and cells diluted into KA buffer (●).

the electrochemical gradient is not coupled to the active transport of glucose in S. mutans.

Counterflow

The results obtained with the above experiments involving the imposition of artificial pH and electrical gradients indicate that glucose transport in not coupled to protonmotive force. These experiments do not, however, address whether non-PTS transport can occur via an alternative mechanism such as facilitated diffusion through a membrane-bound carrier. The fact that 6-deoxyglucose equilibrates across the membrane within one minute suggests this could occur. Counterflow experiments are designed to demonstrate the presence of substrate-specific carrier molecules. For this, cells are loaded by incubation in a high concentration of an unlabelled substrate and then the external medium containing this excess substrate is removed. The loaded cells are then immediately resuspended in buffer containing a low concentration of the same substrate radiolabelled. Equilibration of substrate will occur across the cell membrane until substrate is present in equal concentrations on either side of the membrane. If this process is carrier-mediated, the unlabelled substrate is moved out of the cell and the labelled substrate is brought into the cell because, initially, labelled substrate is predominantly outside the cell and the unlabelled substrate is inside. Once inside the cell, any labelled substrate will

have to compete with unlabelled substrate for exit. Therefore, a transient concentration of labelled substrate is detected in the cells, which in time, dissipates with the exchange and equilibration of substrate molecules across the membrane. Conversely, if transport of the substrate occurs through pores or via diffusion through the membrane, no concentrative uptake of the labelled substrate is observed and rapid equilibration of labelled substrate is seen.

In the experiments with S. mutans Ingbritt, three separate substrates were used: glucose, 2-deoxyglucose and 6-deoxyglucose, and the cells were pretreated with 2-deoxyglucose to deplete 'PEP pools' and fluoride to prevent catabolism. As seen in Figure 15(A), cells loaded with 100 mM 6-deoxyglucose and then resuspended in buffer containing 1 mM H^3-6-deoxyglucose, did not exhibit concentrative uptake of the labelled 6-deoxyglucose, in fact, the in/out ratio of 6-deoxyglucose was less than 1.0 and comparable to unloaded control cells. Cells loaded with 2-deoxyglucose (Figure 15(B)), glucose (Figure 15(C)) gave similar results. Thus, it is apparent, a membrane carrier could not be demonstrated in these experiments.

Figure 16 indicates what occurs with extended incubation of glucose-loaded and unloaded cells with labelled glucose. Both cells demonstrate active transport indicating the incomplete inhibition of metabolism by 10 mM fluoride. The 'loaded' cells displayed greater glucose transport which is not surprising as they had been preincubated with high

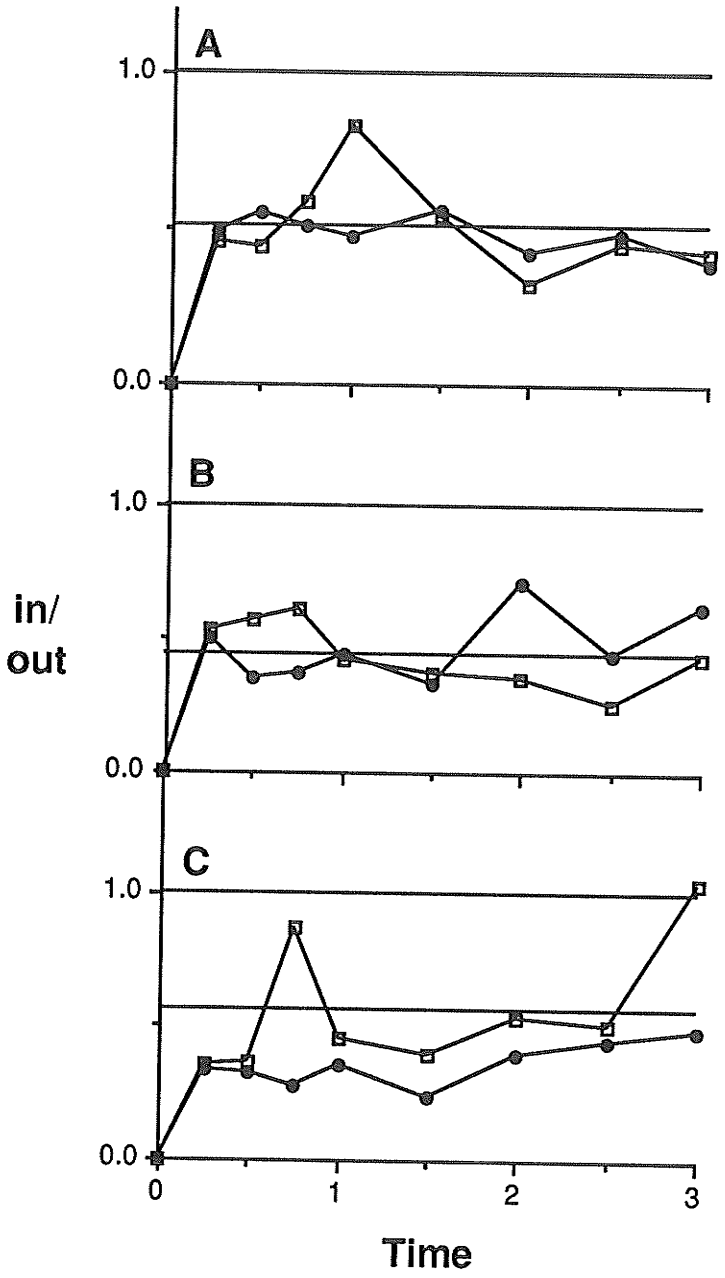

Figure 15. Counterflow experiment with Streptococcus mutans Ingbritt using 6-deoxyglucose (A), 2-deoxyglucose (B) and glucose (C) as substrate. Loaded cells (□) and unloaded (●) cells.

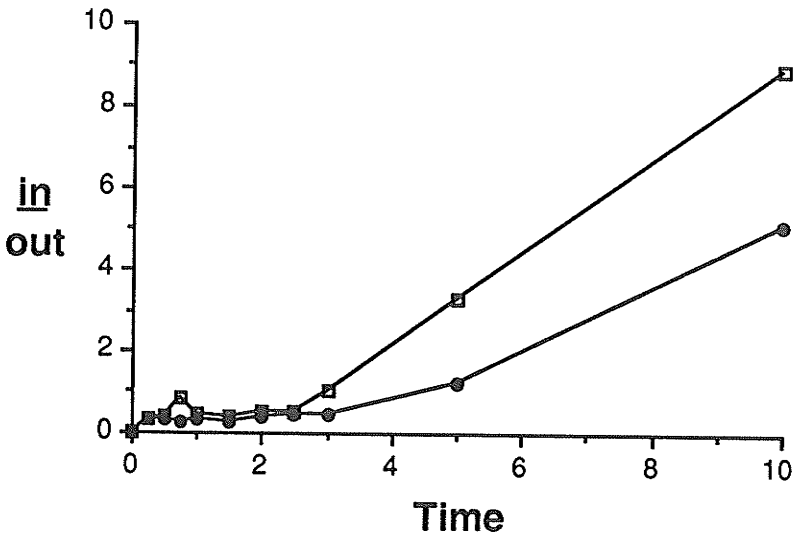

Figure 16. Glucose uptake by 2-deoxyglucose-treated Streptococcus mutans Ingbritt in the presence of 10 mM fluoride. Glucose-loaded cells (□) and unloaded cells (●).

concentrations of unlabelled glucose. If metabolism was not curtailed, any glucose transport systems would have become energized during the glucose loading incubation. The observed uptake was related to incomplete inhibition of metabolism allowing for active transport as none of the cells loaded or challenged with non-metabolizable substrate (2-deoxyglucose or 6-deoxyglucose) displayed active uptake over the same time period (data not shown).

Kinetics of glucose transport

The inability to demonstrate a carrier in the above experiment may have been due to the interference by fluoride with C^{14}-glucose transport and metabolism. Consequently, we examined the initial rate of C^{14}-glucose uptake by PEP-depleted cells of S. mutans Ingbritt incubated in the presence of 10 mM fluoride at varying concentrations of the substrate glucose. The data were plotted in a double reciprocal Lineweaver-Burk plot. As shown in Figure 17, a single transport system with a K_m of 15 μM was detected (r^2= 0.986).

Discussion

Protonmotive force-coupled bacterial transport, first suggested by Mitchell (1962), has now been reported for the uptake of carbohydrates by a variety of bacteria. Such transport systems include: β-galactosides by S. lactis 7962

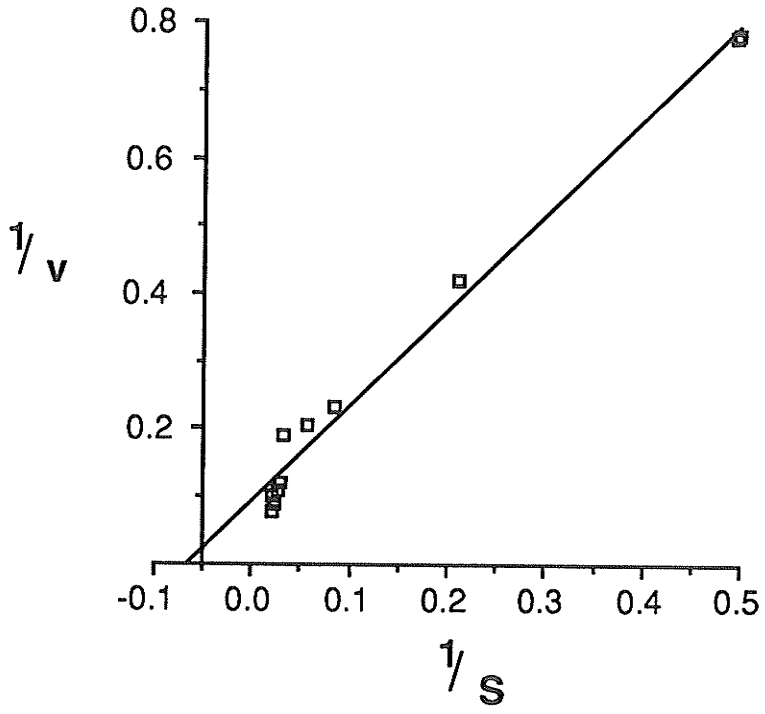

Figure 17. Lineweaver-Burk plot of glucose transport by 2-deoxyglucose-treated Streptococcus mutans Ingbritt exposed to various concentrations of glucose in the presence of 10 mM fluoride.

(Kashket and Wilson, 1973), galactose and gluconate by Clostridium pasteurianum (Booth and Mitchell, 1986) and glucose and galactose by the yeast, Kluyveromyces marxianus (van den Broek et al., 1986). E. coli can transport fucose, rhamnose, lactose, xylose, arabinose and galactose through distinct proton/sugar symporters (Henderson, 1990). Direct proof for proton-carbohydrate symport, with a stoichiometry of 1:1, is based on the demonstration of proton influx concurrent with carbohydrate accumulation or more commonly, by the transport of carbohydrate in direct response to an artificially-imposed electrochemical or pH gradient (Rosen and Kashket, 1978).

The presence of a second glucose transport system in S. mutans has been indicated for some time, based primarily on continuous culture studies (reviewed by Hamilton, 1987). These studies showed that glucose transport activity was higher than could be accounted for by the glucose PTS when S. mutans was grown under a variety of conditions that led to PTS repression. As a consequence, a model for protonmotive force-coupled glucose transport was proposed based on the ionophore sensitivity of glucose transport and metabolism (Hamilton and St. Martin, 1982), although direct evidence for the model was largely lacking. A single, preliminary study reported no proton-glucose symport in S. mutans (Ellwood et al., 1979), however, since metabolic inhibitors were not included in the study and the cells contained significant PTS

activity, it is possible that the presence of the transport system was obscured.

Keevil *et al*. (1986) circumvented the problem of residual PTS activity in assaying non-PTS glucose transport through the use of the glucose analogue, 6-deoxyglucose, which is neither transported via the PTS or metabolized by *S. mutans* (Schachtele and Mayo, 1973). However, their experiments in many ways resembled previous work where the abolition of transport following a variety of protonmotive force-disruptive treatments was taken as direct proof of protonmotive force-coupling (Hamilton and St. Martin, 1982; Keevil *et al*., 1984; Hamilton, 1986). Measurements of protonmotive force were not made in either control cells, or cells treated with a variety of metabolic inhibitors and ionophores. Therefore, an actual relationship between protonmotive force and transport was not shown. They found *S. mutans* could accumulate 6-deoxyglucose only 4-fold and this was only after correcting for significant non-specific binding. In our experiments and others (Dashper and Reynolds, 1990), there was no non-specific binding which has lead to the suggestion that the preparation of radiolabelled 6-deoxyglucose used by Keevil and co-workers was contaminated with other sugars that may have accounted for the low transport activity observed (Dashper and Reynolds, 1990).

We used various techniques to impose protonmotive force in cells by manipulating one of its components, the pH or electrochemical gradients. When manipulating pH gradients,

potassium was maintained at high levels so that concomitant $\Delta\psi$ changes would not occur. By maintaining a constant pH when imposing electrical gradients, we were able to ensure no ΔpH fluctuations during the experiments. Time course trials (Figures 11 and 14) indicated when the applied protonmotive force was at its maximum, therefore, cells exposed to labelled 6-deoxyglucose were sampled at optimum times. Using the acid pulse technique, the size of the acid pulse determined the size of the pH gradient formed across the cellular membrane (Figure 12). Therefore, if ΔpH fuelled transport of the analogue, a linear relationship between the acid pulse and the in/out ratio of 6-deoxyglucose should have been seen. Such a relationship was shown to hold for protonmotive force-coupled transport of galactosides by S. lactis (Kashket and Wilson, 1973). However in S. mutans Ingbritt, no relationship was seen between the size of the acid pulse and 6-deoxyglucose transport. Therefore, the pH gradient was not coupled to the accumulation of the glucose analogue (Figure 13). Indeed, the 6-deoxyglucose concentration inside cells was comparable to control cells pretreated with ionophores to negate pH gradient formation in cells receiving an acid pulse and comparable results were obtained with the 'chemical dilution' technique.

Using the formula $\Delta p = 59 \log \{[A]_{in}/[A]_{out}\}$ (Konings et al., 1987), it is possible to calculate the in/out ratio of 6-deoxyglucose of cells that should have resulted from pH gradients of the sizes generated by either the acid pulse or

chemical dilution techniques. Assuming a 6-deoxyglucose-proton symport was functioning with a proton/substrate ratio of 1.0, the largest pH gradient (50 mV) generated by the acid pulse method had the potential to fuel a 7-fold accumulation of 6-deoxyglucose inside the cell. The smaller gradient of 26 mV seen with manipulations of the pH gradient using the 'chemical dilution' technique should still have resulted in 3-fold concentration of 6-deoxyglucose. In both cases, the glucose analogue equilibrated across the membrane indicating that the pH gradient was not coupled to 6-deoxyglucose transport.

Large electrochemical gradients were reproducibly generated using the 'chemical dilution' technique and similar calculations to the above indicate that a $\Delta\psi$ of 70 mV should have produced a 15-fold accumulation of 6-deoxyglucose by S. mutans Ingbritt if glucose transport were coupled to this gradient. However, as seen in Table 9, 6-deoxyglucose merely equilibrated across the membrane, clearly demonstrating no link between $\Delta\psi$ and glucose transport. Thus, the results demonstrate that the protonmotive force does not fuel glucose transport in S. mutans and are in agreement with a similar study reported by Dashper and Reynolds (1990).

With this result, the question remains whether 6-deoxyglucose passively equilibrates across the membrane or translocation occurs via a specific carrier. The first experiment to answer this involved the loading of cells with unlabelled sugar followed by their exposure to a low external

concentration of labelled sugar. Widdas (1952) predicted that provided transport was mediated through a membrane carrier and did not require energy, a transient accumulation of label inside cells would be observed, as efflux of label would be initially impeded by the high concentration of unlabelled substrate. The term 'counterflow' was applied to this phenomenon. Counterflow experiments have since been used to demonstrate facilitated diffusion of sugars in erythrocytes, yeasts and bacteria (Wong and Wilson, 1970). Active transport systems also display counterflow when uncoupled through the use of metabolic inhibitors or when tested in vesicles (Silhavy et al., 1978).

As it was necessary at this stage to use intact cells for these experiments, fluoride was added at a high concentration to inhibit metabolism by the cells. In addition, test cells were pre-treated with 2-deoxyglucose to deplete the 'PEP pools' to cripple PTS activity. Counterflow was not demonstrated with cells treated with either glucose, 6-deoxyglucose or 2-deoxyglucose. However, the results of these experiments are equivocal based on the lack of uptake seen with control cells that were not pre-treated with 2-deoxyglucose, but were assayed in the presence of high concentrations of fluoride. Sugars did not equilibrate in either loaded or control cells (Figure 15)(i.e., in/out glucose ratios rarely exceeded 0.5) and for the first three minutes, these substrates, did not show active transport. Equilibration in loaded cells would have been predicted by

the 'simple diffusion' model (Dashper and Reynolds, 1990). As seen in Chapter 2, fluoride had a strong effect on transport but not metabolism at neutral pH regardless of the PTS activity of the cells. Thus, on reflection, it is doubtful whether successful loading of cells actually took place in the presence of the high concentration of fluoride used. For these reasons, the lack of counterflow seen here cannot not be taken as proof of diffusion of glucose through membranes.

Hamilton and St. Martin (1982) examined the kinetics of glucose transport by washed cells of S. mutans DR0001 and found a 'high affinity' system and a 'low affinity' system. A PTS-defective mutant, S. mutans DR0001/6, with only 12% of the EII^{glc} activity of the wild type (Vadeboncoeur et al., 1987), possessed only the low affinity system, therefore, the high affinity system was identified as the PTS. When the wild type S. mutans DR0001 was treated with fluoride, the high affinity system was abolished and only the low affinity system was observed. In this study, the K_m of the glucose transport we detected in PEP-depleted, fluoride-treated cells of S. mutans Ingbritt was 65 μM (Figure 17), which is within the range of 62 to 133 μM reported for the low affinity, non-PTS glucose transport system found in both S. mutans DR0001 and DR0001/6 (Hamilton and St. Martin, 1982).

Dashper and Reynolds (1990) propose an alternative mechanism of glucose transfer into cells that they term 'simple diffusion', a combination of passive diffusion

through the plasma membrane and entry through non-specific pores. The hypothesis is not supported by the studies reported here. The rates of glucose uptake they report for S. mutans Ingbritt with 1 and 50 mM glucose were 0.76 and 28.73 nanomoles per mg(dry wt) per min, while the rates in this study were considerably lower, 0.39 and 5.6 nanomoles per mg(dry wt) per min for 1 and 50 mM glucose. The difference may be explained by the fact that the 2-deoxyglucose-pretreatment used by Dashper and Reynolds was less severe than the treatment we used (1 mM 2-deoxyglucose for 20 min versus 25 mM 2-deoxyglucose for 30 min). The low level of 2-deoxyglucose in their experiments may have resulted in some residual PTS activity.

Finally, Dashper and Reynolds determined that following 30 minutes incubation, up to 50 mM glucose equilibrated within cells. In the scenario they describe, initial velocity of diffusion would be high and would slow down as equilibrium of glucose in and outside the cell is reached. In our experiments, the rate of glucose uptake was very low but increased tremendously after the first three minutes until glucose was concentrated in cells (Figure 16). This uptake was related to residual metabolism energizing cells and possibly fueling the non-PTS or PTS transport, as cells loaded and challenged with either 2-deoxyglucose or 6-deoxyglucose did not display any concentrative uptake or even equilibration after 10 minutes (data not shown). Therefore, the equilibration of glucose in S. mutans Ingbritt

following a 30 minute incubation reported by Dashper and Reynolds should be approached with caution since it likely involves energized transport and not 'simple diffusion'.

The 'simple diffusion' of glucose model presupposes that glucose can slowly traverse the membrane. Numerous studies employing various bacteria and vesicles derived from them, have failed to detect appreciable simple diffusion (Kaback, 1968; Postma, 1978; Postma and Stock, 1980; Postma and Lengeler, 1985; Robillard *et al.*, 1987). In 1969, Roseman described the properties of simple diffusion of carbohydrates and concluded that is was not a 'significant physiological process in either entry or exit mechanisms' Dashper and Reynolds cite the work of Gachelin (1970) and Maloney and Wilson (1973) as supporting such transmembrane movement of carbohydrates. However, the work of Gachelin was designed to test the degree of coupling between transport and phosphorylation of the PTS and was attempting to identify free α-methylglucoside inside of cells. When some was detected, the author determined it had entered through the EII but subsequent work showed that the free α-methylglucoside most likely originated from alternative non-PTS mechanisms of glucose transport known to occur in *E. coli* (Postma and Stock, 1980). In the Maloney and Wilson study, lactose transport in *E. coli* was examined. However, a simple diffusion-like movement of carbohydrate could be accounted for by facilitated diffusion mediated by the

residual activity of the uncoupled lactose-proton symporter (Silhavy et al., 1978) .

To date, the glycerol permease is the sole, non-specific pore transporter for carbohydrates although it is present in a wide range of bacteria (Saier, 1985). The concept of S. mutans possessing a glucose-transporting pore is somewhat untenable given the mechanisms it has evolved for the regulation of carbohydrate transport, glycolysis, synthesis of intracellular and extracellular polysaccharides (Carlsson, 1986; Thompson, 1987; Reizer et al., 1989a). For example, S. mutans can experience a 10,000-fold influx of carbohydrates into the oral cavity (Carlsson, 1986) and the unregulated entry of glucose by simple diffusion would likely overwhelm the known regulatory mechanisms. In addition, a system of 'simple diffusion' would not allow for the active transport of free glucose into the cell and yet glucose was detected at over 1000-fold the culture concentration in S. mutans Ingbritt grown under conditions known to repress the PTS (Chapter 2).

The fluoride sensitivity of glucose transport was described in previous studies (Chapter 2; reviewed by Hamilton, 1990) and Dashper and Reynolds present no other data to dispute effects of fluoride at such a high concentration on transport and metabolism of S. mutans. The fluoride sensitivity of transport we describe here could be taken as further support for carrier mediated non-PTS transport. In the absence of fluoride, 6-deoxyglucose equilibrated in cells (Figure 13,

Table 9)(here we are in agreement with Reynolds), and must have done so by non-PTS mechanisms since it is not transported by the PTS. In the presence of high concentrations of fluoride, equilibration was not seen (Figure 15A) as the average in/out ratio was only 0.50. The devastating effect on glucose transport implies a fluoride-sensitive carrier, however, it is difficult to ascribe fluoride sensitivity to a process like 'simple diffusion'.

The lack of active transport in response to artificial pH or electrochemical gradients by cells of S. mutans Ingbritt observed in this study, put to rest the protonmotive force-coupled model of glucose transport. However, whether non-PTS glucose transport occurs via alternative means such as facilitated diffusion is not clear. Counterflow experiments to test for the presence of a carrier were unsuccessful due to the interference with the loading process by the high concentration of fluoride required to inhibit metabolism. However, counterflow would be the method of choice to identify a membrane component involved in glucose transport because it is a general technique that will detect any type of membrane carrier regardless of the mechanism of transport with the exception of group translocation. In addition, EII carriers do not demonstrate appreciable counterflow. Alternative techniques to circumvent metabolism are required to determine finally whether S. mutans possesses an alternative, non-PTS mechanism for glucose transport.

Chapter 4

Glucose Transport by Membrane Vesicles of S. mutans Ingbritt

Introduction

Glucose transport by S. mutans has proven to be somewhat of a puzzle. While the glucose-PTS in S. mutans is well-characterized (Vadeboncoeur 1984; Vadeboncoeur et al., 1987, 1991; Hamilton et al., 1989), an alternative non-PTS glucose transport system has been indicated, but never conclusively proven (Hamilton, 1987). An early model of protonmotive force-linked glucose transport has been disproved but studies with whole cells testing for other mechanisms have led to conflicting results (Chapter 3, Dashper and Reynolds, 1990). In order to examine glucose transport in intact cells, it is necessary to disrupt cellular metabolism, which appears to result in devastating effects on glucose transport, making it impossible to discriminate between glucose transport inhibition due to lack of a transporter or interference with an existing system.

A novel way to attack the problem is through the use of membrane vesicles. In 1960, Kaback first reported that isolated membranes of E. coli strain W could catalyze the uptake of proline and subsequent studies showed that the membrane preparation contained plasma membrane vesicles devoid of cytoplasmic constituents that behaved as

'osmotically intact structures' retaining many transport functions of whole cells (reviewed by Kaback, 1970; 1972). Vesicles have since been isolated from a wide range of bacteria including Staphylococcus aureus, Bacillus species and Veillonella (reviewed by Futai, 1978) and their use has revolutionized the study of bacterial transport. Vesicles of S. mutans afford several advantages over whole cells for the study of carbohydrate transport: (a) the PTS is inactivated by the removal of the general proteins, HPr and EI, as well as the enzymes capable of generating PEP, (b) substrates are not metabolized, and (c) the transport system can be studied in isolation without the use of inhibitors or substrate analogues.

In this chapter, a method for the isolation of membrane vesicles from S. mutans Ingbritt will be described. The vesicles obtained by this method have the same orientation as whole cells and are free of cytoplasmic constituents. To resolve the issue of non-PTS transport and circumvent the need for inhibitors and PTS disruption, vesicles were an ideal alternative to whole cells. Vesicles loaded with unlabelled glucose and exposed to labelled glucose should display counterflow if a glucose carrier exists. No active transport would be expected as vesicles lack ATP and PEP and the enzymatic pathways required to make them and, consequently, are incapable of maintaining electrochemical gradients.

Materials and Methods

Bacterial strain and growth conditions

The S. mutans strain Ingbritt used in this study was kindly supplied by J. Sandham, Toronto Canada. The culture was maintained and assessed for purity as previously described (Chapter 2). Cultures were grown in a chemostat (L.H. Engineering) under two conditions; one previously labelled Condition D (50 mM glucose at a dilution rate of 0.1 h^{-1} at pH 5.5), resulting in repression of the PTS, and the second, previously labelled Condition A (10 mM glucose at a dilution rate of 0.1 h^{-1} at pH 7.0) which results in optimal PTS activity (Hamilton et al., 1979, 1989; Vadeboncoeur et al., 1987, 1991).

Preparation of membranes, vesicles and crude-cell extract

Membrane fragments were prepared with cells harvested from the chemostat overflow using a modification of the technique used by Bender et al. (1986). Cells were harvested from the chemostat overflow, washed twice in cold water and resuspended in 40 ml of osmotic buffer (75 mM Tris HCl (pH 7.5), 2 mM $MgSO_4$, 400 mM sucrose, 40 mM amino-n-caproic acid, 6 mM p-aminobenzamide and 14 mM mercaptoethanol). Cell clumps were dispersed by 2 minute sonication using a Kontes sonifier (Kontes Scientific Glassware, NJ, USA) at a power setting of 4 before being transferred to 160 ml pre-warmed

osmotic buffer containing 1.25 g lysozyme and 7500 units of mutanolysin. Following a 3 hour incubation with stirring at 37°C, the cells were washed and resuspended in 10-15 ml of membrane buffer (50 mM Tris HCl (pH 7.5), 10 mM $MgSO_4$, 40 mM amino-n-caproic acid, 6 mM p-aminobenzamide and 14 mM mercaptoethanol) and passed three times through a pre-cooled French pressure cell with 6000 lbs/inch^2 pressure. The suspension was treated with 10 µg/ml DNase and RNase for 30 min at 37°C and then centrifuged at 20,000 g for 20 minutes to remove protoplasts and any whole cells. The supernatant was centrifuged at 200,000 g for 4 hours and the membrane pellet washed once and resuspended in membrane buffer.

Vesicles were prepared using a combination of the membrane preparation technique described above and the vesicle preparation procedure of Otto et al. (1982). Steady-state cells were collected from the chemostat, washed twice in cold water and resuspended in 40 ml of vesicle buffer (10 mM Tris HCl (pH 7.5), 10 mM $MgSO_4$, 2 µM pepstatin and 14 mM mercaptoethanol). The cell suspension was sonicated for 2 minutes with a Kontes sonifier at a power setting of 4 to disperse the cells before being added to 160 ml prewarmed, sucrose-free osmotic buffer containing 1.25 g lysozyme and 7500 units of mutanolysin. Following a 3-hour incubation at 37°C, the resulting protoplasts were washed and resuspended in 40 ml vesicle buffer and lysed by the addition of 10.8 ml of saturated K_2SO_4. The preparation, a mixture of vesicles and membrane fragments, was treated with 10 µg/ml DNase and

RNase for 15 minutes with stirring at 37°C, after which 9.0 ml of 200 mM EDTA was added and the preparation incubated for a further 15 minutes. The suspension was then washed and resuspended in 60 ml of vesicle buffer. The vesicles were purified by discontinuous density gradient centrifugation by layering 10 ml of the vesicle/membrane supsension onto 10 ml of 60%(w/v) sucrose overlayed with 15 ml of 20% (w/v) sucrose and centrifuging at 67,500 g for 16 hours in a Beckman SW 28 swinging-bucket rotor. The vesicles, located at the interface between the two sucrose layers, were carefully removed, collected and washed by centrifugation at 48,000g for 30 minutes and resuspended in vesicle buffer. The final vesicle pellet was resuspended in buffer, distributed in 200 µl aliquots and stored at -70°C until use.

Crude-cell extract of S. mutans Ingbritt was prepared using a variation of the sonication method described by Khandelwal and Hamilton (1971) for the isolation of cytoplasmic enzymes. Cells grown in Condition A were collected from the chemostat and washed in vesicle buffer and sonicated for 3 minutes at 0°C in the presence of 5 µm beads (cells to beads ratio of 3:1) in a Branson sonifier (Heat Systems Ultrasonics Inc, NY, USA) followed by centrifugation (30,000 g for 15 min). The supernatant was then treated with RNase and DNase (10 µg/ml) for 30 minutes and dialyzed (10,000 MW cutoff) overnight against 4 liter vesicle buffer with one change of buffer during that time.

Enzyme assays

Vesicles, membranes and crude-cell extract prepared from cells grown in Condition A were assayed for the presence of cytoplasmic and membrane-associated enzymes. Vesicles were sonicated for 1 minute at 0°C with the Branson sonifier prior to enzyme assays in order to liberate any trapped cytoplasmic contents.

Glucokinase was assayed using a variation of the glucose-6-phosphate assay described in Chapter 2 by measuring the conversion of the reaction product glucose-6-P to 6-phosphogluconolactone by glucose-6-phosphate dehydrogenase with accompanying reduction of NADP (Porter et al., 1980). Reaction mixtures (1.0 ml) contained (final concentrations): 50 mM Tris-HCl (pH 8.5), 5 mM $MgCl_2$, 5 mM dithiothreitol, 10 mM ATP, 1 mM NADP, 20 mM glucose, 5 units glucose-6-P dehydrogenase and 25-100 µg protein of the crude cell extract, vesicle or membrane preparations. All the reagents, except ATP and glucose, were added to cuvettes at 37°C and endogenous rates established before the addition of glucose. ATP was then added and the rate recorded by measuring the change in absorbance at 340 nm in a UV/visible spectrophotometer (Hewlett Packard model 8452A diode Array Spectrophotometer, Hewlett packard Co., U.S.A.). Enzyme activity was expressed as nanomoles of glucose converted to glucose-6-P per mg protein per minute.

Pyruvate kinase was assayed as described by Yamada and Carlsson (1975a) by measuring the rate of conversion of the

product of the pyruvate kinase reaction, pyruvate, to lactate by lactate dehydrogenase and measuring the accompanying NADH oxidation. The following were combined in a 1.0 ml reaction mixture (final concentrations): 50 mM Tris-HCl (pH 7.0), 100 mM NH_4Cl, 10 mM $MgSO_4$, 0.12 mM NADH, 1 mM ADP, 3 mM PEP, 5 units lactate dehydrogenase and 25-100 µg protein of the crude cell extract, vesicle or membrane preparations. Pyruvate kinase was activated by the addition of 1.6 mM glucose-6-P. Absorbance at 340 nm was recorded with time. The specific activity was expressed as nanomoles of PEP converted to pyruvate per mg protein per minute.

Lactate dehydrogenase was assayed in the presence of its activator, fructose-1,6-diphosphate and saturating amounts of pyruvate by measuring the oxidation of NADH (Brown and Wittenberger, 1972). The 1.0 ml reaction mixtures contained (final concentrations): 50 mM potassium phosphate buffer (pH 7.0), 16 mM $MgSO_4$, 0.12 mM NADH, 4 mM pyruvate, 0.4 mM fructose-1,6-diphosphate and 25-100 µg protein of the crude cell extract, vesicle or membrane preparations.

The membrane-bound, proton-translocating ATPase (H^+/ATPase) was assayed using a variation of the assay of Bender et al. (1986) by measuring the release of inorganic phosphate as described previously (Hamilton and Buckley, 1991). The reaction mixture contained 50 mM Tris-maleate (pH 6.0), 20 mM $MgSO_4$, 5 mM ATP and 50 µg membrane or vesicle protein in a final volume of 500 µl. After 5 min incubation, the reaction was stopped with the addition of 25 µl 2N HCl and the

phosphate released measured by a modified Fiske-Subbarow (1925) method (Sigma Diagnostics, Procedure 670, St. Louis, MO). Units are expressed as nmol of P_i per mg protein per min. Activities were measured in vesicle and membrane preparations and the same preparations treated with sodium dodecyl sulfate (SDS: protein ratio of 1:1) to solubilize the protein.

Measurement of glycolytic activity

The glycolytic activity of vesicles was assayed as described previously (Chapter 2). Vesicles were resuspended at 0.5 mg (protein)/ml in vesicle buffer in a pH stat at a final volume of 5.0 ml. The suspension was constantly mixed under a stream of nitrogen gas and the endpoint pH was maintained at pH 7.0 by the addition of 0.1 M NaOH from a Radiometer Autoburette (model ABU 1a, Radiometer, London, Ontario, Canada). The glycolytic rate was determined after the addition of glucose to a final concentration of 5 mM.

Glucose counterflow

Counterflow was used to test the ability of vesicles derived from cells grown in Condition A (with high PTS activity) and Condition D (with repressed PTS activity) to carry out carrier-mediated facilitated diffusion of glucose. Following incubation with 100 mM glucose in buffer for 1 hour at room temperature, the 'glucose-loaded' vesicles were collected by centrifugation at 48,000 g for 10 min, the

supernatant carefully decanted and any residual fluid in the centrifuge tube removed with cotton swabs. Vesicles were resuspended in 10 ml buffer containing 2 mM C^{14}-glucose (5000 dpm/nmole) and 1 ml samples collected periodically by silicone oil centrifugation and counted with Aquasol in a scintillation counter. Controls consisted of 'unloaded' cells incubated without unlabelled glucose. The internal glucose concentration was determined as described for whole cells (Chapter 3).

Vesicle loading with glucokinase and ATP

To test for active transport by a reconstituted glucose-phosphorylating system, vesicles were loaded with ATP and glucokinase and transport measured by the uptake of C^{14}-glucose. The vesicles were derived from cells grown under Condition D and were resuspended to a final volume of 5 ml in TEA buffer (20 mM triethanolamine buffer (pH 7.0), 14 mM mercaptoethanol) containing 50 mM ATP and 5 units per ml glucokinase. The vesicle suspension was passed once through a pre-cooled French pressure cell and the vesicles washed twice and resuspended in 4 ml buffer. C^{14}-glucose (100 mM, 5000 dpm/nmol) was then added to a final concentration of 1 mM and duplicate 500 µl aliquots were sampled at 1,2,5 and 10 minutes and the vesicles collected by using silicone oil centrifugation. The internal glucose concentration determined as described for whole cells (Chapter 3).

Controls consisted of non-loaded vesicles passed through the French pressure cell.

Electron microscopy

Samples were taken from different stages of vesicle preparation and fixed by the addition of 50% glutaraldehyde directly to the specimen in suspension (40µl fixative per ml sample). They were post fixed with 1% osmium tetroxide and embedded in JemBed 812 (JB EM Services, Dorval, Canada) as described by Yuan and Gulyas (1981). The specimens were sectioned on an LKB Ultratome EIII using a diamond knife and the silver sections were collected on 300 mesh grids. The sections were stained with uranyl acetate as described by Stempek and Ward (1964) and post-stained with lead citrate as described by Venable and Coggeshell (1965). The sections were viewed with a Philips model 201 Electron Microscope at an acceleration voltage of 60 Kev and images were recorded on Kodak positive reverse film 5302. Micrographs were printed on Kodak Polycontrast RC EIII paper.

To determine the orientation of the S. mutans vesicles, preparations were examined by electron microcsopy in the laboratory of T.J. Beveridge, University of Guelph, Guelph, Canada using the freeze-fracture technique of Chapman and Staehelin (1986)(Martin and Beveridge, 1986).

Protein determinations

The protein content of the membranes, vesicles and crude-cell extracts was determined using a modification of the Lowry technique as described by Peterson (1983). Briefly, 10-200 µl aliquots of samples were added to water to a final volume of 1 ml in 1.5 ml Eppendorf tubes and 100 µl of sodium deoxycholate (0.15% w/v) was added and the samples incubated at room temperature for 10 minutes to solubilize the protein. Trichloroacetic acid (100 µl, 72% w/v) was then added and the precipitated protein collected by centrifugation in a microfuge, the pellet resuspended in 1 ml of protein solubilization solution (1 vol CTC reagent(0.1% $CuSO_4 \cdot 5H_2O$, 0.2% Natartrate and 10% Na_2CO_3)/ 2 vol 5% SDS/ 1 vol 0.8 M NaOH) and incubated at room temperature for 90 minutes. Aliquots of samples were transferred to tubes containing protein solubilization solution (final volume 1 ml) and 1 ml water added followed by 0.5 ml of protein assay solution (1 vol 2N Folin-Ciocalteu: 6 vol distilled water) and the tubes incubated at room temperature for 30 min and the absorbance read at 750 nm in a UV/visible spectrophotometer. Bovine serum albumin was used as a protein standard.

Water volume of vesicles

The intravesicular and extravesicular water content of the vesicles was determined as previously described for whole cells (Chapter 2, Hamilton and Buckley, 1991) except that

suspensions were in vesicle buffer and centrifugation was through a 65:35 (vol/vol) silicone oil mixture of fluid 556 to fluid 550 (Dow Corning Corp., Midland, Michigan). The internal vesicle water volume was 4.46 ± 1.50 µl/mg (protein) and the external vesicle water volume was 4.98 ± 2.06 µl/mg (protein). Following single passage of vesicles through a French pressure cell, the intravesicular water volume was 2.34 ± 0.28 µl/mg (protein) and the extravesicular volume was 1.04 ± 0.20 µl/mg (protein).

Chemicals

Radioactive glucose was purchased from ICN Radiochemicals (Irvine, CA., USA). Amino-n-caproic acid, p-aminobenzamide, mercaptoethanol, lysozyme, mutanolysin, and the enzyme assay reagents were purchased from Sigma Chemical (St. Louis, MO, USA). All other reagents were of analytical grade.

Results

Examination of vesicle preparations with electron microscopy

Techniques of electron microscopy were used to aid in the evaluation of the purity and orientation of the vesicle preparations. Samples were removed at different stages in vesicle preparation and subjected to negative staining in order to evaluate each step in the process. As seen in Figure 18(B), the cell wall was completely removed following

mutanolysin and lysozyme treatment and the cytoplasmic contents are clearly visible indicating protoplast formation. The photographs in C and D of Figure 18 indicate that the final vesicles are intact and of high quality as evidenced by the lack of contaminating cytoplasmic debris.

Kaback (1974) was the first to describe the use of freeze-fracture microscopy to demonstrate the sidedness of vesicles based on the observation that the inner (cytoplasmic-facing) side of the membranes has a higher density of particles than the outer surface. In the freeze fracture of S. mutans Ingbritt whole cells (Figure 18(E)), the cell wall and outer layer of the membrane have been removed revealing the hydrophobic, intramembranous region with clustered particles. In other whole cell preparations, intramembranous particles were observed at the midline of the cells along the poles exposed in dividing cells (data not shown). In the vesicles examined by this technique, greater than 90% had the same orientation as the whole cells as identified by the aggregation of particles on the inner surface of the membrane (Fig. 18(F)).

Biochemical evaluation of vesicle preparations

Biochemical techniques were also used to characterize the vesicles. The level of cytoplasmic contamination was evaluated by comparing the activity of glucokinase, pyruvate kinase and lactate dehydrogenase in membranes, vesicles and

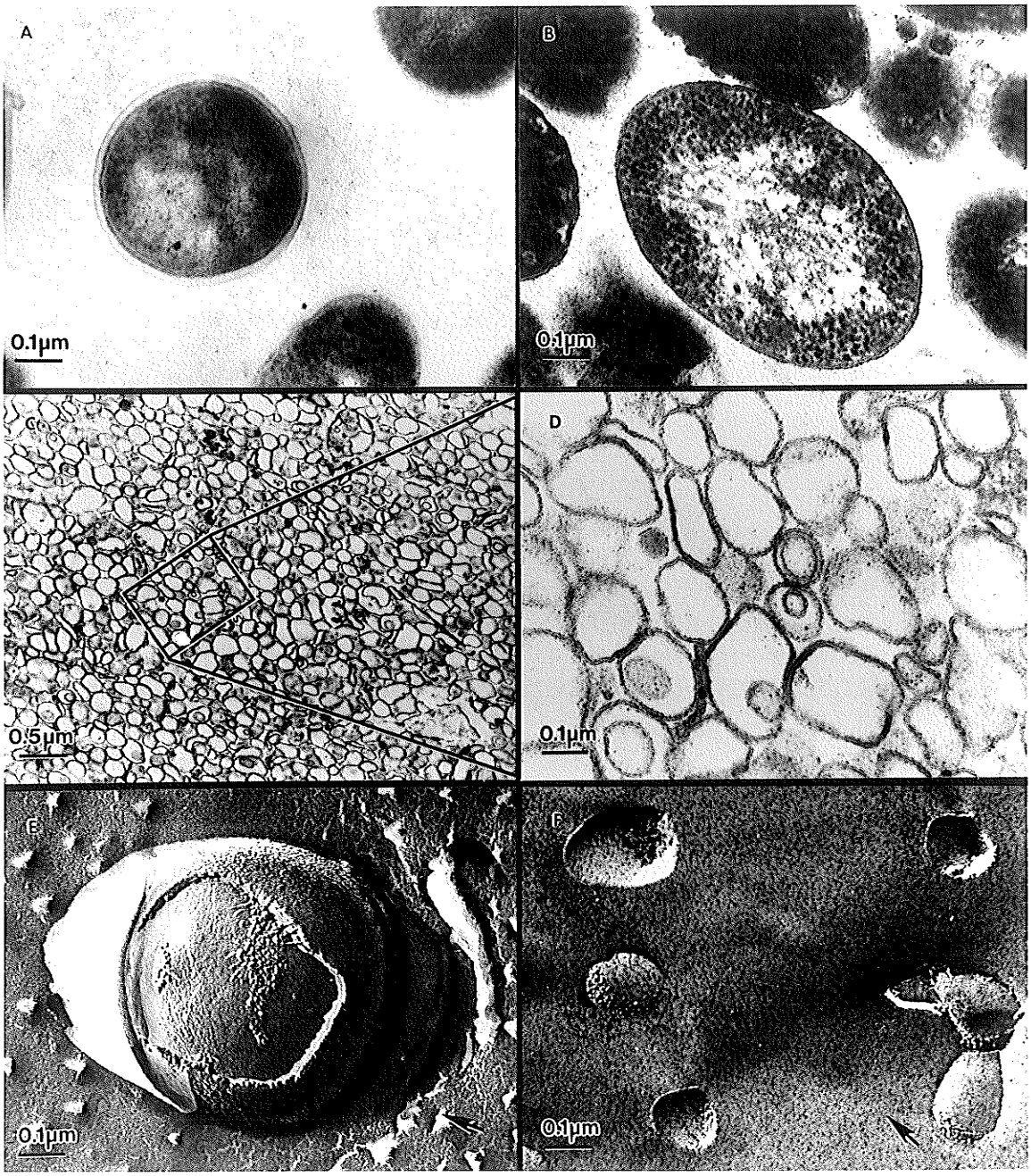

Figure 18. Electron micrographs of Streptococcus mutans Ingbritt. (A-D) Negative stain, (E-F), Freeze-fracture. (A) Intact cell (x100,000), (B) Protoplasts (x100,000), (C) Vesicles (x20,000), (D) Vesicles (x100,000), (E) Intact cell (x98,000), (F) Vesicles (x98,000).

crude-cell extracts. Table 10 shows that the levels of all the cytoplasmic enzymes were greatly reduced in vesicles from those found in crude-cell extracts and were comparable to those seen in a membrane preparation from the same cells. In addition, vesicles incubated with glucose in a pH stat displayed no glycolytic activity indicating efficient removal of the enzymes required for glucose metabolism.

H^+/ATPase is a membrane-bound enzyme whose catalytic portion faces the cytoplasm in intact cells (Futai and Kanazawa, 1983). Therefore, its activity can be used to determine the orientation of vesicles since right-side out vesicles will not display enzyme activity as ATP cannot traverse the membrane. Conversely, activity will be detectable with inside-out vesicles as the catalytic portion will be exposed to the exterior. Untreated vesicles possessed no H^+/ATPase activity (Table 11) therefore, possessed the same orientation as whole cells. To ensure that this result was not due to a lack of activity, intact vesicles were disrupted by treatment with low levels of the detergent SDS. As seen in Table 11, activity was detected indicating that the SDS treatment resulted in exposure of the catalytic portion of the H^+/ATPase to the ATP. Membrane controls treated with equal amounts of SDS were included to demonstrate the effect of SDS on H^+/ATPase activity. Membrane H^+/ATPase activity was reduced 4-fold by SDS suggesting that the vesicles contained significant activity

Table 10

Activity of the cytoplasmic enzymes, glucokinase, pyruvate kinase and lactate dehydrogenase in preparations of Streptococcus mutans

Sample	Glukokinase	Pyruvate kinase	Lactate dehydrogenase
cell-free extract	269 ± 20[a]	75 ± 3[b]	185 ± 16[c]
vesicles	14 ± 3	9 ± 1	13 ± 5
membranes	ND[d]	12	7

[a] Glucose-6-phosphate formed per mg protein per min.
[b]. Pyruvate formed per mg protein per min.
[c]. Lactate formed per mg protein per min..
[d]. Not detected.

Table 11

H^+/ATPase activity in vesicle and membrane preparations of *Streptococcus mutans* Ingbritt

Sample	SDS Treatment[a]	H^+/ATPase
vesicles	-	ND[b]
	+	21.74[c]
membranes	-	45.50
	+	11.56

[a] Nanomoles phosphate released per mg protein per min.

[b]

[c] Vesicle or membrane preparation mixed with SDS in a ratio of 1:1 just prior to assay.

further confirming the right-side-out orientation of the vesicle preparation.

Counterflow

Counterflow experiments were performed on vesicles prepared from cells grown in conditions known to optimize or repress the PTS to test for the presence of a glucose carrier. Counterflow predicts that when the vesicles are loaded with unlabelled glucose and then exposed to labelled glucose, entry through a glucose-specific carrier will be unaltered, but its exit will be inhibited as it competes with unlabelled substrate inside the cell for the carrier. Therefore, if a carrier is present in the vesicle membrane, transient accumulation of the carrier will be observed. If transport occurs through simple diffusion, no concentration of the carrier in the vesicles will be observed. As can be seen in Figure 19(A), glucose accumulated inside 'loaded' vesicles prepared from cells grown in PTS-repressed conditions, while no accumulation was observed with unloaded vesicles, indicating the presence of a glucose-specific carrier. Similar results were obtained with 4 other experiments using 3 different vesicle preparations. Vesicles prepared from cells containing high PTS activity (Condition A) also demonstrated counterflow when loaded with glucose (Figure 19(B)).

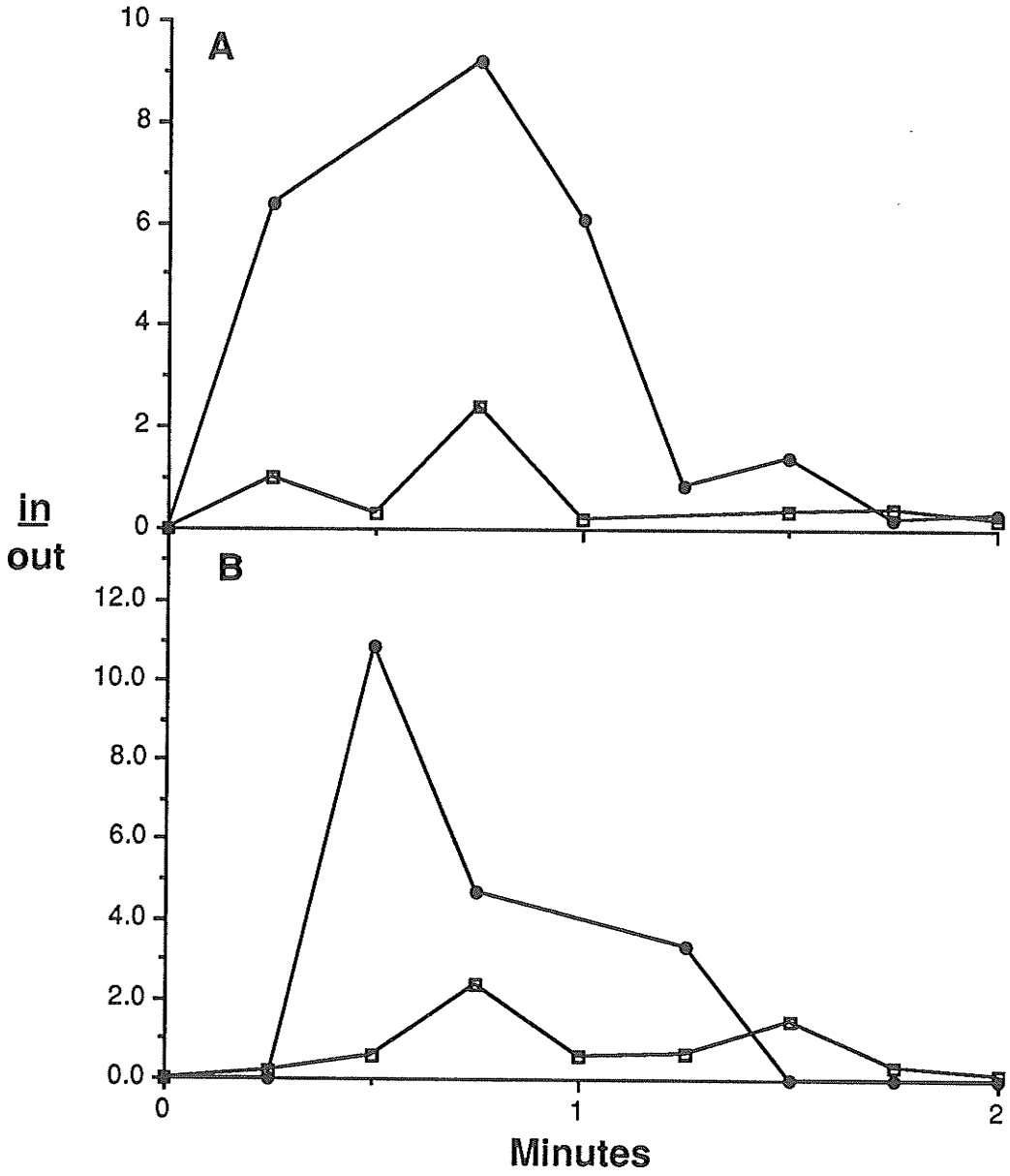

Figure 19. Glucose counterflow with vesicles of Streptococcus mutans Ingbritt grown in Condition D (A) and Condition A (B) with time versus in/out ratio of glucose. Glucose-loaded (●) and unloaded (□) vesicles.

Glucose uptake by vesicles containing ATP and glucokinase

Vesicles disrupted by use of the French pressure cell in the presence of glucokinase and ATP should reassemble with some of the two agents trapped inside. We reasoned that the glucose would accumulate as glucose entering the vesicles by facilitated diffusion would be converted to glucose-6-P causing the entry of more glucose. In spite of a number of such loading experiments, a difference in glucose uptake was not shown between vesicles containing glucokinase and ATP and control vesicles containing no added agents.

Discussion

Typical of other methods (Kaback, 1972), the preparation of membrane vesicles of S. mutans consisted of two stages: protoplast formation followed by lysis and reformation of the membrane as vesicles. Unlike previous reports (Eisenberg and Lillmars, 1975; Schöller et al., 1983; Siegel et al., 1981; Chassy and Giuffrida, 1980), protoplasts generated by lysozyme and mutanolysin in our system showed little lysis in the absence of osmotic stabilizers. Goodman et al. (1981) reported that significant cell wall loss could occur without acquisition of osmotic sensitivity by protoplasts of S. mutans and suggested that strategic cell wall remnants could protect the cell. Nevertheless, electron micrographs of our preparations (Figure 18) indicate complete loss of cell wall material after mutanolysin and lysozyme treatment. The

vesicles, obtained after potassium sulfate lysis of the protoplasts and sucrose density gradient centrifugation, were homogeneous with little contaminating membrane debris.

When presenting a novel method for vesicle preparation, the two most important determinants of its effectiveness are the degree of purity and the orientation of the resulting vesicles. The vesicles were essentially devoid of cytoplasmic contents since they exhibited low activity for the soluble enzymes glucokinase, pyruvate kinase and lactic dehydrogenase compared to cell-free cytoplasmic extracts prepared by sonication (Table 10). Furthermore, vesicles displayed no glycolytic activity as measured by acid formation when incubated with glucose in a pH stat. The lack of active transport by vesicles reflects disruption of the ATP and PEP-generating machinery of the cells (Figure 19(A,B), unloaded cells).

The orientation of our vesicles was shown to be right-side out based on freeze fracture electron microscopy and the location of the H^+/ATPase activity. As seen in Table 11, vesicles possessed no H^+/ATPase activity compared to membrane particles indicating they were right-side-out. This result was not due to the loss of ATPase activity during protoplast lysis with potassium sulphate since vesicle solubilization with SDS to expose the catalytic portion of the enzyme exhibited greater activity than membrane fragments treated in the same manner.

The purpose in isolating vesicles was to resolve the question of non-PTS glucose transport by S. mutans Ingbritt. As seen in Chapter 3, previous attempts to test for counterflow in S. mutans were unsuccessful and we suspected it was because of the high concentrations of fluoride required as a metabolic inhibitor. Vesicles lack metabolic activity therefore the use of potentially widely disruptive inhibitors was unnecessary. In addition, the lack of cellular constituents would mean the absence of PEP, HPr and EI, therefore, the PTS would be inoperative. For these reasons, glucose and not an analogue could be used for the studies with confidence of no PTS-mediated glucose transport occurring..

Vesicles lack the metabolic machinery for ATP or PEP formation. Therefore, active uptake of glucose was not expected or seen (Figure 19, unloaded vesicles). Transient accumulation of labelled glucose was observed in glucose-loaded cells of S. mutans Ingbritt indicating the presence of a glucose carrier. In Chapter 2, cells with glucose PTS repression due to growth conditions (Condition D) and cells where the PTS was inactivated by 2-deoxyglucose-induced depletion of the 'PEP pool' (Condition B, 2-deoxyglucose-treated) displayed comparable levels of glucose transport suggesting constitutive expression of the non-PTS glucose transport system in S. mutans. Vesicles were derived from cells grown in continuous culture in conditions known to result in high PTS activity (Condition A) and conditions

known to repress the PTS (Condition D). Both sets of vesicles displayed counterflow, confirming that unlike the PTS which is under genetic regulation, the expression of the non-PTS carrier is constitutive.

In previous experiments, sampling of vesicles was attempted using filtration instead of centrifugation through silicone oil. Filtration was unsuccessful and resulted in little if any glucose associated with the vesicles. Andersson and Lundahl (1990) reported significant leakage of glucose from liposomes containing reconstituted erythroctye glucose transporter following filtration and resorted to molecular sieve chromatography for sampling. In experiments with silicone centrifugation a carrier was demonstrated. However, the in/out glucose concentration in control vesicles was often less than the value of 1.0, which would have been expected with equilibration of the labelled glucose in the absence of loading. It is possible that some leakage is occurring even using centrifugation through oil and that actual glucose ratios may be even higher than we were able to measure. However, the reproducibility of our results confirms the presence of a glucose carrier capable of facilitated diffusion in S. mutans Ingbritt.

Unlike the PTS, glucose entering the cell through a carrier would appear in the cell in an unaltered state and could then be phosphorylated by glucokinase at the expense of ATP. Glucokinase is under some genetic control and is increased under conditions where the glucose-PTS is repressed and

decreased when the PTS is at its optimum (Hamilton, 1984). This is reasonable since the operation of the non-PTS system would require glucokinase for phosphorylating glucose. However, my attempts to reconstitute a glucose-phosphorylating apparatus inside vesicles by disruption of vesicles in the presence of glucokinase and ATP were unsuccessful. This was likely due to technical problems associated with the French pressure cell technique. One common result from French pressure cell treatment is the inversion of vesicles (Futai, 1978). Examination of French pressure cell-treated vesicles of S. mutans using electron microscopy indicated a mixed spectrum of membrane orientations. The majority (~60%) appeared to be right-side out but were no longer unilamellar (T.J. Beveridge, personal communication). Subtle lipid-packing reorientations were indicated by the tendency of the treated-vesicles to cross-fracture which was not seen in untreated vesicle preparations. Further proof of the disruption of the vesicles following French pressure treatment is indicated by the significant reduction in water volumes of vesicles compared to untreated vesicles. The presence of multilamellar vesicles could seriously compromise the water volume determinations. Disruption of functions such as calcium uptake has been described for vesicles following passage through the French pressure cell (Futai, 1978). Modification of the French pressure cell technique or the use

of alternative means to obtain vesicle loading would be required before attempting the experiment again.

Chapter 5

General Discussion

The purpose of this thesis was to address the issue of non-PTS transport in S. mutans Ingbritt. The existence of a second system of glucose transport had been indicated for some time based on continuous culture studies (Chapter 2). A model of protonmotive force-coupled glucose transport was proposed, based primarily on the ionophore sensitivity of glucose transport in S. mutans (Hamilton and St. Martin, 1982). A closer examination of the transport inhibition, in concert with studies on glucose metabolism (Chapter 2), indicated that many of the previously observed effects (Hamilton and St. Martin, 1982; Keevil et al., 1984; Hamilton, 1986, 1987) were related to the sensitivity of metabolism to low pH and not to direct transport effects. Alternately, the effect of agents such as fluoride at neutral pH, appeared to be directed at transport rather than metabolism (Chapter 2 and 3). The final proof that glucose transport was not fueled by the protonmotive force was the lack of glucose transport in response to artificially-generated protonmotive force (Chapter 3, Figure 13 and Table 9; Dashper and Reynolds, 1990). However, the presence of an alternative glucose transport system was confirmed by the demonstration of low affinity glucose transport in S. mutans with inactivated PTS (Chapter 3, Figure 17) and the

demonstration of glucose counterflow in vesicles of S. mutans (Chapter 4, Figure 19).

The nature of non-PTS glucose transport in S. mutans remains controversial. A brief review of solute transport in Gram-positive bacteria will assist in probing this issue. Solutes, including carbohydrates, can enter bacterial cells by diffusion or active transport. Diffusion is a passive process that does not require energy, thus solutes will not accumulate against a concentration gradient. The two types of diffusion occurring in bacteria are passive and facilitated diffusion. Passive or 'simple' diffusion can occur by either direct translocation through the membrane, as is the case for weak acids and bases and water. Alternately, proteinaceous pores may form channels through which solutes may traverse the membrane. Glycerol is the sole carbohydrate known to be transported through the cytoplasmic membrane in this fashion (Saier, 1985) and glycerol permeases have been found in E. coli, Salm. typhimurium, several species of Pseudomonas, Klebsiella, Shigella, Bacillus and Nocardia (Neidhardt et al., 1990). Whether through pores or directly through the membrane, passive diffusion is characterized as a non-saturable process. With facilitated diffusion, transport is mediated by a specific membrane-bound carrier and is distinguishable from passive diffusion in that it is a saturable process (Maloney et al., 1975). Facilitated diffusion is not a common mode of transport in bacteria and is usually seen with 'uncoupled' active transporters, that

is, active transporters in de-energized cells. One example is the *lac* permease that mediates the active transport of lactose and is fuelled by the protonmotive force. In the absence of an electrochemical gradient, it will mediate the equilibration of the sugar across the membrane (Maloney et al., 1975).

Active transport is an endergonic process involving solute interaction with a specific carrier molecule resulting in accumulation of solute against a concentration gradient (Konings et al., 1987). Active transport systems in Gram-positive bacteria can be further divided into: (1) group translocation, (2) primary transport, and (3) secondary transport systems. Group translocation is not strictly active transport because the solute *per se* does not accumulate in the cell due to its chemical alteration during the translocation process (Neidhardt et al.,1990). The altered solute is usually identical to the product of the first intracellular reaction, therefore, energy is conserved as energy required for modification of the substrate is shared by the transport process. The carbohydrate:PEP phosphotransferase system is an example of group translocation and has been described in detail in Chapter 1.

Primary transport systems convert chemical or light energy into electrochemical energy. These include electrogenic proton pumps, including the H^+/ATPase and electrogenic proton pumps that generate protonmotive force (Konings et al., 1987). Phosphate-bond driven, or ATP-linked transport is an

alternative type of primary transport system that occurs in both Gram-positive and Gram-negative bacteria (Konings et al., 1987). While it has been established that ATP is essential for transport, the actual phosphoryl donor and the underlying mechanism have yet to be established. Examples of this type of transport include the Na^+/ATPase of S. faecalis which accumulates Na^+ in the cell at the expense of ATP (Kobayashi et al.,1978), and transporters for various amino acids in S. faecalis and Lactococcus cremoris mediating the uptake of L-glutamate, L-aspartate and L-asparagine (Poolman et al., 1987; Konings et al., 1987b).

A special family of phosphate-bond driven transporters is the 'binding protein transporters'. First identified in Gram-negative bacteria, the sensitivity of transport to cold shock in these organisms was due to the loss of solute-binding proteins essential for transport from the periplasm (Berger and Heppel, 1974). These transporters occur in both bacteria and eukaryotes and usually do not require a binding protein component. Therefore, the name is somewhat of a misnomer (Higgins et al., 1990). These transporters consist of four protein domains: two integral, hydrophobic regions that are involved in translocation and two membrane-associated, hydrophilic regions that bind ATP. The functional domains may be contained on a single polypeptide or may be separated on up to four polypeptides. One common feature of these transporters is a highly conserved 200 amino acid sequence that binds ATP called the ATP-binding cassette

(ABC), hence, the proposal of Higgins et al. (1990) to rename this family, ABC transporters. There is strong evidence that ATP hydrolysis is essential for solute transport (Bishop et al., 1989; Mimmack et al., 1989), although it is possible that ATP is serving a regulatory or structural role. These systems include maltose transport system of E. coli and antibiotic export in Staphylococcus species.

In secondary transport systems, the accumulation of solutes is fueled by the protonmotive force or one of its components. In streptococci, most amino acids are transported in this fashion, as is the carbohydrate lactose in E. coli (West, 1970) and in S. lactis (Kashket and Wilson, 1972). Secondary active transport can be either uniport, symport or antiport. Uniport occurs when a charged ion is brought into the cell in response to the protonmotive force. Bacillus subtilis contains a uniport system for the uptake of calcium (Konings et al., 1987). Symport occurs when ions move into a cell down an electrochemical gradient which is coupled to the transport of a solute, as is the case for lactose in E. coli which is transported in symport with a proton. Antiport occurs by the simultaneous transport of 2 molecules in opposite directions with transport into the cell fueled by the movement of one down the electrochemical gradient. One example of this is the phosphate/hexose-6-phosphate antiporter in S. lactis (Maloney et al., 1984).

By applying the information provided by my studies together with the current knowledge of mechanisms of bacterial

transport, it is possible to speculate on the type of system in use for the accumulation of glucose in S. mutans when the PTS is repressed. The simplest type of transport known to occur in bacteria is passive diffusion. As stated, this process does not require energy and cannot transport glucose against a concentration gradient. Dashper and Reynolds (1990) have proposed that passive diffusion accounts for non-PTS glucose transport in S. mutans. On teleological grounds, relevant carbohydrate transport by passive diffusion in bacteria is a questionable process or as said by Saier (1985) "as transmembrane permeation represents the first step in the catabolism of exogenous sugars, one would expect the process to be subject to stringent regulatory control". This would exclude carbohydrate transport by passive diffusion since the only regulation of such transport would be through induction and repression. Saccharolytic oral bacteria are exposed to large, rapid carbohydrate fluxes in the oral cavity (Carlsson, 1986) and it is difficult to see how this process would allow for the stringent control suggested by Saier. Experimentally, it has been recognized that passive diffusion of carbohydrates is not a relevant physiological event (Kaback,1968; Roseman, 1969; Postma and Stock, 1980; Saier, 1985; Robillard et al., 1987) with the exception of the glycerol permease which mediates pore-type diffusion in several bacteria but not streptococci (Saier, 1985). This type of transport does not display counterflow, yet counterflow was detected in membrane vesicles of S. mutans

(Chapter 4, Figure 19), therefore, simple diffusion cannot be responsible for non-PTS glucose transport in S. mutans. Facilitated diffusion would be characterized by counterflow in the vesicles, and the presence of such a system would lead to rapid equilibration but not accumulation of glucose within the cell. In Chapter 2 (Table 5), I reported the concentrative uptake of glucose by S. mutans Ingbritt to levels 1000-fold higher than that in the culture medium when cells are grown in PTS-repressive conditions. Neither passive or facilitated diffusion would allow for this observation.

Streptococci already possess a group translocation system, namely the PTS for transport of glucose (Vadeboncoeur, 1984). A group translocation system would only allow for transport of glucose-6-P and would not explain the presence of free glucose reported in continuous culture in S. mutans as reported in Table 5. Iwami and Yamada (1980) also report significant levels of free glucose in S. mutans PK 1 grown in batch culture following incubation with glucose at acidic pH.

It could be argued that despite repression, residual PTS is responsible for the observed transport and that the counterflow seen in the vesicles of S. mutans (Chapter 4, Figure 19) was due to uncoupled EII^{glc} mediating glucose transport. This is also unlikely for several reasons. Studies with Salmonella typhimurium mutants defective in HPr and EI lost all ability to grow on PTS sugars unless they possessed alternative non-PTS mechanisms of transport (Postma

and Stock, 1980, Feldman et al., 1990). Mutants that regained the ability to grow on glucose were shown to harbor mutations in the gene coding for the EII^{glc} that uncoupled transport from phosphorylation and reduced the affinity for glucose 1000-fold (Postma, 1981). Other mutants of E. coli and Salm. typhimurium have been reported that contain EII enzymes able to mediate the facilitated diffusion of substrates, but in all cases, the mutations occur in the genes coding for the EII (Ruijter et al., 1990, 1991, 1992).

Robillard and co-workers (1990) have carried out extensive studies on wild-type EII for mannitol in E. coli both in vesicles and proteoliposomes. These workers have reported that some facilitated diffusion can be observed but at rates too low to sustain the organisms. Finally, S. mutans Ingbritt regulates the glucose-PTS at the genetic level predominantly through the EII enzyme and the EII is repressed 84-fold under conditions of growth similar to those used in our study (Hamilton et al., 1989). Thus, it appears highly unlikely that the carrier mediating transport is a membrane-associated EII enzyme.

Secondary solute transport dictates that solute transport is coupled to an electrochemical gradient and represents the protonmotive force-coupled model of transport. This type of transport process was extensively tested in Chapter 3 and by Dashper and Reynolds (1990) and found not to fuel glucose transport by S. mutans. Other types of secondary transport, that is, coupled to ions other than protons, would have been

detected since they would have responded to the electrical gradient ($\Delta\psi$) if not the pH gradient. No glucose transport in the presence of electrical gradients was seen. Therefore, secondary transport is not responsible for the glucose transport seen in PTS-repressed S. mutans.

The final type of glucose transport possible is a primary transport system of the phosphate-bond driven variety. Poolman and co-workers (1987a) describe this type of transport as the most difficult to demonstrate and study. A phosphate bond-driven model of glucose transport in S. mutans is supported by several observations. The results from Chapter 2 (Table 5) indicate the presence of an active transport system. Other possible modes of coupling have been tested and rejected as discussed above. Previously demonstrated sensitivity of glucose transport to ionophores was used to support the protonmotive force-coupled model but in Chapter 2, I discussed at length the difficulty in separating metabolic effects from direct transport effects. The decreased pH gradient in the presence of ionophores, particularly at acidic pH, strongly inhibited glycolysis and could have led to depletion of intracellular levels of ATP. In an ATP-linked transport system, these metabolic effects would directly inhibit transport by restricting available ATP. It is also ironic that the 2-deoxyglucose treatment used to deplete 'PEP pools' and cripple the PTS, can also deplete 'ATP pools', and as a consequence, the search for non-PTS transport would be inadvertently sabotaged by the

extensive use of pre-treatment of cells with 2-deoxyglucose. In addition, Poolman et al. (1987b) discussed at length the effects of internal and external pH on phosphate-bond driven transport. In S. cremoris, the affinity for L-leucyl-L-leucine increases with increasing pH over a range from pH 5.2 to 7.2 (van Boven and Konings, 1987). Because the substrate is neutral over this pH range, the observed alteration in affinity must be related to allosteric modification of the transporter. All phosphate bond-driven transport systems described to date operate optimally when the cell's internal pH is neutral or slightly alkaline (Poolman et al., 1987b). When grown at acidic pH, S. mutans maintains its internal pH slightly above that of the external environment. Therefore, this may act to restrict glucose transport when the bacterium is in an acidic environment and is exposed to high glucose and explain the decreased glycolytic rates and glucose transport rates at acidic pH described in S. mutans. In conclusion, the results obtained in Chapter 2 concerning pH and ionophore sensitivity of glucose transport in S. mutans, would all fit a model of phosphate-bond driven glucose transport.

The ABC family of phosphate-bond transporters is represented in S. mutans. Russell and co-workers (1992) identified a continuous sequence of genes in S. mutans responsible for the transport of melibiose, raffinose and isomaltotriose and the metabolism of melibiose, sucrose and isomaltosaccharides. An ATP-binding protein was coded for

that showed amino acid homology to the ABC of E. coli confirming the presence of this multiple sugar system in the ABC family. The multiple sugar transport system of S. mutans shows high specificity and does not transport glucose, therefore, it is not the non-PTS glucose transporter. Previous studies in other systems have suggested that the stoichiometry of ATP to solute in the ABC family of transporters may be as high as 2:1 (Higgins et al., 1990). This value is very high for the transport of a monosaccharide such as glucose, therefore, it is unlikely that glucose transport would be of this type. However, this could be resolved by probing the S. mutans chromosome with an ABC sequence probe.

Alternative phosphate-bond driven transport seems the most likely explanation of the present findings. The fluoride sensitivity of glucose transport supports this view. The PTS is fluoride sensitive due to the indirect inhibition of PEP generation by fluoride acting on enolase (Hamilton, 1990). However, the ability of fluoride to disrupt glucose transport in PTS-repressed cells suggests that it also has a detrimental effect on non-PTS transport. The lack of counterflow in whole cell experiments reported in Chapter 3 (Figure 15), may have been related to the ability of fluoride to substitute for ATP leading to the inactivation of transport. In 1982, when performing kinetic studies on glucose transport, Hamilton and St. Martin reported the loss of one glucose transport system following fluoride treatment

(shown to be the PTS) in S. mutans DR0001 and the PTS-defective mutant DR0001/6. In the presence of significantly higher fluoride, the same non-PTS system was identified in S. mutans Ingbritt by its identical Km. The lack of alteration in the K_m despite the greatly increased fluoride added and its obvious inhibitory effects could indicate non-competitive inhibition where fluoride inactivates the enzyme or in this case, the transporter, by binding at a site other than the glucose binding site and would be in keeping with a phosphate-bond driven system.

Russell (1990) reported the presence of a glucose permease in S. bovis. Transport was directly proportional to external sugar concentration to a maximum glucose concentration of 3.6 mM and saturation of the carrier. However he was able to demonstrate the presence of a carrier by performing counterflow studies using 2-deoxyglucose as a substrate. One reason for his success in demonstrating counterflow with whole cells may have been with his choice of iodoacetate and not fluoride as a metabolic inhibitor. Permeases mediating facilitated diffusion are not a common means of solute transport in bacteria and this is the first described. This transporter in S. bovis is not the same as the one identified in S. mutans for two reasons. First, it appears to mediate facilitated diffusion only and not the active transport described in Chapter 2 (Table 5). Secondly, the K_m of the S. bovis transporter is considerably higher (5 mM) than that of

the non-PTS glucose transporter described in S. mutans (62 μM) (Hamilton and St. Martin, 1982; Chapter 3, Figure 17).

Direct evidence for phosphate-bond driven glucose transport could be obtained with vesicle-loading experiments. The preliminary experiments described here involved attempts to reconstitute a glucose-phosphorylating system in vesicles derived from S. mutans. The point was to demonstrate active transport when the glucose entering the cell was converted by glucokinase in the presence of ATP to glucose-6-phosphate (Chapter 4). The lack of success was related to technical difficulties associated with the loading of vesicles. If the problems in loading could be corrected it could be possible to complete the experiment as described and obtain active transport. However, the critical experiment to prove phosphate-bond driven glucose transport in S. mutans would be the demonstration of active transport in vesicles loaded with ATP alone. Provided ATP were the energy donor or essential to the transport process, the ATP-loaded vesicles should accumulate glucose against a concentration gradient. It would be necessary to demonstrate that accumulated glucose was present in an unaltered form and not as a phosphate ester due to any residual glucokinase activity. In addition, while facilitated transport would occur regardless of the orientation of the vesicles, active transport would necessitate the presence of ATP on the cytoplasmic-facing side of right-side-out vesicles. The addition of ATP to everted glucose-loaded vesicles would result in rapid efflux.

If phosphate-bond driven glucose transport is occurring in S. mutans, this would be the first description of phosphate-bond driven glucose transport in bacteria. Streptococci do use this type of transport for accumulation of several other solutes including amino acids and ions (Poolman et al.,1987b).

The PTS system is a high affinity system that can operate to scavenge carbohydrates during fasting periods when carbohydrate availability is limited (Hamilton, 1987). However, when faced with high carbohydrate, S. mutans down-regulates PTS sugar transport to avoid metabolic overload that can be lethal to the bacteria in a process called "sugar-kill" (Carlsson, 1986). It is reasonable to assume that a second system with a lower affinity for glucose would then operate. Such as system was first identified in S. mutans DR0001 and in Chapter 3, was shown to exist in S. mutans Ingbritt. This second system would become of increasing relevance as the PTS is increasingly repressed and one mechanism for this is by increasing external glucose concentration. In the absence of metabolic energy, it mediates facilitated diffusion of glucose. However, the results from Chapter 2, indicate that active transport of glucose can be seen in PTS-repressed S. mutans. Therefore, in vivo, it is likely energy-coupled. Because this system would dominate when glucose is not limiting or when decreased growth rates would be advantageous for the bacterium, energy-coupled non-PTS transport is not unreasonable as it allows

for control of glucose transport at several levels which would not be possible with the model of simple diffusion postulated by others (Dashper and Reynolds, 1990). S. mutans in the oral cavity can face 10,000-fold fluxes in carbohydrate content and transport of this type allows further control on the rate of its transport and metabolism to avoid potential cell-poisoning when challenged with high sugar concentration. The precise mechanism is still unidentified but would work in concert with existing strategies such as PTS repression (Reizer et al., 1989a), regulation of glycolysis and synthesis of intracellular polysaccharides (Carlsson, 1986) to avoid sugar kill.

Chapter 6

Conclusions

Based on the research described in this thesis, I propose a model of phosphate-bond driven glucose transport in S. mutans (Figure 20). It is not clear whether ATP is the the phosphate donor or whether hydrolysis is essential for transport. This model is supported by the following observations:

1. Active transport has been shown to occur in S. mutans Ingbritt when it is grown in continous culture under conditions of excess glucose at neutral or acid pH (Table 5).
2. ATP becomes the dominant phosphate donor to glucose in cells grown in continuous culture with excess glucose at acidic pH or with high dilution (growth) rates (Table 4). When the PTS activity is reduced in S. mutans Ingbritt grown in continuous culture at neutral pH with excess glucose, ATP replaces PEP as the phosphoryl donor to glucose, indicating that a system is in place that could phosphorylate free glucose in the cell.
3. The observed sensitivity of glucose transport to various ionophores and metabolic inhibitors (Chapter 2) could be explained by my proposed model. The sensitivity of phosphate-driven transport systems to decreases in internal and external pH is well documented (Poolman et

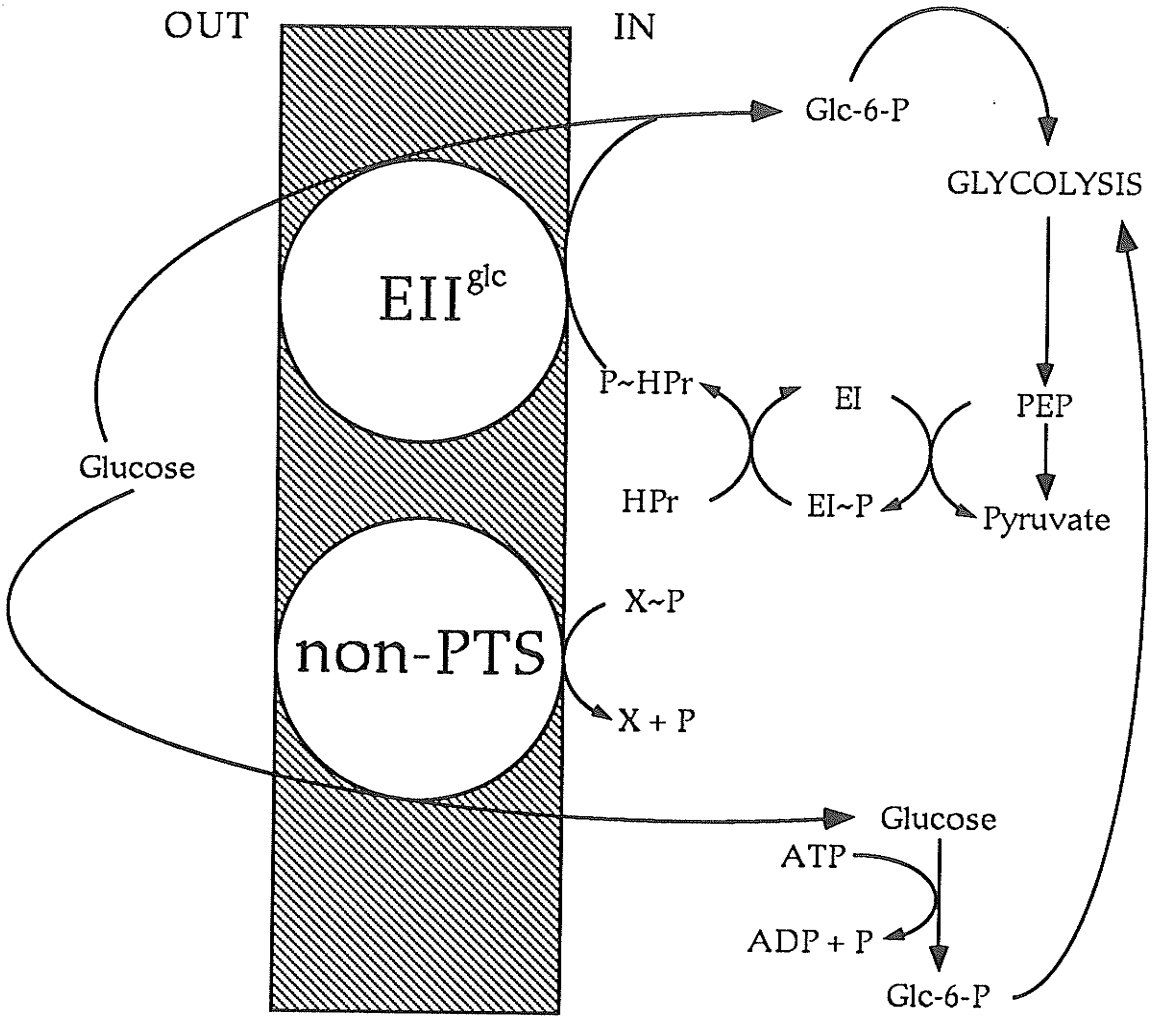

Figure 20. Glucose transport by Streptococcus mutans Ingbritt. Both PTS and phosphate-bond driven glucose transport model indicated.

al., 1987b). The effect of metabolic inhibitors could be explained by associated reductions in 'ATP pools' essential for the non-PTS glucose transport model.

4. Extrapolating from my studies on the kinetics of glucose transport in S. mutans Ingbritt (Figure 17) and previous studies using S. mutans DR0001 (Hamilton and St. Martin, 1982), It is suggested that fluoride may noncompetively inhibit glucose transport by binding at the ATP site in the non-PTS glucose transporter. This inhibition would be in keeping with the proposed model.

5. Other modes of energy coupling to the observed active transport of glucose have been tested and shown not to fuel glucose transport. The protonmotive force does not fuel glucose transport in S. mutans, therefore secondary active transport of glucose in symport with protons does not occur (Figure 13, Table 9). Any other group translocation process distinct from the glucose-PTS would not account for the high levels of free glucose found in the S. mutans Ingbritt grown in continuous culture under PTS repressed conditions excluding alternative group translocation as a possible form of glucose transport.

6. Unlike the PTS, the alternative glucose transport system is constitutive. This is demonstrated by glucose counterflow in vesicles derived from cells of S. mutans Ingbritt grown in continuous culture in conditions optimal for the PTS and conditions where the PTS is

repressed (Figure 19). The constitutive aspect of the non-PTS glucose transport would explain why glucose transport occurs at similar rates in PTS active cells following 2-deoxyglucose treatment and cells grown in PTS-repressed conditions (Figure 9).

7. The observed counterflow indicates that non-PTS carrier mediated glucose transport occurrs (Figure 19) and provides a definitive argument, along with the observed active transport of glucose(Table 5), against a proposed simple diffusion model of glucose transport (Dashper and Reynolds, 1990).

This model proposes that unlike the PTS where glucose transport and phosphorylation will require a single phosphate, one ATP or high energy equivalent may be required for transport and a second ATP to produce glucose-6-phosphate in preparation for catabolism. However, this non-PTS transport system would dominate when glucose is abundant and energy generation is not the sole metabolic imperative of the cell. This is already illustrated by the fact that with excess glucose, S. mutans Ingbritt switches from heterofermentation to the less efficient homofermentation, as maintaining redox balance and avoiding sugar kill become important (Carlsson and Griffith, 1974). The stoichiometry of the glucose to ATP in the proposed model would determine the effective cost to the cell. If a single ATP was coupled to transport of more than one molecule of glucose, the energetic cost would be reduced. By coupling transport to

ATP levels, glucose transport and therefore, metabolism would have an immediate, exquisite link to cell energy levels. The proposed phosphate-bond driven glucose transport would be very important to the pathogenicity of S. mutans since it would be the dominant glucose transport system in use in vivo in conditions of low pH and excess glucose when the bacterium is most cariogenic.

Bibliography

Abbe, K., S. Takahashi and T. Yamada, 1982. Involvement of Oxygen-Sensitive Pyruvate Formate-Lyase in Mixed Acid Fermentation by Streptococcus mutans Under Strictly Anaerobic Conditions. J. Bacteriol. 152: 175-182.

Abelson, D.C. and I.D. Mandel, 1981. The Effect of Saliva on Plaque pH in vivo. J. Dent. Res. 60: 1634-1638.

Abrams, A. and C. Jensen, 1984. Altered Expression of the H^+/ATPase in Streptococcus faecalis Membrane. Biochim. Biophys. Res. Commun. 122: 151-157.

Abrams, A. and J.B. Smith, 1974. Bacterial Membrane ATPases in The Enzymes 10 edited by P.D. Boyar, Academic Press Inc, New York, pp 395-429.

Amaral, D. and H.L. Kornberg, 1975. Regulation of the Fructose Uptake by Glucose in Escherichia coli. J. Gen. Microbiol. 90: 157-168.

Ambudkar, S.V. and P.C. Maloney, 1984. Characterization of a phosphate:hexose 6-phosphate antiport in Membrane Vesicles of Streptococcus lactis. J. Biol. Chem. 259: 2576-2585.

Andersson, L. and P. Lundahl, 1990. Substanial Glucose Leakage from Liposome on Filters and Upon molecular-Sieve Chromotography in Determinations of Reconstituted Glucose-transport Activity and Liposome Volumes. Biochim. Biophys. Acta 1030: 258-268.

Armitage, J.P., 1992. Behavioral Responses in Bacteria. Ann. Rev. Physiol. 54: 683-714.

Arnold,R.R., 1986. Innate Immunity and Streptococcus mutans in Molecular Microbiology and Immunobiology of Streptococcus mutans. Edited by S. Hamada, S.M. Michalek, H. Kiyono, L.Menaker and J. McGhee, Elsevier Science Publishers, Amsterdam, New York and Oxford, pp 421-432.

Atkinson, D.E., 1968. The High Energy Charge of the Adenylate Pool as a Regulatory Parameter. Interaction with Feedback Modifiers. Biochemistry 7: 4030-4034.

Backer-Dirks, O., 1966. Post-eruptive changes in enamel. J. Dent. Res. 45: 503-511.

Baird, J., V. Longyear and R. Ellwood, 1973. Water Soluble and Insoluble Glucans Produced by Extracellular Glucosyl

Tranferases from Streptococcus mutans. Microbios 8: 143-150.

Beckwith, J. R., 1970. Lac: the Genetic System in The Lac Operon. Edited by J.R. Beckwith and D. Zipser, Cold Spring Harbour Laboratory, New York, pp 5-26.

Beighton, D., H. Hayday, R.R.B. Russell and H.A. Whiley, 1984. Streptococcus macacae sp. nov. from Dental Plaque of Monkeys (Macaca fascicularis) Int. J. Syst. Bacteriol. 34: 332-335.

Belli, W.A. and R.E. Marquis, (1991). Adaptation of Streptococcus mutans and Enterococcus hirae to Acid Stress in Culture. Applied and Environ. Microbiol. 57: 1134-1138.

Bender, G.R., S.V.W. Sutton and R.E. Marquis, 1986. Acid Tolerance, Proton Permeabilities and membrane ATPases of Oral Streptococci. Infect. Immun. 53: 331-338.

Berger, E.A. and L.A. Heppel, 1974. Different Mechanisms of Energy Coupling for the Shock-Sensitive and Shock Resistant Amino Acid Permeases of Escherichia coli. J. Biol. Chem. 249: 7747-7755.

Bergey's Manual of Systematic Bacteriology Volume 2, 1986. Gram-Positive Cocci, pp 999-1103. Edited by P.H.A. Sneath. Williams and Wilkins, London, Los Angeles and Sydney.

Bergmeyer, H.U., 1974. Methods of Enzyme Analysis, Verlag Chemie International, Deerfield Beach, Florida.

Berman, K. and R. Gibbons, 1966. Iodiphilic Polysaccharide Synthesis by Rodent and Human Oral Bacteria. Arch. Oral. Biol.11: 533-542.

Bibby, B.G., 1938. The Bacterial Flora in Different Parts of the Mouth. J. Dent. Res. 17: 471-476.

Bibby, B.G., 1942. Preliminary Report on the use of Sodium Fluoride Application in Caries Prophylaxis. J. Dent. Res. 21: 661-668.

Bibby, B.G., 1979. Influence of Diet on the Bacterial Composition of Plaques in Proceedings "Microbial Aspects of Dental Caries". Edited by W.J. Loesche, H.M. Stiles and T.C. O'Brien. Sp. Supp. Microbiol. Abstr. II:477-490.

Birkhed, D. and U. Heintze, 1989. Salivary Secretion Rate, Buffer Capacity and pH in Human Saliva: Clinical Chemistry

and Microbiology, Volume 1. Edited by J.O. Tenovuo, CRC Press, Boca Raton, Florida, U.S.A, pp 26-73.

Bishop, L., R. Agbayani, S.V. Ambudkar, P.C. Maloney, and G.F-L. Ames, 1989. Reconstitution of a Bacterial Periplasmic in Proteoliposomes and Demonstration of ATP Hydrolysis Concomitant with Transport. Proc. Nat'l Acad. Sci. U.S.A. 84: 6953-6957.

Blomfield, J., E. W. van Lennep, E.W. Shorey, A.S., Malin, A.S. Dascalu and J.M. Brown, 1974. Ultrastructure of the in vitro formation of hydroxy-apatite in submandibular saliva of children with cystic fibrosis. Archs. Oral Biol. 19:1153-1157.

Booth, I.R. and W.J. Mitchell, 1986. Sugar Transport and Metabolism in the Clostridia in Sugar Transport and Metabolism in Gram-positive Bacteria. Edited by J. Reizer and A. Peterkofsky, Ellis Horwood, Chichester, U.K., pp 270-294.

Botsford, J.L. and J.G. Harman, 1992. Cyclic AMP in Prokaryotes. Microbiol. Rev. 56: 100-122.

Bowden, G.H.W., 1991. Which Bacteria are Cariogenic in Humans? in Dental Caries Vol.1, Markers of High and Low Risk Groups and Individuals. Edited by N.W. Johnson, Cambridge University Press, Cambridge,England, pp 266-286.

Bowden, G.H.W., D.C. Ellwood, and I.R. Hamilton, 1979. Microbial Ecology of the Oral Cavity, in Advances in Microbial Ecology, Volume 3. Edited by M. Alexander, Plenum Publishing Corporation, pp 135-217.

Bowden, G. H., J. M. Hardie and E. D. Fillery, 1976. Antigens from Actinomyces species and their value in identification. J. Dent. Res. 55: Special Issue A, A192-204.

Bowden, G.H.W., J.M. Hardie, A.S. McKee, P.D. Marsh, E.D. Fillery and G.L. Slack. 1976. The Microflora Associated with Developing Carious Lesions on the Distal Surfaces on the Upper First Premolars in 13-14-year-old Children; in Microbial Aspects of Dental Caries, Vol. 1, Microbiol. Abstr. Spec. Suppl: 223-241.

Bowden, G.H.W., A.R. Milnes and R. Boyar, 1984. Streptococcus mutans and Caries: State of the Art 1983 in Cariology Today. Edited by B. Guggenheim, Basel:Karger, pp 173-181

Boyar, R.M. and G.H. Bowden, 1985. The Microflora Associated with the Progression of Incipient Carious Lesions in Teeth of Children Living in a Water Fluoridated Area. Caries Res 19: 1298-1302.

Boyar, R.M., A. Thylstrup, L. Holman and G.H. Bowden, 1985. The Microflora Associated with the Development of Initial Enamel Decalcification below Orthodontic Bands in vivo in Children Living in a Fluoridated-Water Area. J. Dent. Res. 68: 1734-1738.

Boyd, W. and H. Sheldon, 1980. Introduction to the Study of Disease 8th Edition, Henry Kimpton Publishers, London.

Brady, L.J., D.A. Piacentini, P.J. Crowley, P.C.F. Oyston and A. S. Bleiweis, 1992. Differentiation of Salivary Agglutinin-Mediated Adherence and Aggregation of Mutans Streptococci by Use of Monoclonal Antibodies Against the Major Surface Adhesin P1. Infect. Immun. 60: 1008-1017.

Brandtzaeg, P., 1971. Human Secretory Immunoglobulins 7. Concentrations of Parotids IgA and Other Secretory Proteins in Relation to Rate of Flow and Duration of Secretory Stimulus. Arch. Oral Biol. 16: 1295-1304.

Brandtzaeg, P., 1989. Salivary Immunoglobulins in Human Saliva: Clinical Chemistry and Microbiology, Volume 2, edited by J.O. Tenovuo, CRC Press, Boca Raton, Florida, U.S.A., pp 2-40

Bratthall, D., 1991. The Global Epidemiology of Mutans Streptococci in Dental Caries Vol.1, Markers of High and Low Risk Groups and Individuals. Edited by N.W. Johnson, Cambridge University Press, Cambridge,England, pp 287-312.

Bridge, S.N. and P.H.A. Sneath, 1982. Streptococcus gallinarum sp. nov. and Streptococcus oralis sp. nov. Int. J. Syst. Bacteriol. 32: 410-415.

Brown A.T. and C.L. Wittenberger, 1972. Fructose-1,6-Diphosphate-Dependent Lactate Dehydrogenase from a Cariogenic Streptococcus: Purification and Regulatory Properties. J. Bacteriol. 110: 604-615.

Brown A.T. and C.L. Wittenberger, 1973. Mannitol and Sorbitol Catabolism in Streptococcus mutans. Arch. Oral Biol. 18: 117-126.

Calmes, R. and A.T. Brown, 1979. Regulation of Lactose Catabolism in Streptococcus mutans: Purification and Regulatory Properties of Phospho-beta-galactosidase. Infect. Immun. 23: 68-79.

Carlsson, J., 1967. A Medium for the Isolation of Streptococcus mutans. Archs. Oral Biol. 12: 1657-1658.

Carlsson, J. , 1986. Metabolic Activities of Oral Bacteria in Textbook of Cariology. Edited by A. Thylstrup and Ole Fejerskov, Munksgaard, Copenhagen, pp 74-106.

Carlsson, J., G. Grahmen, G. Jonsson and S. Wikner, 1970. Early Establishment of S. salivarius in the Mouths of Infants. Scand. J. Dent. Res. 49: 415-418.

Carlsson, J. and C.J. Griffith, 1974. Fermentation Products and Bacterial Yields in Glucose-Limited and Nitrogen-Limited Cultures of Streptococci. Archs. Oral Biol. 19: 1105-1109.

Carlsson, J. and U. Kujala, 1984. Pyruvate oxidase activity dependent on thiamine pyrophosphate, flavin adenine-dinucleotide and ortho-phosphate in Streptococcus sanguis. FEMS Microbiol. letters 25: 53-56.

Carlsson, J., U. Kujala and M.K. Edlund, 1985. Pyruvate Dehydrogenase Activity in Streptococcus mutans. Infect. Immun. 49: 674-678.

Chapman, R.L. and L.A. Staehelin, 1986. Freeze-fracture (-etch) Electron Microscopy in Ultrastructure Techniques for Microorganisms. Edited by H.C. Aldrich and W.J. Todd, Plenum Press, New York, pp 213-240.

Chassey, B., 1983. Sucrose Metabolism and Glucosyltransferase Activity in Oral Streptococci in Proceeding 'Glucosyltransferases, Glucans, Sucrose and Dental Caries, Editors: R.J.Doyle and J.E. Ciardi. Sp. Supp. Chemical Senses, pp 3-10.

Chassey, B. and A. Giuffrida, 1980. Method for the Lysis of Gram-Positive, Asporogenous Bacteria with Lysozyme. Appl. Environ. Microbiol. 39: 153-158.

Chia, J., T. Hsu, L. Teng, J. Chen, L. Hahn and C. Yang, 1991. Glucosyltransferase Gene Polymorphism among Streptococcus mutans Strains. Infect. Immun. 59: 1656-1660.

Clarke, J.K., 1924. On the Bacterial Flora in the Aetiology of Dental Caries. Brit. J. Exp. Path. 5: 141-147.

Collins and Dawes, 1987. The Surface Area of the Adult Human Mouth and Thickness of the Salivary Film Covering the Teeth and Oral Mucosa. J. Dent. Res. 66: 1300-1302.

Cowan, M., K.G. Taylor and R.J. Doyle, 1987. Energetics of the Initial Phase of Adhesion of Streptococcus sanguis to Hydroxylapatite. J. Bacteriol. 169: 2995-3000.

Coykendall, A.L., 1977. Proposal to Elevate the Subspecies of Streptococcus mutans to Species Status, Based on their Molecular Composition. Int. J. Syst. Bacteriol. 27: 26-30.

Coykendall, A.L. and M.L. Freedman, 1981. Colonization and Cariogenicity of S. ferus in Rats. Infect. Immun. 32: 80-85.

Coykendall, A.L. and K.B. Gustafson, 1986. Taxonomy of Streptococcus mutans in Molecular Microbiology and Immunobiology ofStreptococcus mutans. Edited by S. Hamada, S.M. Michalek, H.Kiyono, L. Menaker and J. McGhee, Elsevier Science Publishers, Amsterdam, New York and Oxford, pp 21-28.

Dashper, S.G. and E.C. Reynolds, 1990. Characterization of Transmembrane Movement of Glucose and Glucose Analogues in Streptococcus mutans Ingbritt. J. Bacteriol. 172: 556-563.

Dashper, S.G. and E.C. Reynolds, 1992. pH Regulation by S. mutans. J. Dent. Res. 71: 1159-1165.

Dawes, C., 1987. Physiological Factors Affecting Salivary Flow Rate, Oral Sugar Clearance, and the Sensation of Dry Mouth in Man. J. Dent. Res. (Spec. Iss) 66: 623-627.

Dawes, C., S. Watanabe, P. Bigelow-LeComte and G.H. Didbin, 1989. Estimation of the Velocity of the Salivary Film at Some Different Locations in the Mouth. J. Dent. Res. 68: 1479-1482.

de Jong, M.H., J.S. Van der Hoeven and J.H. van Os, 1986. Growth of Micro-Organisms from Supragingival Dental Plaque on Saliva Agar. J. Dent. Res. 65: 85-88.

de Soet, J.J., C. van Loveren, A.J. Lammens, M.J.A.M.P. Pavicic, C.H.E. Homburg, J.M. ten Cate, J. de Graaff, 1991. Differences in Cariogenicity Between Fresh Isolates of Streptococcus sobrinus and Streptococcus mutans. Caries Res.25:116-122.

Delmuth, D.R., E.E. Golub and D. Malalmud, 1990. Streptococcal-Host Interactions. J. Biol. Chem. 265:7120-7126.

Deutscher, J. and H. Sauerwald, 1986. Stimulation of Dihydroxyacetone/Glycerol Kinase Activity in Streptococcus faecalis by Phosphoenolpyruvate-Dependent Phosphorylation Catalyzed by EI and HPr of the Phosphotransferase System. J. Bacteriol. 166: 829-836.

Didbin, G.H. and R.P. Shellis, 1988. Physical and Biochemical Studies of Streptococcus mutans Sediments Suggest New Factors Linking the Cariogenicity of Plaque with its Extracellular Polysaccharide Content. J. Dent. Res. 67: 890-895.

Dills, S.S. and S. Seno, 1983. Regulation of Hexitol Catabolism in Streptococcus mutans. J. Bacteriol. 166: 861-866.

Dirksen, T.R., M.F. Little, B.G. Bibby and S.L. Crump, 1962. The pH of Carious Cavities-1, the Effect of Glucose and Phosphate Buffer on Cavity pH. Arch. Oral Biol. 7: 49-58.

Downie J.A., F. Gibson, and B.C. Graeme, 1979. Membrane Adenosine Triphosphatases of Prokaryotic Cells. Ann. Rev. Bioch. 48: 103-131.

Edgar, W.M., 1976. The Control of pH Changes in Human Dental Plaque. Caries Res. 10: 241-256.

Edgar, W.M. and S.M. Higham, 1991. Diet as a Determinant of Caries Risk in Dental Caries Vol.1, Markers of High and Low Risk Groups and Individuals. Edited by N.W. Johnson, Cambridge University Press, Cambridge,England, pp 218-251.

Edwardsson, S., 1968. Characteristics of Caries-inducing Human Streptococci Resembling Streptococcus mutans. Archs. Oral Biol. 13: 637-646.

Eisenberg, R.J. and K. Lillmars, 1975. A Method for the Gentle Lysis of Streptococcus sanguis and Streptococcus mutans. Biochim. Biophys. Res. Comm. 65: 378-384.

Elferink, M.G.L., A.J.M. Driessen and G.T. Robillard., 1990 Functional Reconstitution of the Purified Phosphoenolpyruvate-Dependent Mannitol Specific Transport System of Escherichia coli in Phospholipid Vesicles: Coupling Between Transport and Phosphorylation. J. Bacteriol. 172: 7119-7125.

Ellen, R.P., 1985. Specificity of Attachment as a Tissue-Tropic Influence on Oral Bacteria In Molecular Basis of Oral Microbial Adhesion. Edited by S.E. Mergenhagen and B. Rosen, Amer. Soc. Microbiol., Washington, D.C., pp 33-39.

Ellwood, D.C. and I. R. Hamilton, 1982. Properties of Streptococcus mutans Ingbritt Growing on Limiting Sucrose in a Chemostat: Repression of the Phosphoenolpyruvate Phosphotransferase Transport System. Infect. Immun. 36: 576-581.

Ellwood, D.C. P.J. Phipps and I. R. Hamilton, 1979. Effect of the Growth Rate and Glucose Concentration on the Activity of the Phosphoenolpyruvate Phosphotransferase System in Streptococcus mutans Ingbritt grown in Continuous Culture in the Chemostat. Infect. Immun. 23: 224-231.

Emilson, C.-G. and I. Thorselius, 1988. Prevalence of Mutans Streptococci and Lactobacilli in Elderly Swedish Individuals. Scan. J. Dent. Res. 96: 14-21.

Enright J.J., H.E. Friesell, and M.O. Trescher, 1932. Studies of the Cause and Nature of Dental Caries. J. Dent. Res. 12; 759-827.

Ferguson, D.B., 1989. Salivary Electrolytes in Human Saliva: Clinical Chemistry and Microbiology, Volume 1, edited by J.O. Tenovuo, CRC Press, Boca Raton, Florida, U.S.A., pp 76-99.

Fiske, C.H. and Y Subbarow, 1925. The Colorometric Determination of Phosphorous. J. Biol. Chem. 66: 375-400.

Fitzgerald, R.J., 1968. Gnotobiotic Contribution to Oral Microbiology. J. Dent. Res. 42: 549-552.

Fitzgerald, R.J., 1973. Brief History of Streptococcus mutans in *Streptococcus mutans* and Dental Caries, Proceedings of a Round Table Discussion, pp 1-2, DHEW Pub. No. (NIH) 74-286.

Fitzgerald, R.J., B.O. Adams and D.B. Fitzgerald, 1981. Cariogenicity of Human Plaque Lactobacilli in.the Gnotobiotic Rat. J. Dent. Res. 60: 919-926.

Fitzgerald, R.J., H.V. Jordan and H.R. Stanley, 1960. Experimental Caries and Gingival Pathologic Changes in the Gnotobiotic Rat. J. Dent. Res.: 923-935.

Fitzgerald, R.J. and P.H. Keyes, 1960. Demonstration of the Etiological Role of Streptococci in Experimental Caries in the Hamster. Jour. Am. Dent. Assoc. 61: 9-19.

Foster, J.W., 1991. Salmonella Acid Shock Proteins are Required for the Acid Tolerance Response. J. Bacteriol. 173 : 6896-6902.

Foster, J.W. and H.K. Hall, 1990. Adaptive Acidification Tolerance Response of Salmonella typhimurium. J. Bacteriol. 172: 771-778.

Foster, J.W. and H.K. Hall, 1991. Inducible pH Homeostasis and the Acid Tolerance Response of Salmonella typhimurium. J. Bacteriol. 173: 5129-5135.

Fox, D.K., N.D. Meadow and S. Roseman, 1986. Phosphate Transfer Between Acetate Kinase and EI of the Bacterial Phosphotransferase System. J. Biol. Chem. 261: 13498-13503.

Fraenkel, D.G., F. Falcoz-Kelly and B.L. Horecker, 1964. Utilization of Glucose 6-Phosphate by Glucokinaseless and Wild-type Strains of E. coli. Proc. Nat'l Acad. Sci. U.S.A. 52: 1207-1213.

Freedman, M.L., J.M. Tanzer and R.L. Eifert, 1976. Isolation and Characterization of Mutants of Streptococcus mutans with Defects Related to Intracellular Polysaccharide in Proceedings "Microbial Aspects of Dental Caries". Edited by H.M. Stiles, W.J. Loesche and T.C. O'Brien, Sp. Supp. Microbiol Abstr 3; 583-596.

Friberg, S., 1977. Colloidal Phenomena Encountered in the Bacterial Adhesion to the Tooth Surface. Swed. Dent. J. 1: 207-214.

Fukoshima, K., T. Ikeda and H. Kuramitsu, 1992. Expression of Streptococcus mutans *gft* Genes in Streptococcus milleri. Infect. Immun. 60: 2815-2822.

Futai, M., 1978. Experimental Systems for the Study of Active Transport in Bacteria in Bacterial Transport. Edited by B.P. Rosen, Marcel Dekker, Inc., New York, pp 7-41.

Futai, M. and H. Kanazawa, 1983. Structure and Function of the Proton-Translocating Adenosine Triphosphatase (F_0F_1): Biochemical and Molecular Biological Approaches. Microbiol. Rev. 47: 285-312.

Gachelin, G., 1970. Studies on the a-Methylglucoside Permease of Escherichia coli. Eur. J. Bioch. 16: 342-357.

Gauthier, L., D. Mayrand and C. Vadeboncoeur, 1984. Loss of Sensitivity to Xylitol by Streptococcus mutans LG-1. Caries Res. 18: 289-295.

Geddes, D.M., 1991. Methods for the Determining the Cariogenicity of Foodstuffs and their Use in Risk Determination in Dental Caries Vol.1, Markers of High and Low Risk Groups and Individuals. Edited by N.W. Johnson, Cambridge University Press, Cambridge,England, pp 252-265.

Gibbons, R.J., 1964. Bacteriology of Dental Caries. J. Dent. Res. 43: 1021-1028.

Gibbons, R.J., 1979. On the Mechanisms of Bacterial Attachment to Teeth. Proceedings "Saliva and Dental Caries". Edited by I. Kleinberg, S.A. Ellson and I.D. Mandel. Sp Supp. Microbiol. Abstracts, pp 267-273.

Gibbons, R.J., 1983. Importance of Glucosyltransferase in the Colonization of Oral Bacteria in Proceedings: Glucosyltransferases, Glucans, Sucrose and Dental Caries, Editors: R.J.Doyle and J.E. Ciardi. Sp. Supp. Chemical Senses, 1983, pp 11-20.

Gibbons, R.J., 1989. Bacterial Adhesion to Oral Tissue: A Model for Infectious Diseases. J. Dent. Res. 68: 750-760.

Gibbons, R.J., K.S. Berman, P. Knoettner and B. Kapsimalis, 1966. Dental Caries and Alveolar Bone Loss in Gnotobiotic Rats Infected with Capsule Forming Streptococci of Human Origin. Archs. Oral Biol. 11: 549-560.

Gibbons, R.J. and D.I. Hay, 1988. Human Salivary Acidic Proline-Rich Proteins and Statherin Promote the Attachment of Actinomyces viscosus LY7 to Apatic Surfaces. Infect. Immun. 56: 439-445.

Gibbons, R.J. and D.I. Hay, 1989. Adsorbed Salivary Acidic Proline-Rich Proteins Contribute to the Adhesion of Streptococcus mutans JBP to Apatic Surfaces. J. Dent. Res. 68: 1303-1307.

Gibbons, R.J., D.I. Hay and D.H. Schlesinger, 1991. Delineation of a Segment of Adsorbed Salivary Acidic Proline-Rich Proteins Which Promotes Adhesion of Streptococcus gordonii to Apatic Surfaces. Infect. Immun. 59: 2948-2954.

Gibbons, R.J., and B. Kapsimalis, 1963. Synthesis of Intracellular Iodophilic Polysaccharide by Streptococcus mitis. Archs. Oral Biol. 8: 319-329.

Gilbert, W. and B. Müller-Hill, 1970. The Lactose Repressor in The Lac Operon. Edited by J.R. Beckwith and D. Zipser, Cold Spring Harbour Laboratory, New York, pp 93-110.

Gilmour, M.N., T.S. Whittam, M. Kilian and R.K. Selander, 1987. Genetic Relationships among the Oral Streptococci. J. Bacteriol. 169: 5247-5257.

Goodman, H., J.J. Pollack, V.J. Iacono, W.Wong and G. Shockman, 1981. Peptidoglycan Loss During Hen Egg White Lysozyme-Inorganic Salt Lysis of Streptococcus mutans. J. Bacteriol. 146: 755-763.

Grübl, G., A.P. Vogeler, J.W. Lengeler, 1990. Involvement of the Histidine Protein (HPr) of the Phosphotransferase System in Chemotactic Signaling of Escherichia coli K12. J. Bacteriol. 172: 5871-5876.

Guggenheim, B., 1976. Ultrastructure and Some Biochemical Aspects of Dental Plaque: A Review in "Microbial Aspects of Dental Caries" edited by H.M. Stiles, W.J. Loesche and T.C. O'Brien, Sp. Supp. Microbiology Abstracts 1: 89-108.

Hamada, S. and H.D. Slade, 1980. Biology, Immunology and Cariogenicity of Streptococcus mutans. Microbiol. Rev. 44: 331-284.

Hamilton, I.R., 1976. Intracellular Polysaccharide Synthesis by Cariogenic Microorganisms in "Microbial Aspects of Dental Caries" edited by H.M. Stiles, W.J. Loesche and T.C. O'Brien, Sp. Supp. Microbiology Abstracts 3 683-701.

Hamilton, I. R. , 1984. Growth of the Oral "Pathogen", Streptococcus mutans, in Continuous Culture Reveals two Glucose Transport Systems in Continuous Culture 8: Biotechnology, Medicine and the Environment Edited by A.C.R. Dean, D.C. Ellwood and C.G.T. Evans, Ellis Harwood, Chichester, U.K, pp 58-71.

Hamilton, I. R. , 1986. Growth, Metabolism and Acid Production od Streptococcus mutans in Molecular Microbiology and Immunobiology of *Streptococcus mutans*. Edited by S. Hamada, S.M. Michalek, H. Kiyono, L. Menaker and J.R. McGhee, Elsevier Science Publishers, Amsterdam, pp145-155.

Hamilton, I. R., 1987. Effects of Changing Environment on Sugar Transport and Metabolism by Oral Bacteria in Sugar Transport and Metabolism in Gram-positive Bacteria. Edited by J. Reizer and A. Peterkofsky, Ellis Horwood, Chichester, U.K., pp 94-131.

Hamilton, I. R., 1990. Maintenance of Protonmotive Force by Streptococcus mutans and Streptococcus sobrinus during Growth in Continuous Culture. Oral Microbiol. Immunol. 5: 280-287.

Hamilton, I.R. and G. H. Bowden, 1982. Response of Freshly Isolated strains of Streptococcus mutans and Streptococcus mitior to Change in pH in the Presence and Absence of Fluoride during Growth in Continuous Culture. Infect. Immun. 36: 255-262.

Hamilton, I.R. and G. H. Bowden, 1992. Oral Microbiology in Encyclopedia of Microbiology, ed. J. Lederberg, Academic Press, Inc, San Diego, USA, pp 269-282.

Hamilton, I.R. and N.D. Buckley, 1991. Adaptation of Streptococcus mutans to Acid Tolerance. Oral Microbiol. Immun. 6 : 65-71.

Hamilton, I. R., L. Gauthier, B. Desjardins and C. Vadeboncoeur, 1989. Concentration-Dependent Repression of the Soluble and Membrane Components if the Streptococcus mutans Phosphoenolpyruvate:Sugar Phosphotransferase System by Glucose. J. Bacteriol. 171: 2942-2948.

Hamilton, I.R. and H. Lebtag, 1979. Lactose Metabolism by Streptococcus mutans: Evidence for Induction of the Tagatose 6-Phosphate Pathway. J. Bacteriol. 140: 1102-1104.

Hamilton, I.R. and G.Y.C. Lo, 1978. Co-induction of β-galactosidase and the Lactose-P-Enolpyruvate Phosphotransferase System in Streptococcus salivarius and Streptococcus mutans. J. Bacteriol. 136: 900-908.

Hamilton, I.R., P.J. Phipps and D.C. Ellwood, 1979. Effect of Growth Rate and Glucose Concentration on the Biochemical Properties of Streptococcus mutans Ingbritt in Continuous Culture. Infect. Immun. 26: 861-869.

Hamilton, I. R. and E.J. St. Martin, 1982. Evidence for the Involvement of Protonmotive Force in the Transport of Glucose by a Mutant of Streptococcus mutans Strain DR0001 defective in the Glucose Phosphoenolpyruvate Phosphotransferase System. Infect. Immun. 36: 567-575.

Hanada, N. and H.K. Kuramitsu, 1988. Isolation and Characterization of the Streptococcus mutans *gftC* gene, coding for synthesis of both soluble and insoluble glucans. Infect. Immun. 56: 1999-2005.

Hanada, N. and H.K. Kuramitsu, 1989. Isolation and Characterization of the Streptococcus mutans *gftD* gene, coding for primer-dependent soluble glucan synthesis. Infect. Immun. 57: 2079-2085.

Hardie, J.M. and G.H. Bowden, 1976. The Microflora of Dental Plaque: Bacterial Succession and Isolation Considerations in "Microbial Aspects of Dental Caries" edited by H.M. Stiles, W.J. Loesche and T.C. O'Brien, Sp. Supp. Microbiology Abstracts 1: 63-87.

Hardie, J.M., P.L. Thomson, R.J. South, P.D. Marsh, G.H. Bowden, A.S. McKee, E.D. Fillery and G.L. Slack, 1977. A Longitudinal Epidemiological Study on Dental Plaque and the Development of Dental Caries-Interim Results After Two Years. J. Dent. Res. Special Issue C: C90-C98.

Harold, F.M., 1977. Membranes and Energy Transduction in Bacteria. Curr. Topics in Bioenergetics 6: 83-149.

Harold, F.M. and Y. Kakinuma, 1985. Primary and Secondary Transport of Cations in Bacteria. Annals. New York Acad. Sci. 456: 375-383.

Harris, G.S., 1992. Characterization of a Glycogen Gene Locus Which Contributes to Streptococcus mutans Virulence. 92nd General Meeting of the Amer. Soc. Microbiol. D-111.

Harris, G.S., S.M. Michalek and R. Curtiss III, 1992. Cloning of a Locus Involved in Streptococcus mutans Intracellular Polysaccharide Accumulation and Virulence Testing of an Intracellular Polysaccharide-Deficient Mutant. Infect. Immun. 60: 3175-3185.

Hay, D.I. and E.C. Moreno, 1979. Macromolecular Inhibitors of Calcium Phosphate Precipitation in Human Saliva. Their Roles in Providing a Protective Environment for the Teeth. Proceedings "Saliva and Dental Caries". Edited by I. Kleinberg, S.A. Ellson and I.D. Mandel. Sp Supp. Microbiol. Abstracts, pp 45-58.

Henderson, P.J.F., 1990. Proton-Linked Sugar Transport Systems in Bacteria. J. Bioener. Biomemb. 22: 525-569.

Herbert, D., 1958. Some Principles of Continuous Culture in Recent Progress in Microbiology Edited by G. Tunewall, Almquist and Wikseil, Stockholm, p 381-396.

Higgins, C.F., S.C. Hyde, M.M. Mimmack, U. Gileadi, D.R. Gill and M.P. Gallagher, 1990. Binding Protein-Dependent Transport Systems. J. Bioenerg. Biomemb. 22: 571-592.

Hughes, C.V., R.N. Andersen and P.E. Kolenbrander, 1992. Characterization of Veillonella atypica PK1910 Adhesin-mediated Coaggregation with Oral Streptococcus spp. Infect. Immun. 60: 1178-1186.

Hughes, C.V., P. E. Kolenbrander, R.N. Andersen and L.V.H. Moore, 1988. Coaggregation Properties of Human Oral Veillonella spp.: Relationship to Colonization Site and Oral Ecology. Appl. Environ. Microbiol. 54: 1957-1963.

Huis in 't Veld, J.H.J. and O. Backer-Dirks, 1978. Intracellular Polysaccharide Metabolism in Streptococcus mutans. Caries Res. 12: 243-249.

Huis in 't Veld, J.H.J., W.H. van Palenstein Helderman and O. Backer Dirks, 1979. Streptococcus mutans and Dental Caries in Humans: a Bacteriological and Immunological study. Antonie van Leeuwenhoek 45: 25-33.

Hunter, J.R., J.K. Baird and D.C. Ellwood, 1973. Effect of Fluoride on the Transport of Sugars into Chemostat-Grown Streptococcus mutans. Abstr. Annu. Meet. Br. Assoc. Dent. Res., 99: 954.

Ikeda, T., T. Kurita and M. Hirasawa, 1988. Suppression of Streptococcus sobrinus 6715(g) in plaques by Streptococcus mutans 32K (c). J. Oral Pathol. 17: 471-474.

Ikeda, T., H.J. Sandham and E.L. Bradley, Jr., 1973. Changes in Streptococcus mutans and Lactobacilli in Relation to the Initiation of Dental Caries in Negro Children. Arch. Oral Biol.18: 555-566.

Inoue, M. and E.E. Smith, 1976. Extracellular Polysaccharide Formed by Dextransucrase Isozymes of Cariogenic Microorganisms in "Microbial Aspects of Dental Caries" Edited by H.M. Stiles, W.J. Loesche and T.C. O'Brien, Sp. Supp. Microbiology Abstracts 3 665-682.

Iwami, Y., 1988. Influence of Environment on Glycolysis in Streptococci. The Kitasato Archives Exp. Med. 61: 1-20.

Iwami, Y., T. Yamada and S. Araya, 1975. Glycolytic Intermediates in Streptococcus mutans PK 1. Archs. Oral Biol. 20: 695-697.

Iwami, Y. and T. Yamada, 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. Archs. Oral Biol. 25: 163-169.

Izumiya, N, T. Kato, H. Aoyagi, M. Waki and M. Kondo, 1979. Modes of Action of Gramicidin S and Tyrocidines in Synthetic Aspects of Biologically Active Cyclic Peptides-Gramicidin S and Tyrocidins, A Halsted Press Book, John Wiley and Sons, New York, pp 99-108.

Jenkinson, H.F., 1992. Adherence, Coaggregation, and Hydrophobicity of Streptococcus gordonii Associated with Expression of Cell Surface Lipoproteins. Infect. Immun. 60: 1225-1228.

Jones, C.W., 1980. Unity and Diversity in Bacterial Energy Conservation in Contemporary Microbial Ecology. Edited by D.C. Ellwood, M.J. Lantern, J.N. Hedger, J.M. Lynch and J.W. Slater. Academic Press, London, U.K, pp 193-213.

Kaback, H.R., 1960. Uptake of Amino Acids by "Ghosts" of Mutant Strains of E. coli. Fed. Proc. 19: 130.

Kaback, H.R., 1968. The Role of the Phosphoenolpyruvate-phosphotransferase System in the Transport of Sugars by Isolated Membrane Preparations of Escherichia coli. J. Biol. Chem. 243: 3711-3724.

Kaback, H.R., 1970. Transport. Ann. Rev. Bioch. 39: 561-598.

Kaback, H.R., 1972. Transport Across Isolated Bacterial Cytoplasmic Membranes. Biochim. Biopys. Acta 265: 367-416.

Kaback, H.R., 1983. The *lac* Carrier Protein in Escherichia coli. J. Memb. Biol. 76: 95-112.

Kashket, E., 1985. The Protonmotive Force in Bacteria: A Critical Assessment of Methods. Ann. Rev. Microbiol.39: 219-242.

Kashket, E.R. and S.L. Barker, 1977. Effects of Potassium Ions on the Electrical and pH Gradients Across the Membrane of Streptococcus lactis cells. J. Bacteriol. 130: 1017-1023.

Kashket, E.R.and T.H. Wilson, 1972. Galactoside Accumulation Associated with Ion Movements in Streptococcus lactis . Bioch. Biophys. Res. Commun. 49: 615-620.

Kashket, E.R. and T.H. Wilson, 1973. Proton-Coupled Accumulation of Galactoside in Streptococcus lactis 7962. Proc. Nat'l Acad. Sci. U.S.A. 70: 2866-2869.

Keevil, C.W. and I.H. Hamilton, 1984. Comparison of the polyvinyl chloride membrane electrodes sensitive to alkylphosphonium ions for the determination of the electrical difference ($\Delta\psi$) of Streptococcus mutans and Lactobacillus casei. Anal. Bioch. 139: 228-236.

Keevil, C.W., A.S. McDermid, P.D. Marsh and D.C. Ellwood, 1986. Protonmotive Force driven 6-deoxyglucose Uptake by the Oral Pathogen, Streptococcus mutans Ingbritt. Arch. Microbiol. 146: 118-174.

Keevil, C.W., M.I. Williamson, P.D. Marsh and D.C. Ellwood, 1984. Evidence that Glucose and Sucrose Uptake in Oral Streptococcal Bacteria Involves Independent Phosphotransferase and Proton-Motive Force-Mediated Mechanisms. Archs. Oral. Biol. 29: 871-878.

Kennedy, E.P., 1970. The Lactose Permease System of E. coli in The Lactose Operon. Edited by J.R. Beckwith and D. Zipser, Cold Spring Harbour Laboratory, New York, pp 49-92.

Khan, S. and H.C. Berg, 1983. Isotope and Thermal Effects in Chemiosmotic Coupling to the Membrane ATPase of Streptococcus. J. Biol. Chem. 258: 6709-6712.

Khandelwal, R.J. and I.R. Hamilton, 1971. Purification and Properties of Adenyl Cyclase from Streptococcus salivarius. J. Biol. Chem. 246 : 3297-3304.

Khandelwal, R.J., T.N. Spearman and I.R. Hamilton, 1973. Purification and Properties of Glycogen Phosphorylase from Streptococcus salivarius. Arch. Bioc. Biop. 154: 295-305.

Kingsley, G.R. and G. Getchell, 1960. Direct Ultra Micro Glucose Oxidase Method for Determination of Glucose in Biological Fluids. Clin. Chem. 6: 466-475.

Kinoshita, N., T. Unemoto and H. Koyabashi, 1984. Sodium-stimulated ATPase in Streptococcus faecalis. J. Bacteriol. 158: 844-848.

Kishimoto, E., D.I. Hay and R. Kent, 1990. Polymorphism of Submandibular-Sublingual Salivary Proteins which Promote Adhesion of Streptococcus mutans Serotype-c Strains to Hydroxyapatite. J. Dent. Res. 11: 1741-1745.

Kleinberg, I., J.A. Kanapka and D. Craw, 1976. Effects of Saliva and Salivary Factors on the Metabolism of the Mixed Oral Flora. In Proceedings "Microbial Aspects of Dental Caries". Edited by H.M. Stiles, W.J. Loesche and T.C. O'Brien, Sp. Supp. Microbiol. Abstr. 2: 433-464.

Kligler, I.J., 1915. A Biochemical Study and Differentiation of Oral Bacteria with Special Reference to Dental Caries. J. Allied Dent. Sci. 10: 282-332.

Kobayashi, H. and T. Unemoto, 1980. Streptococcus faecalis Mutants Defective in Regulation of Cytoplasmic pH. J. Bacteriol. 143: 1187-1193.

Kobayashi, H., J. Van Brunt and F.M. Harold, 1978. ATP-linked Calcium Transport in Cells and Membrane Vesicles of Streptococcus faecalis. J. Biol. Chem. 253: 2085-2092.

Konings, W.N., A. Bisshop, M. Veenhuis and C.A. Vermeulen, 1973. New Procedure for the Isolation of Membrane Vesicles of Bacillus subtilis and an Electron Microscopy Study of their Ultrastructure. J. Bacteriol. 116 : 1456-1465.

Konings, W.N., W. de Vrij, A.J.M. Driessen and B. Poolman, 1987. "Primary and Secondary Transport in Gram-Positive Bacteria" in Sugar Transport and Metabolism in Gram Positive Bacteria, edited by J. Reizer and A. Peterkofsky, Ellis Horwood Ltd, Chichester, England, pp 270-294.

Köhler, B. and S. Bjarnason, 1987. Mutans Streptococci, Lactobacilli and Caries Prevalence in 11 and 12-year-old Icelandic children. Comm. Dent. Oral. Epidemiol. 15: 332-335.

Kundig, W., S. Ghosh and S. Roseman, 1964. Phosphate Bound to a Histidine in a Protein as an Intermediate in a Novel Phospho-Transferase System. Proc. Nat'l Acad. Sci. U.S.A. 52: 1067-1074.

Lebet and J.B Rottenstein, 1872. Dental Caries and its Causes. An Investigation into the Influence of Fungi in the Destruction of the Teeth. Translated by T.H. Chandler, Philadelphia, Lindsay and Blakiston, Buffalo.

Lehninger, A., 1982. Principles of Biochemistry, Worth Publishers Inc., New York, U.S.A.

Leingruber, R.M., C. Jensen and A. Abrams, 1981. Purification and Characterization of the Membrane Adenosine Triphosphatase Complex for the Wild-type and N,N'-dicyclohexylcarbodiimide Resistant Strains of Streptococcus faecalis. J. Bacteriol. 147: 363-372.

Lengeler, J.W., 1990. Molecular Analysis of the Enzyme II-complexes of the Bacterial Phosphotransferase System (PTS) as Carbohydrate Systems. Biochim. Biophys. Acta 1018: 155-159.

Lengeler, J. and H Steinberger, 1978. Analysis of the Regulatory Mechanisms Controlling the Activity of the Hexitol Transport Systems in Escherichia coli K12. Mol. Gen. Genet. 167: 75-82

Liberman, E.S. and A.S. Bleiweis, 1984a. Role of the Phosphoenolpyruvate-Dependent Glucose Phosphotransferase System of S. mutans GS5 in the Regulation of Lactose Uptake. Infect. Immun. 43: 536-542.

Liberman, E.S. and A.S. Bleiweis, 1984b. Transport of Glucose and Mannose by a Common Phosphoenolpyruvate-dependent Phosphotransferase System in Streptococcus mutans GS5. Infect. Immun. 43: 1106-1109.

Liljemark, W.F. and S.V. Schauer, 1977. Competitive Binding Among Streptococci to Hydroxyapatite. J. Dent. Res. 56: 157-165.

Lindquist, B. and C.G. Emilson, 1991. Interactions Between and Within Streptococcus mutans and Streptococcus sobrinus Isolated from Humans harboring Both Species. Scand. J. Dent. Res. 99: 498-504.

Littleton, N.W., R.M. McCabe and C.H. Carter, 1967. Studies of the Oral Health in Persons Nourished by Stomach Tube II- Acidogenic Properties andSelected Bacterial Components of Plaque Material. Archs. Oral. Biol. 12: 601-609.

Lodge, J. and G.R. Jacobson, 1988. Starvation-Induced Stimulation of Sugar Uptake in Streptococcus mutans is due to an Effect on the Activities of Preexisting Proteins on the Phosphotransferase System. Infect. Immun. 56: 2594-2600.

Loesche, W.J. 1986. Role of Streptococcus mutans in Human Dental Decay. Microbiol. Rev. 50: 353-380.

Loesche, W.J., J. Rowan, L.H. Straffon and P.J. Loos, 1975. Association of Streptococcus mutans with Human Dental Decay. Infect. Immun. 11:1252-1260.

Loesche, W.J. and L.H. Straffon, 1979. Longitudinal Investigation of the Role of Streptococcus mutans in Human Fissure Decay. Infect. Immun. 26:498-507.

Loesche, W.J. and S.A. Syed, 1973. The Predominant Cultivatable Flora of Carious Plaque and Carious Dentine. Caries Res. 7: 201-216.

Lolkema, J.S., D.S. Dijkstra and G.T. Robillard, 1992. Mechanics of the Solute Translocation Catalyzed by Enzyme II^{mtl} of the Phosphoenolpyruvate-Dependent Phosphotransferase System of Escherichia coli. Biochemistry 31: 5514-5521.

Lolkema, J.S. and G.T. Robillard, 1990. Subunit Structure and Activity of the Mannitol-Specific Enzyme II of the Escherichia coli Phosphoenolpyruvate-Dependent Phosphotransferase System Solubilized in Detergent. Biochemistry. 29: 10120-10125.

Macpherson, L.M.D., T.W. MacFarlane, D.M. Geddes, K.W. King, 1992. Assessment of the Cariogenic Potential of Streptococcus mutans Strains and its Relationship to in vivo Caries Experience. Oral. Microbiol. Immunol. 7: 142-147.

Macpherson, L.M.D., T.W. MacFarlane and K.W. Stephen,1991. An in Situ Microbiological Study of the Colonisation of Human Enamel Surfaces. Microbial Ecology in Health and Disease 4: 39-44.

Magasanik, B., 1970. Glucose Effects: Inducer Exclusion and Repression in The Lac Operon. Edited by J.R. Beckwith and D. Zipser, Cold Spring Harbour Laboratory, New York, pp 189-220.

Makman, R. and E. Sutherland, 1965. Adenosine 3'-5' phosphate in Escherichia coli. J. Biol. Chem. 240: 1309-1314.

Maloney, P.C., 1977. Obligatory Coupling Between Proton Entry and the Synthesis of Adenosine 5'-Triphosphate in Streptococcus lactis J. Bacteriol. 132: 564-575.

Maloney, P.C., 1982. Coupling between H^+-entry and ATP Synthesis in Bacteria. Curr. Topics Memb. Trans. 16: 175-193.

Maloney, P.C., 1983. Relationship Between Phosphorylation Potential and Electrochemical H^+ Gradient During Glycolysis in Streptococcus lactis. J. Bacteriol. 153: 1461-1470.

Maloney, P., S.V. Ambudkar, J. Thomas and L. Schiller, 1984. Phosphate/Hexose 6-Phosphate Antiport in Streptococcus lactis. J. Bacteriol. 158: 238-245.

Maloney, P.C., E.R. Kashket and T.H. Wilson, 1975. Methods for Studying Transport in Bacteria. Methods Memb. Biol. 5: 1-49.

Maloney, P.C., and T.H. Wilson, 1973. Quantitative Aspects of Active Transport by the Lactose Transport System of E. coli. Biochim. Biophys. Acta 330: 196-205.

Maloney, P.C. and T.H. Wilson, 1975. ATP Synthesis Driven by a Protonmotive Force in Streptococcus lactis. J. Membrane Biol. 25: 285-310.

Mandel, I.D., 1987. The Functions of Saliva. J. Dent. Res. (Spec. Iss) 66 : 623-627.

Marsh P.D and C. W. Keevil, 1986. The Metabolism of Oral Bacteria in Health and Disease, Chapter 11 in Microbial Metabolism in the Digestive Tract, edited by M.J. Hill, CRC Press, Florida, U.S.A., pp 155-182.

Marsh, P.D. and M. Martin, 1992. Oral Microbiology, Aspects of Microbiology, Thomas Nelson and Sons, Canada.

Martin, N.L. and T.J. Beveridge, 1986. Gentamicin Interaction with Pseudomonas aeruginosa Cell Envelope. Antimicrob. Agents Chemother. 29: 1079-1087.

McIntire, F.C., 1985. Specific Surface Components and Microbial Coaggregation. In Molecular Basis of Oral Microbial Adhesion eds. S.E. Mergenhagen and B. Rosen, Amer. Soc. Microbiol., Washington, D.C., pp 153-159.

Meadow, N.D, D.K Fox, and S. Roseman, 1990. The Bacterial Phosphoenolpyruvate:Glycose Phosphotransferase System. Ann. Rev. Biochem. 59: 497-542.

Meckel, A.H., 1965. The Formation and Properties of Organic Films on Teeth. Arch. Oral Biol. 10: 585-598.

Michels, P.A.M., J.P.J. Michels, J. Boonstra and W.N. Konings, 1979. Generation of an Electrochemical Proton Gradient in Bacteria by the Excretion of Metabolic End-Products. FEMS Microbiol. Lett. 5: 357-364.

Mikkelsen, L. and S. Poulsen, 1976. Microbiological Studies on Plaque in Relation to Development of Dental Caries in Man. Caries Res. 10: 178-188.

Miller, W.D., 1889. Mikrooganismen der Mundhöhle, Georg Thieme, Leipzig.

Mimmack, M.L., M.P. Gallagher, S.C. Hyde, S.R. Pearce, I.R. Booth and C.F. Higgins, 1989. Energy coupling to Periplasmic Binding Protein-Dependent Transport Systems:

Stoichiometry of ATP Hydrolysis During Transport in vivo. Proc. Nat'l Acad. Sci. U.S.A. 86: 8257-8261.

Mitchell, P., 1962. Molecule, Group and Electron Translocation through Natural Membranes. Biochem. Soc. Symp. 22: 142-169.

Mitchell, P., 1972. Chemiosmotic Coupling in Energy Transduction: a Logical Development of Biological Knowledge. Bioenergetics: 3: 5-24.

Monod, J., 1942. Recherches sur la Croissance des Cultures Bactériennes. Hermann et Cie, Paris.

Mooser G. and C. Wong, 1988. Isolation of a Glucan-Binding Domain of Glucosyltranferase (α1,6-Glucan Synthase) from Streptococcus sobrinus. Infect. Immun. 56: 880-884.

Mouton, C., H. Reynolds and R.J. Genco, 1977. Combined Micromanipulation, Culture, and Immunofluorescent Techniques for the Isolation of the Coccal Organisms Comprising the "Corn-cob" Configuration of Human Dental Plaque. J. Biol. Buccale 5: 321-332.

Munoz, E., 1982. Polymorphism and Conformational Dynamics of F_1-ATPases for Bacterial Membranes: A Model for the Regulation of these Enzymes on the Basis of Molecular Plasticity. Biochim. Biophys. Acta 650 : 233-265.

Munro, C., S.M. Michalek, and F.L. Macrina, 1991. Cariogenicity of Streptococcus mutans V403 Glucosyltransferase and Fructosyltransferase Mutants Constructed by Allelic Exchange. Infect. Immun. 59: 2316-2323.

Murakami, Y.,H. Nagata, A.Amano, M. Takagaki, S. Shizukuishi, A. Tsunemitsu and S. Aimoto, 1991. Inhibitory Effects of Human Salivary Histatins and Lysozyme on Coaggregation between Porphyromonas gingivalis and Streptococcus mitis. Infect. Immun. 59: 3284-3286.

Murray Prokobphol, T. Lee, C.I. Hoover and S.J. Fisher, 1992. Adherence of Oral Streptococci to Salivary Glycoproteins. Infect. Immun. 60: 31-38.

Neidhart, F.C., J.L. Ingraham and M. Schaechter, 1990. Physiology of the Bacterial Cell: A Molecular Approach, Sinauer Associates, Inc., Sunderland, Mass., U.S.A.

Nelson, S.O., J.K. Wright and P.W. Postma, 1983. The Mechanism of Inducer Exclusion. Direct Interaction Between Purified III^{Glc} of the Phosphoenolpyruvate:sugar

Phosphotransferase System and the Lactose Carrier from Escherichia. coli. EMBO J. 2: 715-720.

Newbrun, E. 1976. Polysaccharide Synthesis in Plaque. In Proceedings "Microbial Aspects of Dental Caries". Edited by H.M. Stiles, W.J. Loesche and T.C. O'Brien, Sp. Supp. Microbiol. Abstr. 3: 649-664.

Newbrun, E. 1989. Cariology , second edition, Quintessence Publishing Co, Chicago, USA.

Newman, M.J. and T.H. Wilson, 1980. Solubilization and Reconstitution of the Lactose Transport System from Escherichia. coli. J. Biol. Chem. 255: 10583-10586.

Néron, S. and C. Vadeboncoeur, 1987. Two Functionally Different Glucose Phosphotransferase.Transport Systems in Streptococcus mutans and Streptococcus sobrinus. Oral Microbiol. Immunol. 2: 171-177.

Nicholls, D., 1982. Bioenergetics: An Introduction to the Chemiosmotic Theory. Academic Press, London and New York.

Nyvad, B. and M. Kilian, 1987. Microbiology of the Early Colonization of Human Enamel and Root Surfaces in vivo. Scand. J. Dent. Res. 95: 369-380.

Oppenheim, F.G., T. Xu, F.M. McMillan, S.M. Levitz, R.D. Diamond, G.D. Offner and R.F. Troxler, 1988. Histatins, A Novel Family of Histidine-Rich Proteins in Human Parotid Secretion. Isolation, Characterization, Primary-Structure and Fungistatic Effects on Candida albicans. J. Biol. Chem. 263: 7472-7477.

Orland, F.J., J.R. Blayney and W.R. Harvison, 1954. Use of the Germ-free Animal Technique in the Study of Experimental Dental Caries. I. Basic Observations in Rats Reared Free from all Microorganisms. J. Dent. Res. 33: 147-174.

Ostrom, C.A., T. Koulourides, F. Hickman and J. McGhee, 1977. Microbial Characterization of an Experimental Cariogenic Plaque in Man. J. Dent. Res. 56: 550-558.

Otto, R., B. Klont, B. ten Brink, and W.N. Konings, 1984. The Phosphate Potential, Adenylate Charge and Protonmotive Force in Growing Cells of Streptococcus cremoris. Arch Microbiol. 139: 338-343.

Otto, R., R.G. Lageveen, H. Veldkamp, and W.N. Konings, 1982. Lactate Efflux-Induced Electrical Potential in Membrane

Vesicles of Streptococcus cremoris. J. Bacteriol. 149: 733-738.

Örstavik, D. and F.W. Kraus, 1974. The Acquired Pellicle. Enzyme and Antibody Activities. Scand. J. Dent. Res. 82: 202-205.

Padan, E., 1984. Adaptation of Bacteria to External pH in Current Perspectives in Microbial Ecology. Edited by M.J. Klug and C.A. Reddy. Amer. soc. for Microbiol., Washington, D.C., pp 49-55

Padan, E., D. Zilberstein and S. Schuldiner, 1981. pH Homeostasis in Bacteria. Biochim. Biophys. Acta 650: 151-166.

Pastan, I. and S. Adhya, 1976. Cyclic-Adenosine 5'-monophosphate in Esherichia. coli. Bacteriol. Rev. 40: 527-551.

Pastan, I. and R.L. Perlman, 1970. Cyclic Adenosine Monophosphate in Bacteria. Science 169: 339-344.

Peterson, G.L., 1983. Determination of Total Protein. Methods in Enzymology, 91: 95-119.

Pearce, E.I.F., 1991. Salivary Inorganic and Physical Factors in the Aetiology of Dental Caries, and their Role in Prediction, pp 358-381 in Dental Caries Vol.1, Markers of High and Low Risk Groups and Individuals. Edited by N.W.Johnson, Cambridge University Press, Cambridge,England.

Poolman B., A.J.M. Driessen and W.N. Konings, 1987a. Regulation of Solute Transport in Streptococci by External and Internal pH Values. Microbiol. Rev. 51: 498-508.

Poolman B., A.J.M. Driessen and W.N. Konings, 1987a. Regulation of Solute Transport in Streptococci by External and Internal pH Values. Microbiol. Rev. 51: 498-508.

Poolman B., K.J. Hellingwerf and W.N. Konings, 1987b. Regulation of the Glutamate/Glutamine Transport System by the Intracellular pH in Streptococcus lactis. J. Bacteriol. 169: 2272-2276.

Porter, V.E. , B.M. Chassey and C.E. Holmlund, 1980. Partial Purification and Properties of a Specific Glucokinase from Streptococcus mutans SL-1. Biochim. Biophys. Acta 611: 289-298.

Postma, P.W., 1981. Defective Enzyme II-B^{Glc} of the Phosphoenolpyruvate:Sugar Phosphotransferase System Leading to the Uncoupling of Transport and Phosphorylation in Salmonella. typhimurium. J. Bacteriol. 147: 382-389.

Postma, P.W. and J.W. Lengeler, 1985. Phosphoenolpyruvate:Carbohydrate Phosphotransferase System of Bacteria. Microbiol. Rev. 49: 232-269.

Postma, P.W. and S. Roseman, 1976. The Bacterial Phosphoenolpyruvate:Sugar Phosphotransferase System. Biochim. Biophys. Acta 457: 213-257.

Postma, P.W. and J.B. Stock, 1980. Enzymes II of the Phosphotransferase System do not Catalyze Sugar Transport in the Absence of Phosphorylation. J. Bacteriol. 141: 476-484.

Reizer, J., J. Deutscher, S. Sutrina, J. Thompson and M. Saier, Jr., 1985. Sugar Accumulation in Gram Positive Bacteria: Exclusion and Expulsion Mechanisms. Trends. Biochem. Sci. 10: 32-35.

Reizer, J., M.J. Novotny, W. Hengstenberg, C. Panos and M.J. Saier Jr., 1983. Mechanism of Inducer-Expulsion in Streptococcus pyogenes: a Two-Step Process Activated by ATP. J. Bacteriol. 160: 354-361.

Reizer, J. and C. Panos, 1980. Regulation of β-Galactosidase Phosphate Accumulation in Streptococcus pyogenes by an Expulsion Mechanism. Proc. Nat'l Acad. Sci. U.S.A. 77: 5497-5501

Reizer, J., M. H. Saier Jr., J. Deutscher, F. Grenier, J. Thompson and W. Hengstenberg, 1989a. The Phosphoenolpyruvate Phosphotransferase System in Gram-positive Bacteria: Properties, Mechanism and Regulation. CRC Crit. Rev. Microbiol. 15: 297-338.

Reizer, J., S.L. Sutrina, M.H. Saier, G.C. Stewart, A. Peterkofsky and P. Reddy, 1989b. Mechanistic and Physiological Consequences of HPr(ser) Phosphorylation on the Activities of the Phosphoenolpyruvate:Sugar Phosphotransferase system in Gram-Positive Bacteria: Studies with Site-Specific mutants of HPr. EMBO J. 8: 2111-2120.

Ritz, H.L., 1967. Microbial Population Shifts in Developing Human Dental Plaque. Arch. Oral Biol. 12: 1561-1568.

Rodrigue, L., L. Lacoste, L. Trahan and C. Vadeboncoeur, 1988. Effect of Nutritional Constraints on Biosynthesis

of the Components of the Phosphoenolpyruvate:Sugar Phosphotransferase System in a Fresh Isolate of Streptococcus mutans. Infect. Immun. 56: 518-522.

Roseman, S., 1969. The Transport of Carbohydrates by a Bacterial Phosphotransferase System. J. Gen. Physiol. 54: 138-184.

Rottenberg, H., 1979. The Measurement of Membrane Potential and pH in Cells, Organelles, and Vesicles. Meth. Enzym. 55: 547-569.

Rölla, G., J.E. Ciardi and W.H. Bowen, 1983. Identification of IgA, IgG, lysozyme, albumin, α-amylase and glucosyltransferase in the protein layer adsorbed to hydroxyapatite from whole saliva. Scand. J. Dent. Res. 91: 186-190.

Rölla, G., A.A. Scheie and J.E. Ciardi, 1985. Role of Sucrose in Plaque Formation. Scand. J. Dent. Res. 93: 105-111.

Rosen, B.P. and E.R. Kashket, 1978. Energetics of Active Transport. In Bacterial Transport. Edited by B.P. Rosen, Marcel Dekker, Inc., New York, 559-620.

Rugg-Gunn, A.J., W.M. Edgar and G.N. Jenkins, 1981. The Effect of Altering the Position of a Sugary Food in a Meal Upon Plaque pH in Human Subjects. J. Dent. Res. 60: 867-872.

Ruijter, G.C.G., P.W. Postma and K. van Dam, 1990. Adaptation of Salmonella typhimurium Mutants Containing Uncoupled Enzyme II^{Glc} to Glucose-limited Conditions. J. Bacteriol. 172: 4783-4789.

Ruijter, G.C.G., P.W. Postma and K. van Dam, 1991. Energetics of Glucose Uptake in a Salmonella. typhimurium mutant Containing Enzyme II^{Glc}. Arch. Microbiol. 155: 234-237.

Ruijter, G.C.G., G. van Meurs, M.A. Verwey, P.W. Postma and K. van Dam, 1992. Analysis of Mutations that Uncouple Transport from Phosphorylation in Enzyme II^{Glc} of the Escherichia. coli Phosphoenolpyruvate Phosphotransferase System. J. Bacteriol. 174: 2843-2850.

Russell, J.B., 1990. Low-Affinity, High-Capacity System of Glucose Transport in the Ruminal Bacterium Streptococcus bovis: Evidence for a Mechanism of Facilitated Diffusion. Appl. Environ. Microbiol. 56: 3304-3307.

Russell, R.R.B., J. Aduse-Opoku, I. C. Sutcliffe, L. Tao and J. Ferretti, 1992. A Binding Protein-dependent Transport System in Streptococcus mutans Responsible for Multiple Sugar Transport. J. Biol. Chem. 267: 4631-4637.

Sabo, D.L., E.A. Bocker, B. Byers, H. Waron and E.H. Fischer, 1974. Purification and Physical Properties of Inducible Escherichia coli Lysine Decarboxylase. Biochemistry 13: 662-669.

Saier Jr., M., 1985. Mechanisms and Regulation of Carbohydrate Transport in Bacteria. Academic Press, Inc., New York.

Saier Jr., M., 1989. Protein Phosphorylation and Allosteric Control of Inducer Exclusion and Catabolite Repression by the Bacterial Phosphoenolpyruvate:Phosphotransferase System. Microbiol. Rev. 53: 109-120.

Saier Jr., M., and J. Reizer, 1992. Proposed Uniform Nomenclature for the Proteins and Protein Domains of the Bacterial Phosphoenolpyruvate:Sugar Phosphotransferase System. J. Bacteriol. 174: 1433-1438.

Saier Jr., M., M. Yamada, B. Erni, K. Suda, J. Lengeler, R. Ebner, P. Argos, B. Rak, K. Schnetz, C.A. Lee, G.C. Stewart, F. Breidt Jr., E.B. Waygood, K.G. Peri and R.F. Doolittle, 1988. Sugar Permeases of the Bacterial Phosphotransferase System: Sequence Comparisons. FASEB J. 2: 199-208.

Schachtele, C.F., A.E. Loken, M.K. Schmitt, 1973. Use of Specifically Labeled Sucrose for the Comparison of extracellular Glucan and Fructan Metabolism by Oral Streptococci. Infect. Immun. 5: 263-266.

Schachtele, C.F.and J.A. Mayo, 1973. Phosphoenolpyruvate-Dependent Glucose Transport in Oral Streptococci. J. Dent. Res. 52: 1209-1215.

Schilling, K.M. and W.H. Bowen, 1992. Glucans Synthesized in Situ in Experimental Salivary Pellicle Function as Specific Binding Sites for Streptococcus mutans. Infect. Immun. 60: 284-295.

Schöller, M., J.P. Klein, P. Sommer and R. Frank, 1983. Protoplast and Cytoplasmic Membrane Preparations from Streptococcus sanguis and Streptococcus mutans. J. Gen. Microbiol. 129: 3271-3279.

Schroeder, H.E., and J. De Boever, 1970. The Structure of Microbial Dental Plaque in Dental Plaque. Edited by W.D. McHugh, Edinburgh, E. &S. Livingstone, Ltd, pp 49-74.

Schroeder, V.A., S.M. Michalek and F.L. Macrina, 1989. Biochemical Characterization and Evaluation of Virulence of a Fructosyltransferase-Deficient Mutant of Streptococcus mutans V403. Infect. Immun. 57: 3560-3569.

Scully, C., 1992. Oral Infections in the Immunocompromised Patient. Br. Dent. J. 6: 401-407.

Shellis, R.P. and G.H. Dibdin, 1988. Analysis of the Buffering Systems in Dental Plaque. J. Dent. Res. 67: 438-446.

Shiroza, T. and H.K. Kuramitsu, 1988. Sequence Analysis of the Streptococcus mutans fructosyltransferase gene and flanking regions. J. Bacteriol. 170: 810-816.

Shiroza, T., S. Ueda and H.K. Kuramitsu, 1987. Sequence Analysis of the *gtfB* Gene from Streptococcus mutans. J. Bacteriol. 169: 4263-4270.

Shklair, I.L. and H. J. Keene, 1976. Biochemical characterisation and distribution of Streptococcus mutans in three diverse populations in Microbial Aspects of Dental Caries, 1. Edited by M. Stiles, W. J. Loesche and T. O'Brien, Special Supplement to Microbiology Abstracts, New York: Information Retrieval Inc., (eds), pp 201-210.

Shovlin F.E. and R.E. Gillis, 1969. Biochemical and Antigenic Studies of Lactobacilli Isolated from Deep Dental Lesions I. Biochemical Aspects. J. Dent. Res. 48: 356-360.

Siegel, J.L., S.F. Hurst, E.S. Liberman, S.E. Coleman and S. Bleiweis. Mutanolysin-Induced Spheroplasts of Streptococcus mutans are True Protoplasts. Infect. Immun. 31: 808-815.

Silhavy, T.J., T. Ferenci and W. Boos, 1978. Sugar Transport in Escherichia coli in Bacterial Transport. Edited by B.P. Rosen, Marcel Dekker, Inc., New York, pp 127-169.

Simoni, R.D., M. Levinthal, F.D. Kundig, W. Kundig, B.E. Anderson, P.E. Hartman and S. Roseman, 1967. Genetic Evidence for the Role of Bacterial Phosphotransferase System in Sugar Transport. Proc. Nat'l Acad. Sci. U.S.A. 58: 1963-1969.

Simonsson, T., S. Edwardsson and P. Glantz, 1987. Biophysical and Microbiologic Studies of "Heavy" and "Light" Plaque Formers. Scand. J. Dent. Res. 95: 43-48.

Singh, S.P., C.J. Bishop, R. Vink and P.J. Rogers, 1985. Regulation of the Glucose Phosphotransferase System in Brochothrix therosphacta by Membrane Energization. J. Bacteriol. 164: 367-373

Slots J.and T.E. Rams, 1991. New Views on the Periodontal Microbiota in Special Patient Categories. J. Clin. Periodontol. 18: 411-420.

Smith D.J., 1992. Ontogeny of Immune Mechanisms in the Oral Cavity in Contemporary Oral Microbiology and Immunology. Edited by J. Slots and M.A. Taubman, Mosby Yearbook, St. Louis, U.S.A., pp 513-541.

Smith, D.J. W.F. King and M.A. Taubman, 1991. Isotype, Subclass and Molecular Size of Immunoglobulins in Salivas from Young Infants. Clin. Exper. Immunol. 76: 97-102.

Smith, D.J. and M.A. Taubman, 1991. Association of Specific Host Immune Factors with Dental Caries Experience in Dental Caries Vol.1, Markers of High and Low Risk Groups and Individuals. Edited by N.W. Johnson, Cambridge University Press, Cambridge,England, pp 513-541.

Socransky, S.S , A.D. Haffajee, J.L. Dzink and J.D. Hillman, 1988. Associations Between Microbial Species in Subgingival Plaque Samples. Oral. Microbiol. Immunol. 3: 1-7.

Socransky, S.S and S.D. Manganiello, 1971. The Oral Microbiota of Man from Birth to Senility. J. Periodont. 42 : 485-496.

Sonju, T. and G. Rolla, 1973. Chemicla Analysis of the Acquired Pellicle Formed in Two Hours on Cleaned Human Teeth in vivo. Caries Res. 7: 30-38.

Spearman, T.N., R.L. Khandelwal, and I.R. Hamilton, 1973. Some Regulatory Properties of Glycogen Phosphorylase from Streptococcus salivarius. Arch. Biochim. Biophys. 154: 306-313.

Sreebny, L.M., 1982. Sugar Availability, Sugar Consumption and Dental Caries. Comm. Dent. Oral Epidemiol. 10: 1-7.

Staat, R.H., T.H. Gawronski, D.E. Cressey, R.S. Harris and L.E.A. Folke, 1975. Effects of Dietary Sucrose Levels on

the Quantity and Microbial Composition of Human Dental Plaque. J. Dent. Res. 54: 872-880.

Staat, R.H. and C.F. Schachtele, 1974. Evaluation of Dextranase Activity Produced by the Cariogenic Bacterium Streptococcus mutans. Infect. Immun. 9: 467-469.

Staat, R.H. and C.F. Schachtele, 1975. Characterization of Dextranases Produced by an Oral Strain of Actinomyces israelii. Infect. Immun. 12: 556-561.

Stempek, J.G. and Ward, R.T., 1964. An Improved Method for Electron Microscopy. J. Cell Biol. 22: 697-701, 1964.

Stephan, R.M., 1944. Intra-oral Hydrogen-ion Concentration Associated With Dental Caries Activity. J. Dent. Res. 23: 257-266.

Sund, M., C. Ericsson, L.E. Linder and C. Branting, 1987. Degradation of Streptococcus mutans Water-Soluble Glucans by Dextran-Glucosidase from Streptococcus mitis. Caries Res. 21: 1-6.

Sutton, S.T.W., G.R. Bender and R.E. Marquis, 1987. Fluoride Inhibition of Proton-Translocating ATPases of the Oral Bacteria. Infect. Immun. 55 : 2597-2603.

Sutton, S.T.W. and R.E. Marquis, 1987. Membrane-Associated and Solubilized ATPases of Streptococcus mutans and Streptococcus sanguis. J. Dent. Res. 66: 1095-1098.

Syed, S.A., W.J. Loesche, H.L. Pape and E. Grenier, 1975. Predominant Cultivable Flora Isolated From Human Root Surface Plaque. Infect. Immun. 11: 727-731.

Tanzer, J.M., J. Fisher and M.L. Freedman, 1982. Preemption of Streptococcus mutans 10440S Colonization by its Mutant 805. Infect. Immun. 35: 138-142.

Tanzer, J.M., M.L. Freedman, F.N. Woodiel, R.L. Eifert and L.A. Rinehimer, 1976. Association of Streptococcus mutans Virulence with Synthesis of Intracellular Polysaccharide in Proceedings "Microbial Aspects of Dental Caries". Edited by H.M. Stiles, W.J. Loesche and T.C. O'Brien, Sp. Supp. Microbiol. Abstr. 2: 597-616.

Tempest, D.W., 1970. The Place of Continuous Culture in Microbiological Research. Adv. Microbiol. Phys. 4: 223-249.

ten Brink, B., R. Otto, U.-P. Hansen and W.N. Konings, 1985. Energy Recycling by Lactate Efflux in Growing and

Nongrowing Cells of Streptococcus cremoris. J. Bacteriol. 162: 383-390.

Tenovuo, J.O., 1989. Nonimmunoglobulin Defense Factors in Human Saliva, in Human Saliva: Clinical Chemistry and Microbiology, Volume 2. Edited by J.O. Tenovuo, CRC Press, Boca Raton, Florida, U.S.A., pp 55-92.

Thibodeau, E.A. and R.E. Marquis, 1983. Acid Sensitivity of Glycolysis in Normal and Proton-permeable Cells of Streptococcus mutans GS-5. J. Dent. Res. 62: 1174-1178.

Thomas, E.L. and K.S. Pera, 1983. Oxygen Metabolism of Streptococcus mutans: Uptake of Oxygen and Release of Superoxide and Hydrogen Peroxide. J. Bacteriol. 154: 1236-1244.

Thompson, J., 1987. Sugar Transport in the Lactic Acid Bacteria in Sugar Transport and Metabolism in Gram Positive Bacteria, edited by J. Reizer and A. Peterkofsky, Ellis Horwood Ltd, Chichester, England, pp 13-38.

Thompson, J. and B.M. Chassey, 1982. Novel Phosphoenolpyruvate-Dependent Futile Cycle in Streptococcus lactis: 2-Deoxy-D-Glucose Uncouples Energy Production From Growth. J. Bacteriol. 151: 1454-1465.

Thompson, J. and B.M. Chassey, 1983. Intracellular Hexose-6-Phosphate:Phosphohydrolase from Streptococcus lactis: Purification, Properties and Function. J. Bacteriol. 156: 70-80.

Thomson, L.A., W.A. Little, W.A. Bowen, W.H. Sierra, L.I. Aguirrer, M. and G. Gillespie, 1980. Prevalence of Streptococcus mutans serotypes, Actinomyces, and other Bacteria in the Plaque of Children. J. Dent. Res. 59: 1581-1589.

Tukeuchi, D., 1961. Epidemiological Study on Dental Caries in Japanese Children, Before, During and After World War II. Int. Dent. J. 11: 443-457.

Ueda, S., T. Shiroza and H.K. Kuramitsu, 1988. Sequence Analysis of the *gftC* gene from Streptococcus mutans GS-5. Gene 69: 101-109.

Ullman, A. and A. Danchin, 1983. Role of cyclic AMP in Bacteria. Adv. Cycl. Nucl. Res. 15: 1-53.

Ullman, A. and J. Monod, 1968. Cyclic AMP as an Antagonist of Catabolite Repression in Escherichia coli. FEBS Letters 2: 57-60.

Vadeboncoeur, C., 1984. Structure and Properties of the Phosphoenolpyruvate:Glucose Phosphotransferase System of Oral Streptococci. Can. J. Microbiol. 30: 495-502.

Vadeboncoeur, C. and L. Trahan, 1983. Comparative Study of the Streptococcus mutans laboratory Strains and Fresh Isolates from Carious and Caries-free Tooth Surfaces and from Subjects with Hereditary Fructose Intolerance. Infect. Immun. 40: 81-90.

Vadeboncoeur, C., L. Thibault, S. Neron, H. Halvorson and I. R. Hamilton, 1987. Effect of Growth Conditions on Levels of Components of the Phosphoenolpyruvate:Sugar Phosphotransferase System in Streptococcus mutans and Streptococcus sobrinus Grown in Continuous Culture. J. Bacteriol. 169: 5686-5691.

Vadeboncoeur, C., M. Proulx and L. Trahan, 1983. Purification of Proteins Similar to HPr and EI from the Oral Bacterium Streptococcus salivarius. Can. J. Microbiol. 29: 1694-1705.

Vadeboncoeur, C., S. St. Martin, D. Brochu and I. R. Hamilton, 1991. Effect of Growth Rate and pH on Intracellular Levels and Activities of the Components of the Phosphoenolpyruvate;Sugar Phosphotransferase System in Streptococcus mutans Ingbritt. Infect. Immun. 59: 900-906.

van Beelen, P., J.S. van der Hoeven, M.H. de Jong and H. Hoogendoorn, 1986. The Effect of Oxygen on the Growth and Acid Production of Streptococcus mutans and Streptococcus sanguis. FEMS Microbiol. Ecology 38: 25-30.

van Boven, A. and W.N. Konings, 1987. A Phosphate-bond Driven Dipeptide Transport System in Streptococcus cremoris is Regulated by the Internal pH. Appl. Environ. Microbiol. 53: 2897-2902.

van den Broek, J., C.C. van Leewen and J. van Steveninck, 1986. Characterization of 2-deoxyglucose and 6-deoxyglucose Transport in Kluveromyces marxianus: Evidence for Two Different Transport Mechanisms. Biochim. Biophys. Acta 860: 626-631.

van Houte, J., 1980. Bacterial Specificity in the Etiology of Caries. Int. Dent. J. 30: 305-326.

van Houte, J. and D.B. Green, 1974. Relationship between the Concentration of Bacteria in Saliva and the Colonization of Teeth in Humans. Infect. Immun. 9: 624-630.

van Houte, J., C. Sansone, K. Joshipura and R. Kent, 1991a. In Vitro Acidogenic Potential and Mutans Streptococci of Human Smooth-surface Plaque Associated with Initial Caries Lesions and Sound Enamel. J. Dent. Res. 70: 1497-1502.

van Houte, J., C. Sansone, K. Joshipura and R. Kent, 1991b. Mutans Streptococci and Non-mutans Streptococci Acidogenic at Low pH, and in vitro Acidogenic Potential of Dental Plaque in Two Areas of the Human Dentition. J. Dent. Res. 70: 1503-1507.

van Houte, J., J. Russo and K.S. Prostak, 1989. Increased pH-Lowering Ability of Streptococcus mutans Cell Masses Associated with Extracellular Glucan-Rich Matrix Material and the Mechanisms Involved. J. Dent. Res. 68: 451-459.

Venable, J.H. and R. Coggeshell, 1965. A Simplified Lead Citrate Stain for Use in Electron Microscopy. J. Cell Biol. 25: 407-412.

Walker, G.J., M.D. Hare and J.G. Morry-Jones, 1983. Activity of Fructanase in Batch Cultures of Oral Streptococci. Carbohydr. Res. 113: 101-112.

Wallace, B.A., 1990. Gramicidin Channels and Pores. Annu. Rev. Biophys. Chem. 19: 127-157.

Watson, N., D.S. Dunyak, E.L. Rosey, J.L. Slonczewski, E.R. Olson, 1992. Identification of Elements Involved in Transcriptional Regulation of the Escherichia coli *cad* Operon by External pH. J. Bacteriol. 174: 530-540.

Weeghel, R.P., G.H. Meyer, W. Keck and G.T. Robillard, 1991a. Phosphoenolpyruvate-Dependent Mannitol Phosphotransferase System of Escherichia coli: Overexpression, Purification, and Characterization of the Enzymatically Active C-Terminal Domain of Enzyme II^{mtl} Equivalent to Enzyme III^{mtl}. Biochemistry 30: 1774-1779.

Weeghel, R.P.,Y.Y. van der Hoek, H.H. Pas, M. Elferink, W. Keck and G.T. Robillard, 1991b. Details of Mannitol Transport in Escherichia coli Elucidated by Site-Specific Mutagenesis and Complementation of Phosphorylation Site Mutants of the Phosphoenolpyruvate-Dependent Mannitol-Specific Phosphotransferase System. Biochemistry 30: 1768-1773.

West, I.C, 1970. Lactose Transport Coupled to Proton Movements in Escherichia coli. Biochem. Biophys. Res. Commun. 41: 655-661.

Widdas, W.F., 1952. Inability of Diffusion to Account for Placental Glucose Transfer in the Sheep and the Consideration of the Kinetics of a Possible Carrier Transfer. J. Physiol. 118: 23-29.

Whiley, R.A., R.R.B. Russel, J.M. Hardie and D. Beighton, 1988. Streptococcus downei sp. nov. for Strains Previously Described as Streptococcus mutans serotype h. Int. J. Syst. Bacteriol. 38: 25-29.

Yamada, T. 1987. Regulation of Glycolysis in Streptococci in Sugar Transport and Metabolism in Gram Positive Bacteria. Edited by J. Reizer and A. Peterkofsky, Ellis Horwood Ltd, Chichester, England, pp 69-93.

Yamada, T. and J. Carlsson., 1975a. Regulation of Lactate Dehydrogenase and Change of Fermentation Products in Streptococci. J. Bacteriol. 124: 55-61.

Yamada, T. and J. Carlsson., 1975b. Glucose-6-Phosphate Dependent Pyruvate Kinase in Streptococcus mutans. J. Bacteriol. 124: 562-563.

Yamada, T. and J. Carlsson., 1976. The Role of pyruvate formate-lyase in Streptococcus mutans, In Proceedings "Microbial Aspects of Dental Caries" edited by H.M. Stiles, W.J. Loesche and T.C. O'Brien, Sp. Supp. Microbiology Abstracts 3; 809-819.

Yamada, T., K. Endo and S.Araya, 1976. A Fructose 1,6-diphosphate-independent l-Lactate Dhydrogenase in a Strain of Streptococcus mutans. Arch. Oral Biol. 21: 233-236.

Yamashita, Y., W.H. Bowen and H.K. Kuramitsu, 1992. Molecular Analysis of a Streptococcus mutans Strain Exhibiting Polymorphism in the Tandem *gftB* and *gftC* Genes. Infect. Immun. 60: 1618-1624.

Yuan, L.C. and Gulyas, B.J. An Improved Method for Processing Single Cells for E.M. Utilizing Agarose. Anat. Record 201: 273-281.

Zero, D.T., J. van Houte and J. Russo, 1986a. Enamel Dimeneralization by Acid Produced from Endogenous Substrate in Oral Streptococci. Archs. Oral Biol. 31: 229-234.

Zero, D.T., J. van Houte and J. Russo, 1986b. The Intra-Oral Effect on Enamel Dimeneralization of Extracellular Matrix Material Synthesized from Sucrose by Streptococcus mutans. J. Dent. Res. 65: 918-923.

Zinner, D.D. and J.M. Jablon, 1969. Cariogenic Streptococci in Infants. Archs. Oral Biol. 14: 1429-1431.

Zinner, D.D., J.M. Jablon, A.P. Aran and M.S. Saslaw, 1965. Experimental Caries Induced in Animals by Streptococci of Human Origin. Proc. Soc. Exp. Biol. Med. 118: 766-770.