STUDIES ON THE METABOLISM OF SUCROSE BY THE BACTERIA IN SALIVARY SEDIMENT AND DENTAL PLAQUE

By

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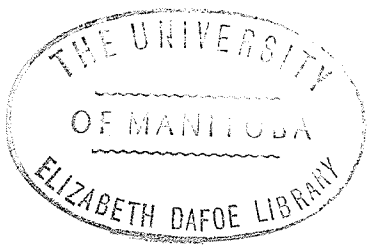

To

THORAYA and KHALID

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ABSTRACT

Sucrose is the sugar found in greatest abundance in human diets and has been implicated both in human and animal studies as a causative agent in the initiation and progression of dental caries. Information on its metabolism particularly that by the mixed bacterial populations found in dental plaque and saliva, is fragmentary. The studies reported in this thesis have examined those aspects of the metabolism of sucrose by the bacteria in salivary sediment and dental plaque likely to be applicable to the ability of the latter to form acid on the tooth surface and the development of the caries lesion.

A basic study was carried out to compare the metabolism of sucrose by the microorganisms in salivary sediment to that of its constituent monosaccharides, glucose and fructose. The parameters examined were decrease in pH, sugar utilization and polysaccharide, carbon dioxide, lactic acid and hetero-acid formation. Comparison was made at several sugar concentrations in mixtures incubated for four hours at $37^{o}C$ and in which the concentration of salivary sediment was 16.7 per cent (V/V).

With all the sugars tested, a fall and rise in the pH occurred at low sugar concentration and a fall without the subsequent rise at high concentration. The rates of utilization at each of the sugar concentrations examined, acid and carbon dioxide formation, were almost identical. Paper chromatography demonstrated that sucrose was rapidly hydrolyzed extracellularly to glucose and fructose. The amount of carbohydrate stored by the bacteria in the sediment was most with sucrose and least with fructose. Sucrose was found to be the main sugar

from which extracellular polyglucose and polyfructose could be synthesized.

The next study was restricted to sucrose and attention was focused on the effect of sucrose concentration and pH on the formation of extracellular polysaccharide, released into the medium and that remained with the sediment.

It was evident from this study that; (i) extracellular polysaccharide synthesis requires the presence of sucrose molecule; (ii) synthesis increases with increase in sucrose concentration; (iii) that free and attached polysaccharide and the polyglucose and polyfructose they contain are dependent upon the sucrose concentration; (iv) synthesis and the types of polymers formed are also dependent upon the pH, and; (v) the fructose polymers formed from sucrose were more soluble and homogenous than the glucose polymers.

Utilization of a variety of levans and dextrans by salivary sediment and dental plaque was then examined. Also examined was whether the presence of sucrose or such factors as salivary supernatant and pH might affect the degradation of extracellular polysaccharide.

Bacteria in salivary sediment and dental plaque utilized all of the several dextrans and levans tested. Acid formation from the various dextrans and levans were dependent upon their concentration and on the pH. It was also shown in this study that the ability of the bacteria in plaque to utilize dextrans or levans was comparable to that of the bacteria in salivary sediment. The extracellular polysaccharides syn-

thesized in salivary sediment mixtures incubated with C^{14}-sucrose when isolated and supplied as substrate, were utilized by the bacteria in the same system. A most important observation was that sucrose inhibited utilization of both extracellular polyfructose and polyglucose. This inhibition was evidently an effect due to substrate, rather than an effect due to pH.

Finally, the effect of salivary supernatant, fluoride and the pH on the metabolism of sucrose was examined in both salivary sediment and dental plaque.

Salivary supernatant stimulated utilization of sucrose, formation of plaque carbohydrate and a slight decrease in the pH. Both in the presence and absence of salivary supernatant, fluoride inhibited the same three parameters of sucrose metabolism. Both in plaque and sediment mixtures incubated with sucrose, salivary supernatant stimulated while fluoride inhibited acid formation. The stimulatory effect of salivary supernatant was greater at pH 6.0 than 5.0, whereas the inhibitory effect of fluoride was more at pH 5.0 than pH 6.0. More sediment carbohydrate was formed in the presence than in the absence of supernatant and more formed at pH 6.0 than at pH 5.0. On the other hand, fluoride inhibited formation of sediment carbohydrate. Both the supernatant and sediment components of the extracellular carbohydrate decreased in the absence of supernatant and increased with fluoride whether supernatant was present or not.

Metabolism of fructose was affected in the same way by salivary

supernatant and fluoride as the metabolisms of sucrose and glucose. This plus the fact that sucrose is hydrolyzed extracellularly, indicates that the effect of salivary supernatant and fluoride on sucrose metabolism is essentially through their effects on its glucose and fructose monomers.

Several aspects of the metabolism of sucrose by the oral microflora associated with the formation of glucose and fructose polymers acid formation, and factors regulating the metabolism of sucrose have been identified and their relation to the caries process are discussed.

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CHAPTER I

INTRODUCTION

Numerous studies have been carried out to determine the role of sucrose and other carbohydrates in the initiation and progression of dental caries, one of the most prevalent diseases of man. Miller in 1890 proposed that acid formed during degradation of dietary carbohydrate by the bacteria found in the mouth dissolves the mineral portion of the enamel and initiates the caries lesion. The regions of the dentition where both food and bacteria collect are those surfaces of the teeth protected from abrasion by the oral musculature. Williams (1897) and Black (1898) named the bacterial deposits, dental plaque.

Subsequent studies have shown that acid formation occurs as soon as carbohydrate enters the mouth (Stephan and Miller, 1943) and as long as there is still carbohydrate available as substrate. The carbohydrate available to the bacteria for acid formation can be classified according to origin into two types, exogenous and endogenous.

Exogenous carbohydrate is the carbohydrate externally available as substrate for acid formation and consists mostly of sucrose and the starchy foodstuffs of the diet. The latter upon hydrolysis by the enzymes of the bacteria and saliva provide the bacteria with maltose and glucose (Manners, 1955). Small amounts of other sugars such as lactose are also present in the human diet but relative to sucrose the amounts are generally of minor

significance in providing substrate for acid formation. A small amount of fermentable carbohydrate is also available from the mucoprotein of the saliva (Leach, 1965) but its significance as a source of substrate is not known.

Endogenous carbohydrate is mainly the carbohydrate synthesized and stored by the bacteria. Storage may be intracellular or extracellular and the polymers stored may serve as reserves for acid formation during those periods that exogenous carbohydrate is not available.

The Microbial Flora of the Dental Plaque

The microflora of the dental plaque consists of a wide variety of microorganisms, the relative proportions of which vary from dentition site to dentition site, from day to day and from person to person. Samples of plaque removed from the human dentition without regard to these variables (Gibbons *et al*, 1964) showed the predominant cultivable flora in plaque to consist of the following: facultative streptococci, 27 per cent; facultative diptheroids, 23 per cent; anaerobic diptheroids, 18 per cent; peptostreptococci, 13 per cent; veillonella, 6 per cent; bacteroides, 4 per cent; fusobacteria, 4 per cent; neisseria, 3 per cent; vibrios, 2 per cent and lactobacilli, less than 0.01 per cent.

Ritz (1967) has found that as plaque forms on the labial surfaces of newly cleaned maxillary or mandibular incisors, the microbial composition changes in an orderly manner; *Neisseria* gradually declined from an average of 9 per cent at day 1 to 3 per cent at day 9; *Nocardia* com-

prising 6 per cent of the total population at day 1, declined sharply to 0.1 per cent at day 9; Fusobacteria , which accounted for only 0.02 per cent of the total on day 1 increased to 1 per cent on day 9; Veillonella increased from 1.5 to 12 per cent and Actinomyces from 3 per cent to 23 per cent over the same period.

Streptococci, the predominant microorganisms at each sampling interval varied between 30-70 per cent of the cultivable microflora with the highest counts on the third day. From this study it was evident that the microorganisms in early plaque are mainly aerobic and that as plaque thickens, the proportion of anaerobic to aerobic microorganisms in the flora increases.

Plaques in regions of the dentition protected from saliva show a higher incidence of acidogenic and aciduric microorganisms than those on the more accessible surfaces. Pits and fissures, approximal surfaces and caries lesions show much higher levels of lactobacilli than the other regions of the dentition (Bahn and Quillman, 1963). Insertion of appliances causes a sharp rise in the acidogenic component of the plaque flora. For example, the fixing of orthodontic bands results in a sharp increase in the incidence of such microorganisms as S. salivarius, S. mitis and Lactobacilli (Balenseifen and Madonia, 1970).

A. Role of Sucrose in the Caries Process

1. Availability as substrate for acid formation

Lanke (1957) carried out an extensive investigation on the clearance

of a large variety of sweets and starchy foodstuffs from the salivas of human subjects. Following ingestion of a fixed amount of a particular carbohydrate, the concentration sharply rose and then slowly fell in an exponential fashion. The extent of the rise and the subsequent clearance was affected by a variety of factors which included the sugar content of the foodstuff. Of particular interest was the observation that the salivary sugar concentration rose more sharply with sweets than with bread, but the clearance of the latter was slower. With the more soluble sweets, sugar was available at a higher concentration for a shorter time period, whereas with the less soluble starchy foodstuffs, substrate was available at lower concentration but for a longer time.

An extensive study on the effect of carbohydrate on caries in human subjects was carried out in the Vipeholm hospital in Sweden particularly to determine the effect of between meal eating (Gustafsson et al, 1954). Eating of various types of sweets between meals sharply increased caries activity; unfortunately, because between meal eating of starchy foodstuffs was not tested, starch components of the human diet could not be evaluated.

2. Animal studies

Shafer (1949) observed in hamsters that the caries producing capacity of a diet containing 61 per cent sucrose was much higher than that of the same diet containing glucose. The greater cariogenicity of sucrose was also observed by Krasse in hamsters (1965a) and by Grenby (1963) in rats.

Experiments have also been carried out to determine if the unhydrolyzed sucrose molecule is more cariogenic than the glucose-fructose mixture one would obtain upon sucrose hydrolysis. Steinman and Haley (1957) found that such a glucose-fructose mixture administered to rats was less cariogenic than comparable amounts of sucrose. Gustafson et al (1955) noted that sucrose and fructose caused more caries activity in golden hamsters than glucose. On the other hand, Grenby and Hutchinson (1969) reported that there was no consistent difference in cariogenicity between glucose and fructose. Similar results were also obtained by Green and Hartles (1969) using albino rats, Campbell and Zinner (1970) using hamsters and Rosen (1969) using gnotobiotic rats inoculated with different oral microorganisms.

The animal studies in general seem to support the view that the sucrose of the diet rather than starches is the carbohydrate mainly responsible for the cariogenicity of the modern human diet. This does not appear to agree with human data particularly that showing similarity of acid formation in experiments carried in vivo and in vitro (see section B). One explanation may be that the difference in cariogenicity observed between the sugars and starches in animal studies arises from a difference in their retentivity (Grenby, 1967). Another, particularly in those studies where the animals are inoculated with various types of cariogenic bacteria, the difference may arise from the absence of flora having the enzymes needed for initial hydrolysis of starch to the simpler sugars which can be fermented by many of the bacteria of the oral flora

(see section B).

3. Effect of sucrose on the composition of the salivary and plaque microfloras

The role of carbohydrates in the establishment and survival of certain oral microbes within the oral cavity have been examined by a number of investigators. Individuals on diets high in fermentable carbohydrate show a higher incidence of acidogenic microorganisms such as lactobacilli (Appleton, 1950). On the other hand, restricting the intake of carbohydrate greatly reduced the lactobacillus populations (Jay, 1947).

Interest in specific microorganisms lagged until the 1960's when the concept of the role of bacteria in the caries process was revived. Because of their much higher numbers in the plaque, particular attention was paid to the streptococci. Attention focused on the ability of the streptococci to form polysaccharide and the role of these polymers in; (i) providing reserves for acid production and; (ii) adhesion of microorganisms to tooth surfaces.

Bowen and Cornick (1967) observed that although the total population of streptococci was unaffected, the population of extracellular polysaccharide-forming streptococci declined decidedly in plaque from monkeys when the level of sucrose in the diet was drastically reduced.

Van Houte (1964) showed that restriction of the carbohydrate content of the diet produced a marked reduction in the number of polysaccharide-storing microorganisms in the dental plaque. Streptococcus salivarius increased in both saliva and in dental plaque after adding either sucrose

or glucose to the diet (Carlsson and Sundstrom, 1968). The increase in population was more with sucrose than with glucose.

A high sucrose diet supports the implantation of caries-inducing streptococci in hamsters, while with the same amounts of glucose, few of the implanted organisms can be recovered (Krasse, 1965b). Studying the effect of carbohydrate restriction on the presence of some oral bacteria, De Stoppelaar et al (1970), have shown that during carbohydrate free periods, both Streptococcus mutans and iodophilic-polysaccharide producing bacteria decrease in population. Reinstitution of sucrose or glucose into the diet, resulted in an increase in the number of these bacteria in the dental plaque. Jordan et al (1969) reported that sucrose, glucose and fructose diets supported the implantation of Odontomyces viscosus in hamsters.

4. Effect on plaque formation

The relation between dietary carbohydrate and plaque formation has received considerable attention in recent years. More plaque is formed in humans on diets containing sucrose rather than glucose (Carlsson and Egelberg, 1965). The carbohydrate content of the plaque was shown to be higher with a sucrose than with a glucose diet (Carlsson and Sundstrom, 1968). On the other hand, less plaque formation occurred with either fructose or glucose or a mixture of the two compared to the extensive coronal plaque formed following frequent exposure to a sucrose diet (Frostell et al, 1967).

B. Carbohydrate Metabolism of the Salivary and Plaque Flora

The oral microflora can ferment a wide variety of carbohydrates, including the common monosaccharides, glucose, galactose, fructose and mannose and the common disaccharides lactose, sucrose and maltose (Manly and Walborn, 1956). The carbohydrates (hexoses, hexosamines, fucose and sialic acid) that are part of carbohydrate-protein complexes, can also be fermented by the oral microorganisms (Critchley and Leach, 1965).

1. Acid formation

The ability of mouth organisms to ferment carbohydrates was observed by Miller (1890) and later many others by incubating saliva with the various common sugars.

In studies with pure cultures, Stephan and Hemmens (1947) found that microorganisms isolated from dental plaque not only produce acid at different rates, but the amount of acid produced by a particular microorganism varied with the type of carbohydrate. The monosaccharides, glucose, fructose and galactose produced more acid than the disaccharides, maltose, lactose and sucrose, which in turn produced more acid than starch. Galactose and lactose produced slightly less acid than their corresponding mono- and disaccharides. The mixed microorganisms of the salivary and plaque flora reduced the differences between the mono- and disaccharides (Miller, Muntz and Bradel, 1940; Volker and Pinkerton, 1947; Kleinberg, 1970a). Presumably, the presence of invertase in some microorganisms would enable others to utilize the resulting monosaccharides.

When incubations were with wax stimulated whole saliva, except for a slightly slower rate with galactose and lactose, the differences in acid formation between the various sugars and between the sugars and starch disappeared (Volker and Pinkerton, 1947). The disappearance of the difference in rate of fermentability has been attributed to the presence of amylase in saliva which would facilitate more rapid hydrolysis of the starches to the more easily fermented maltose and glucose.

Lilienthal and Reid (1959) in studies with saliva-carbohydrate mixtures found that acid production from glucose could either be equal to or less than that from sucrose. They suggested that for acid formation to be equal from sucrose and glucose, the salivary microorganisms would first have to be exposed to a high sucrose diet. One effect of such exposure would be to enable microorganisms in the oral microflora to adapt to the sucrose by increasing their level of bacterial invertase. This would enable the microorganisms to produce more acid from sucrose than they might otherwise be able to do.

Manly and Walborn (1956) found no differences in acid formation when salivary sediment was exposed to equal concentrations of glucose, fructose or sucrose. The same results were obtained by Frostell (1964) with plaque in vitro, by Ranke (1968) with streptococci isolated from dental plaque, and by Neff (1967) in studies on the pH changes in dental plaque in vivo.

The acids produced by the oral bacteria have been explored in a number of different experimental systems. All have pointed to the fact that lactic acid is one of the main acids formed.

Neuwirth and Klosterman (1940) found that lactic acid was rapidly produced in vivo following introduction of carbohydrate into the oral cavity. Also, rapid formation occurred in saliva incubated with different carbohydrates. With glucose and sucrose, lactic acid formation was approximately equal.

Miller et al (1940) examined the formation of lactic acid from a variety of carbohydrates in incubated mixtures containing dental plaque. Their data suggested that slightly more lactic acid was produced from sucrose than from either glucose or fructose. Using plaque material in vitro, Muntz (1943) demonstrated in addition to lactic acid, that other acids were formed from glucose amongst which were acetic, propionic and formic acids.

Neuwirth and Baerger (1957) observed in saliva-glucose mixtures that in addition to lactic acid, malic, oxaloacetic, α-ketoglutaric, succinic, fumaric and pyruvic acids were also formed. Andlaw (1968) studying acid production in whole human saliva incubated with several substrates demonstrated the formation of succinic, fumaric and malic acids. Drucker and Melville (1968) examined the acids formed from glucose by cariogenic and non-cariogenic streptococci and found that these were formic, acetic and butyric acids.

Sandham and Kleinberg (1970a) found in salivary sediment mixtures that lactic, acetic and propionic acids are the main acids formed from glucose during its breakdown and that lactic acid is only formed as long as glucose is still present in the medium. They showed that once the

glucose was used up, the lactic acid that had accumulated during the period of glucose utilization was converted immediately to acetic and propionic acids. When glucose was not used up, the lactic acid accumulated as though it were an end product rather than an intermediate.

Combining microorganisms alters the metabolism of the individuals. For example, streptococci prevent lactobacilli from reaching as low a pH as that reached by lactobacilli when present alone (Stephan and Hemmens, 1947). The lactic acid produced by streptococci can be rapidly converted by Veillonella to the weaker propionic and acetic acids and to carbon dioxide; the two microorganisms act as though they are a single metabolic unit.

Hu and Sandham (1969) have shown that of the streptococci isolated from plaque and tested for cariogenicity in animal experiments, most form lactic acid as their final end product, but some such as strain AHT, produce acetic acid. However, in mixed populations, such as salivary sediment or dental plaque, lactic acid is an intermediate and not an end product of glucose catabolism (Muntz, 1943; Sandham and Kleinberg, 1970a)

2. Polysaccharide formation

In addition to their ability to form acid from carbohydrate, many of the bacteria found in plaque and saliva can synthesize intra- and extracellular polysaccharides.

a. Intracellular polysaccharide

Many bacteria, isolated from dental plaque, e.g., streptococci, diptheroids, fusobacteria and bacteriodes, are capable of forming intracellular polysaccharides from glucose, maltose or sucrose (Gibbons and Socransky, 1962; Berman and Gibbons, 1966). Staining of smears of dental plaque with iodine solution disclosed iodophilic polysaccharide in many of the organisms, especially the streptococci (Gibbons and Socransky, 1962). Critchley et al (1967), demonstrated that rapid in vivo production of intracellular polysaccharide occurs in different types of plaque organisms following exposure to glucose or sucrose.

In experiments on Streptococcus mitis, a microorganism isolated from human dental plaque, Gibbons et al (1962, 1963) examined the synthesis and degradation of polysaccharide formed from glucose. The polysaccharides formed were mainly intracellular and of the glycogen-amylopectin type. When microorganisms containing this polysaccharide were placed in a medium free of exogenous carbohydrate the microorganisms utilized the stored carbohydrate and lowered the pH of the medium. Thus the plaque could continue forming acid long after the environmental carbohydrate was depleted.

Similar observations were reported by Sandham and Kleinberg (1969a) in salivary sediment mixtures. In their studies they showed that sediment carbohydrate accumulates asymptotically as long as there is glucose in the medium. Once the glucose is used up, the cells in the sediment switch to the utilization of stored carbohydrate.

b. Extracellular polysaccharides

In addition to producing intracellular polysaccharides and polysaccharides that are integral constituents of cell structure, many microorganisms also synthesize abundant amounts of extracellular polysaccharide. The most important of these in relation to caries are two types of homopolysaccharides, the dextrans and levans.

Dextrans are glucans comprised exclusively of the monomeric unit α-D-glucopyranose coupled mainly by α-1, 6-linkages. Branching occurs by means of α-(1,4) or α-(1,3) glucosidic bonds, the proportions of which can differ considerably in different bacteria (Jeanes et al, 1954). Levans are polyfructans composed of D-fructo-furanose residues linked by β-2, 6 bonds and with branching occurring by means of β-2, 1 linkages.

Gibbons et al (1966, 1967), demonstrated that streptococci, shown by several investigators to be cariogenic in experimental animals, form dextran-like polymers from sucrose. The organisms tested included human streptococcal strains PK1, LM7 and GS5, hamster streptococcal strains HS-6 and E49, rat streptococcus strains GF 71 and Lactobacillus acidophilus strain 108 T.

Wood and Critchley (1966) investigated the polysaccharides formed by the cariogenic streptococcus FA-1 and confirmed the formation of dextran from sucrose. Formation of dextrans have also been demonstrated in Streptococcus mutans (Guggenheim and Schroeder, 1967) and in a human oral strain of Lactobacillus casei (Hammond, 1969). Human dental plaque was examined for the presence of dextran-forming bacteria by De Stoppelaar

et al (1967). They showed that the number of dextran forming streptococci in human dental plaque varies between 1-40 per cent of the total flora (average 11.6 per cent). Dextransucrase, the enzyme involved in the formation of dextran from sucrose, has been isolated by Wood (1967a) from streptococcal strain FA-1. Dextransucrase was also isolated from Streptococcus sanguis, a microorganism originating from human dental plaque (Carlsson et al, 1969).

Evidence for levan synthesis by the oral microflora has been presented in a number of reports. McDougall (1964) and Manly et al (1966), have demonstrated that dental plaque when provided with sucrose is capable of synthesizing an extracellular levan-like polysaccharide. Formation of this polysaccharide has also been shown in bacterial isolates. Niven et al (1941) and Snyder et al (1955), have shown that levan formation from sucrose occurs in Streptococcus salivarius. Gibbons and Nygaard (1968) demonstrated levan formation by streptococal strains GS5 and LM7, Howell and Jordan (1967) by Odontomyces viscosus, an organism that predominates in subgingival plaque, and Carlsson (1970) by Streptococcus mutans.

3. Polysaccharide degradation

a. Intracellular

The intracellular polysaccharide formed by the oral flora appears to be mainly of the glycogen-amylopectin type (Gibbons and Socransky, 1962). The enzyme phosphorylase acts on this polymer (Dawes and Ribbons, 1964) by attacking terminal α-(1,4) glucosidic bonds in a step-wise manner. Removal of D-glucose residues occurs in the presence of inorganic

phosphate and glucose-1-phosphate is formed. When α-1, 6-linkages are reached, hydrolysis occurs by a debranching enzyme which specifically attacks these bonds and permits phosphorylase to continue its hydrolysis of α-1, 4 bonds (Van Houte, 1967). A debranching enzyme, isoamylase, has been described in brewer's yeast. This enzyme hydrolyzes only outer α-(1,6) glucosidic linkages of both glycogen and amylopectin (Gunja et al, 1961).

b. Extracellular

i. Dextran degradation

Two dextranase systems have been described by several investigators. Hultin and Nordstrom (1949) demonstrated that dextranases were produced by various moulds and that the dextranases formed attacked 1,6-linkages and hydrolyzed high molecular weight dextrans into various sizes. Jeanes et al (1953), have shown that the dextranase from Penicillium funiculosum hydrolyzes dextran B-512 into the di- and trisaccharides, isomaltose and isomaltotriose.

Tsuchiya et al (1952), suggested that more than one enzyme can degrade dextran and that a microorganism may produce more than one type of dextranase. He showed that both the pH and composition of the media play an important role in both the formation and activity of dextranases. Using dextrans B-512, B-523 and B-742 they noticed that these polysaccharides were not degraded at the same rate with a particular dextranase.

Ingelman (1948) extracted dextranase from bacterium Cellvibrio

fulva which was able to hydrolyze a dextran derived from Leuconostoc mesenteroides. This enzyme hydrolyzed the dextran into large fragments rather than into glucose or disaccharides. Hehre and Sery (1952) demonstrated that anaerobically grown gram negative intestinal bacteria can breakdown dextrans, whereas those that grow under aerobic conditions can not. The microorganisms implicated were of the bacteroides genus, and of the strains tested, all were able to form acid from dextran; they could form acid from monosaccharides as well. Using different strains of bacteroides and different dextrans Sery and Hehre (1956) observed that the difference in degradability between the various dextrans was related to the percentage of the linkages that were 1, 6. Their results indicated that the bacteroides genus contains two different dextranases, one that lowers the viscosity by degrading the large molecular weight dextran into smaller fragments, which occurred most readily at pH 5.0-5.5; a second that caused rapid release of glucose by endwise degradation which occurred most readily at pH 7.0-7.5. Unlike Bacteroides, Lactobacillus bifidus formed a dextranase that hydrolyzed dextran by random cleavage of the molecule.

With regard to the oral microflora, Gold, Brest and Bleckman (1969) reported that filamentous and diptheroid microorganisms from subgingival plaque produced acid from dextran under aerobic, while bacteroides showed the same activity under anaerobic, conditions. Dextrans can also be catabolized by dental plaque (Wood, 1967b, 1969) and salivary sediment (Halhoul and Kleinberg, 1969); these findings conflict with the belief

of a number of investigators that dextrans are biologically inert in the mouth and not easily hydrolyzed by the salivary and plaque microorganisms (Gibbons and Banghart, 1967).

ii. Levan degradation

Loewenberg and Reese (1957) tested a total of 50 fungi and bacteria for their ability to utilize fructans. They found that the ability to utilize these polymers differed amongst the different microorganisms, and that fructanases were inducible enzymes.

A fructanase that hydrolyzes levan into oligofructosides has been demonstrated in levan-forming bacteria grown on levan (Hestrin and Goldblum, 1953). When the same bacteria were grown on sucrose, a fructosidase was produced that was capable of completing the hydrolysis of the oligofructosides into fructose.

In studies on the oral microflora, Da Costa and Gibbons (1968) have shown that 37 per cent of 57 bacterial isolates from human dental plaque are capable of hydrolyzing the levan synthesized by Streptococcus salivarius. All of the levan hydrolyzing microorganisms proved to be streptococci. The fructan hydrolase produced by streptococci (strain 6) was shown to be inducible and appeared to act by cleaving terminal fructose units from the levan molecule.

Significant amounts of the enzyme levanase are present in both dental plaque and saliva (Manly, 1967). Levan synthesized by a strain of S. salivarius can be hydrolyzed by the bacteria in both dental plaque

and saliva (Wood, 1964; Gibbons et al, 1967). Using levans from Ps. auroefacies and S. salivarius, Van Houte (1967) demonstrated that only some strains of streptococci isolated from human dental plaque are capable of hydrolyzing these polysaccharides while other streptococci are not. Van Houte also found that S. salivarius strain SS_9 was able to utilize the levan it synthesized. This is in contrast to the belief of many investigators that microorganisms cannot utilize their own extracellular polysaccharide (Wilkinson, 1958).

Effect of Saliva on the Carbohydrate Metabolism of the Oral Microflora

Hartles and Wasdell (1955) and Hartles (1963) have reported that salivary supernatant contains a factor that stimulates the glycolytic activity of the bacteria found in wax-stimulated saliva. Kleinberg and Craw (1969) have confirmed that salivary supernatant stimulates glycolysis of the salivary bacteria. It was also shown that salivary supernatant stimulates glycolysis of the microorganisms in dental plaque (Komiyama and Kleinberg, 1971). Yet, saliva is protective against dental caries. Studies have also shown that greater acidity occurs in dental plaque when sucrose is applied to the plaque and free access of saliva to the dental plaque is prevented (Englander et al, 1959). Eggers-Lura (1954) found that the bacteria in the saliva of caries free subjects (who usually have a more rapid salivary flow rate) actually produce more acid from glucose than the bacteria in the saliva of subjects who are caries active.

This discrepancy has been resolved by Kleinberg and Craw (1971) who found in addition to stimulating acid formation, salivary supernatant stimulates the formation of base. As a result, carbohydrate is used up faster and the pH fall is less than would otherwise occur (Kleinberg, 1970b).

Measurement of the pH of plaques located in different regions of the human dentition disclosed a pattern that was related to the flow of saliva to the various regions of the dentition (Kleinberg and Jenkins, 1964). Plaques to which saliva had greater access showed a higher pH than those where saliva access was less. Individuals with a more rapid resting salivary flow rate showed higher plaque pH levels than those with slower rates (Kleinberg and Jenkins, 1964).

Saliva may also affect the carbohydrate metabolism of the plaque by its effect on the pH. Saliva contains urea which is rapidly broken down by the ureolytic bacteria in the plaque to form ammonia and carbon dioxide; since ammonia is a stronger base than carbon dioxide is an acid, a rise in pH results. Consequently, regions of the dentition to which saliva has greater access will be exposed to more urea in a given period of time and thus would be more prone to a higher plaque pH. Although hydrolysis of salivary protein and the deamination of the resulting amino acids will also produce ammonia, the simultaneous formation of keto-acids would tend to lower the pH of the dental plaque under these circumstances (Kleinberg, 1970b).

Effect of Fluoride on the Carbohydrate Metabolism of the Oral Microflora

Fluoride inhibition of dental caries appears to result from the effect of fluoride on two processes. First, in a number of ways, fluoride makes the enamel of the tooth more resistant to solution by the acids formed by the bacteria in the dental plaque during their fermentation of ingested carbohydrate (Jenkins, 1963). Second, fluoride can inhibit acid formation by the microorganisms of the plaque, particularly if the pH falls near or below 5.0 (Jenkins, 1959).

With regard to the latter, Bibby and Van Kesteren (1940) carried out experiments to determine the effect of fluoride on acid formation from glucose by a number of oral bacteria. Whereas concentrations of fluoride up to 100 ppm had no bactericidal effect on the microorganisms examined, they found that concentrations of only 0.9 ppm caused a slight reduction in the formation of acid. In experiments with incubated saliva, Wright and Jenkins (1954) and Jenkins (1959) showed that fluoride at levels as low as 0.5 ppm inhibited the formation of acid from glucose and that the acid formation was more sensitive to fluoride at acidic than at neutral pH. The greater inhibition of acid formation at acidic pH has been confirmed in salivary sediment (Sandham and Kleinberg, 1964 and 1969b), where it was also shown that fluoride inhibits glucose uptake and synthesis of storage carbohydrate.

Sandham and Kleinberg (1969b) suggested that the site of fluoride action on the cells may be in or in association with the cell membrane rather than at enzymes in the pathway leading to acid and polysaccharide

formation. In addition to altering membrane permeability they suggested that fluoride might alter the solubility of phosphate containing complexes in bacterial cell membranes, and thus alter the availability of inorganic phosphate for intracellular processes such as the phosphorylation of glucose by hexokinase. Such a restriction on hexokinase activity would account for decrease in glucose uptake and as a consequence decreased polyglucose synthesis and acid production.

Weiss et al (1965), using Streptococcus mitis have shown that extremely low concentrations of fluoride inhibit intracellular polysaccharide storage from glucose, maltose and sucrose but not its degradation. They also suggested that fluoride may act by preventing substrate utilization rather than by inhibiting intracellular enzyme systems. Hamilton (1967 and 1969) observed the same effects of fluoride with Streptococcus salivarius.

Jenkins et al (1969), have shown that plaque bacteria cultured on media containing fluoride, contained high concentrations of fluoride and showed less capacity for acid production than controls. This suggested that stored fluoride was active within the bacteria. Edgar et al (1970), compared acid formation from sucrose by plaque obtained from subjects before and after fluoridation of water supply. The results showed a reduction in the pH in plaque after fluoridation, supporting the view that the effect on the pH is produced directly by the fluoride.

Effect of the pH on the Carbohydrate Metabolism of the Oral Microflora

The pH of the environment is one of the major factors that affect the growth and metabolic activity of microorganisms. During the utilization of carbohydrate, the pH falls in plaque *in situ*, in incubated saliva and in a number of other *in vitro* systems containing pure or mixed microorganisms from the mouth.

In studies with glucose and urea and measurements of plaque pH *in situ*, it was clearly demonstrated that the pH can vary from a value as low as approximately 4.0 to one as high as approximately 9.5 (Stephan, 1940 and 1944; Stralfors, 1950; Kleinberg, 1967). Over this wide range of pH, one would expect within the dental plaque both inhibition and activation of most enzymic processes. pH would therefore, be a major determinant of which processes would proceed or not and at what rates.

Surprisingly little is known, however, about the effect of pH on the overall carbohydrate metabolism of the mixed bacterial floras in both saliva and in plaque. Korayem and Kleinberg (1970 and 1971) have reported recently that the effect of pH on glucose utilization and acid formation by salivary sediment and dental plaque are virtually the same. In both, at pH 4.0 and 4.5, hardly any glucose was utilized or acid formed. At pH 5, glucose utilization and acid formation occurred at a slow rate. But, as the pH was raised above 5.0, both glucose uptake and acid formation increased to maxima at approximately pH 7.0, before showing a progressive decrease as the pH was raised further.

Salivary Sediment as a Model for the Study of the Metabolism of Dental Plaque

It is apparent from many of the above studies that investigations on the metabolism of the common sugars with pure cultures often give a different picture from that with the mixed populations in saliva and plaque. Studies on dental plaque are necessarily limited because of the small amounts of plaque available from human subjects. Saliva, on the other hand, is not only more plentiful than plaque but also contains a microbial flora which extensive studies have shown has a carbohydrate metabolism like that of plaque (Singer and Kleinberg, 1970; Kleinberg, 1970c).

The concentration of the bacteria in wax-stimulated whole saliva, i.e., salivary sediment is normally about 1-2 per cent and at such low concentrations, acid formation is too slow to produce the Stephan type of pH-curve seen in plaque in situ after a sugar rinse (Stephan, 1940). However, when the cellular elements in stimulated saliva are re-combined with the supernatant so that the cell concentration is 16.7 per cent (V/V) (or higher) pH curves are produced with characteristics like those seen in plaque in situ (Kleinberg, 1961, 1967; Singer and Kleinberg, 1970).

Eichel and Lisanti (1964) have proposed that leukocytes in salivary sediment are responsible for its glycolytic activity. However, the low osmolarity of stimulated saliva results in the rapid bursting of any intact leukocytes that are present (Dawes, 1968). Tonzetich and Friedman (1965) have proposed that the large number of desquamated epithelial

cells present in salivary sediment are the main source of its glycolytic activity rather than the bacteria. That the epithelial cells do not contribute to the glycolytic activity of salivary sediment is evident from the fact that salivary sediment can but mammalian cells cannot readily metabolize either sucrose or urea, or form levans and dextrans from sucrose. Moreover, a recent comparison of the glycolytic activity of epithelial cells derived from cheek scrapings to that of salivary sediment indicated that the activity resides in the bacteria in salivary sediment (Tatevossian and Jenkins, 1969).

In contrast to the belief that the microorganisms in salivary sediment arise mainly from the oral soft tissues (Gibbons et al, 1964), Carlsson (1967) has pointed out that Streptococcus sanguis found mainly in the plaque on the teeth is found in increasing number in the salivas of persons with poor oral hygiene. He concluded that the microbiota on the teeth may contribute as much as that of the tongue to the pool of salivary streptococci.

Purpose and Outline of this Thesis

The review of the literature has disclosed that although numerous studies have been carried out to determine the properties of sucrose which are responsible for its cariogenicity, information on its metabolism particularly by the mixed bacterial populations found in the dental plaque and saliva, is fragmentary.

The purpose of this thesis is to examine those aspects of the meta-

bolism of sucrose by the bacteria in salivary sediment and dental plaque which are likely to be applicable to the ability of the latter to form acid on the tooth surface and cause development of carious lesions.

Sucrose during its metabolism by the oral bacteria can be hydrolyzed into its constituent monosaccharides, glucose and fructose, or it can be synthesized into polymers of these sugars. In addition, the bacteria found in the human mouth can degrade sucrose or polymers formed from sucrose into acidic end products.

The studies that have been carried out are; (i) establishment of a single and relatively simple analytical procedure for the estimation of glucose and fructose, either free as monomers or as residues of sucrose or polymers of glucose and fructose (Chapter II); (ii) a basic study to compare the metabolism of sucrose by the microorganisms in salivary sediment with that of its constituent monosaccharides, glucose and fructose. The parameters examined were decrease in pH, sugar utilization and polysaccharide, carbon dioxide, lactic acid and hetero-acid formation (Chapter III); (iii) the next study was restricted to sucrose and attention was focused on the effect of sucrose concentration and pH on the formation of extracellular polysaccharide (Chapter IV); (iv) the utilization by salivary sediment of extracellular polysaccharide formed by the same system was examined and compared with its utilization of known bacterial dextrans and levans (Chapter V); (v) in the last study, the effect of salivary supernatant and fluoride on the metabolism of sucrose by dental plaque and salivary sediment was examined (Chapter VI).

Methods used in the different studies are described in the methods section within the appropriate chapters.

A summary and statement of the conclusions in Chapter VII complete the thesis.

CHAPTER II

USE OF ANTHRONE FOR THE QUANTITATIVE DETERMINATION OF GLUCOSE AND FRUCTOSE IN SUCROSE AND IN A VARIETY OF DEXTRANS AND LEVANS

Anthrone has been used for the qualitative (Dreywood, 1946) and quantitative (Morris, 1948) estimation of a large variety of carbohydrates. The reaction of anthrone with carbohydrates has been examined by numerous investigators because of the sensitivity, simplicity and wide application of the anthrone test.

In a study carried out to adapt the anthrone method to the analysis of polysaccharide mixtures, Koehler (1952) found distinctive curves for the reaction of anthrone with different classes of sugars in boiling water. For example, aldohexoses such as glucose reacted more slowly with anthrone than ketohexoses, such as fructose. He also showed that the anthrone reaction curves for carbohydrate polymers resembled closely those for the corresponding simple sugars.

During examination of the suitability of the anthrone test for the estimation of μg quantities of glucose in culture media, Bonting (1954) found that anthrone reacted with fructose at room temperature but not with glucose. Additional experiments suggested that anthrone could be used to differentiate between glucose and fructose in mixtures.

However, Bonting did not examine whether the amount of heating necessary to produce maximum colour with glucose increased or decreased the colour contributed by fructose present in the same mixture. Koehler's

study had shown that a rise and fall in colour occurs with fructose if heated with anthrone for the period of time necessary to obtain maximum colour development with glucose.

Van Handel (1967) demonstrated that fructose in polymers could be measured at room temperature with anthrone without prior hydrolysis. Also, that fructose could be determined in the presence of glucose and other carbohydrates that do not react with anthrone at room temperature.

Difference in reactivity of glucose and fructose with anthrone and the fact that glucose and fructose in polymers can be determined without prior treatment, suggested that differential heating with anthrone could provide a test particularly suited for metabolic studies on the metabolism of sucrose by the bacteria found in the oral cavity. Sucrose, the main sugar of the human diet can undergo several changes. It can be hydrolyzed into its constituent monosaccharides, glucose and fructose, or it can be synthesized into polymers of glucose and fructose. The bacteria found in the human mouth can rapidly degrade sucrose, its constituent monomers, or the polymers formed from sucrose to acidic end products. Being able to follow these different processes where mixtures are likely to be present and with a single and relatively simple analytical procedure is obviously of advantage.

To adapt the anthrone method for this purpose, experiments were carried out in the present study to determine the following; (i) the most suitable heating conditions for estimating glucose and fructose in mixtures; (ii) the most suitable and the minimum number of glucose and

fructose standards required to analyze a series of unknowns where glucose and fructose concentrations vary widely in ratio, and; (iii) whether a variety of dextrans and levans, glucose and fructose polymers formed from sucrose, could be analyzed.

METHODS AND RESULTS

A. Preparation of Anthrone Reagent

Anthrone reagent was prepared by dissolving 150 mg anthrone in 100 ml of 26.2 N sulfuric acid (analytical grade) at room temperature (Van Handel, 1967). After all anthrone particles had dissolved, the reagent was stored at 4^{o}C for at least 1 hour prior to use.

B. Heating Conditions for Analysis of Glucose and Fructose Free in Solution and as Residues of Sucrose

In this series, experiments were carried out to determine the appropriate heating conditions for estimating solutions containing glucose, fructose, sucrose and glucose-fructose in 1:1 ratio.

Four solutions were prepared; sucrose, glucose and fructose solutions, each at a concentration of 1.11 mM, and a solution containing both glucose and fructose, with the concentration of each at 1.11 mM. Twenty seven aliquots (50 µl) were removed from each solution and transferred to the same number of 10 x 75 mm test tubes sitting in cracked ice. Anthrone reagent previously chilled in ice was added (1.5 ml) to

each sample with a 2 ml syringe fitted with a teflon tip (Monostat Corp., N. Y.). The sugar sample and anthrone reagent in each tube was mixed immediately and vigorously on a vortex mixer (Scientific Industr. Inc., N. Y.). The tubes were left standing for approximately 10 to 15 minutes at room temperature before proceeding with development of colour which was done by incubating the tubes in a water bath at either 25°C, 40°C, 60°C or 95°C for various times. At intervals up to 4 hours when the incubation was at 25°C, and up to 30 minutes when heating was at the other temperatures, tubes were transferred to cracked ice to stop the sugar reaction with the anthrone reagent from proceeding further. The optical density of each sample was then measured at 620 mμ in a Beckman DU spectrophotometer. Because even heating of samples was necessary to obtain reproducible results (cf. Seifter et al, 1950), tubes were either continually hand-shaken or rotated in a rack fixed to the shaft of an air-driven rotary mixer (Thomas, Philadelphia, U.S.A.).

The results of these experiments are shown in Figs. 2.1 and 2.2. Only solutions containing fructose (which includes those containing sucrose) reacted with anthrone when the reaction proceeded at 25°C (Fig. 2.1). Maximum colour intensity was reached in about 2 to 2½ hours. Glucose did not form its characteristic blue chromogen with anthrone even up to 4 hours of incubation.

When the reaction was carried out at 40°C, maximum colour development in solutions containing fructose was reached in about 20-25 minutes

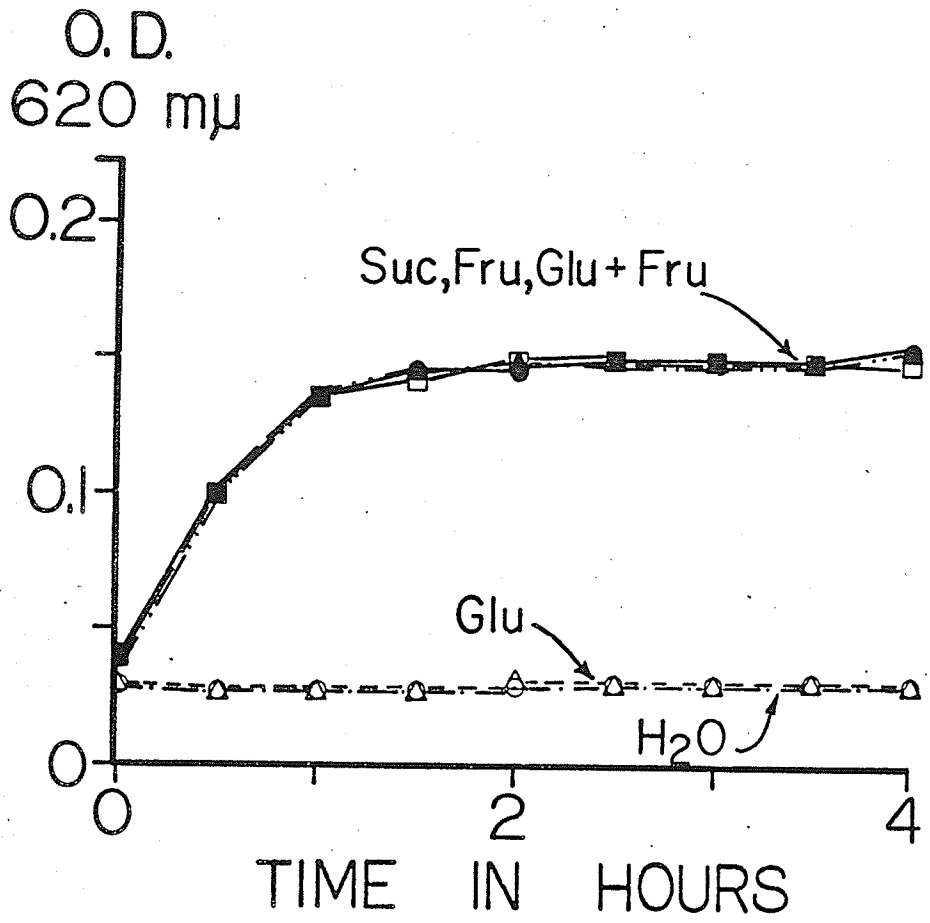

Figure 2.1. Anthrone reaction curves with different sugars at 25°C. Δ - glucose (55.6 nmoles); □ - fructose (55.6 nmoles); ● - sucrose (55.6 nmoles); ▲ - glucose (55.6 nmoles) + fructose (55.6 nmoles) in 1:1 ratio; o - distilled water.

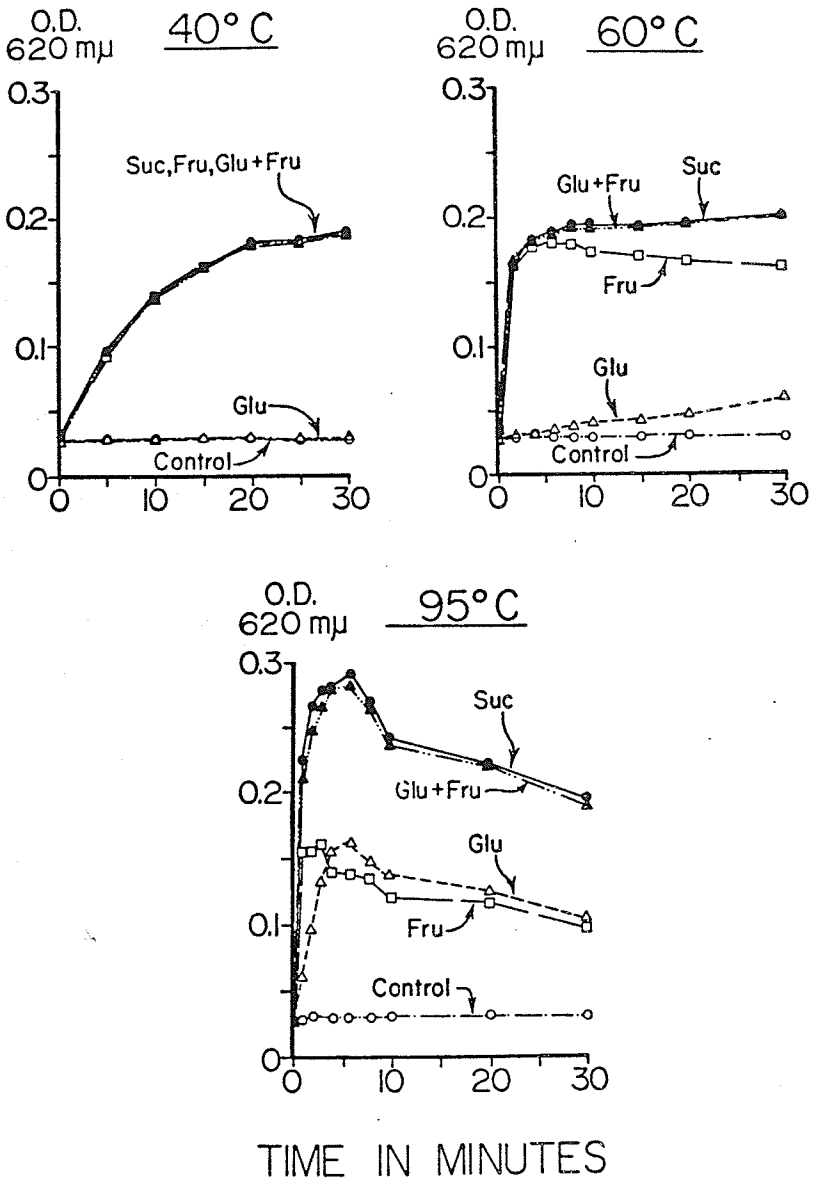

Figure 2.2. Anthrone reaction curves with different sugars at; (a) 40°C; (b) 60°C, and; (c) 95°C. Δ - glucose (55.6 nmoles); □- fructose (55.6 nmoles); ● - sucrose (55.6 nmoles); ▲ - glucose (55.6 nmoles) + fructose (55.6 nmoles) in 1:1 ratio; o - distilled water.

(Fig. 2.2a). In experiments at this temperature (not shown) where heating was continued for longer than 30 minutes, the optical density showed a small further increase up to 1 hour. This can be attributed to glucose, which did not react with anthrone for about 30 minutes, but did show a slow, small increase thereafter.

At 60°C, samples containing fructose developed maximum colour between 4-8 minutes. Glucose samples showed a slow, continuous increase in colour (Fig. 2.2b). Optical density changes in the sucrose and glucose-fructose solutions were similar and were approximately equal to the sum of the optical densities of the solutions containing only glucose or fructose. The optical density decreased in the solution containing only fructose and this occurred after about 6 minutes of heating.

Upon heating the samples at 95°C, the optical densities of the different sugar solutions rapidly reached a maximum and then slowly decreased. An earlier maximum was reached with fructose than with glucose. Optical densities were similar in the sucrose and glucose-fructose samples which were again approximately equal to the sum of the optical densities of the solutions containing only glucose or fructose (Fig. 2.2c).

From these experiments, the conditions selected for determining fructose and glucose in unknown samples were as follows. First, samples were incubated at 25°C for 2 hours to determine the optical density (OD_1) of the anthrone reagent with fructose. Samples were then heated at 95°C for 6 minutes, immediately cooled in an ice water bath for 2

minutes and kept at room temperature for 15 minutes before measuring the optical density again (OD_2). The optical density only due to glucose can be obtained by subtracting OD_1 from OD_2 after correcting for the change in OD_1 arising from the decrease in O.D. that occurs with fructose (see next section).

C. Determination of the Glucose and Fructose Standards Needed for Analysis of Unknowns Containing Various Ratios of Glucose and Fructose

For the method to be of value, it would be necessary to be able to determine glucose and fructose in mixtures where glucose and fructose can be present more or less at any ratio and yet not require the large number of corresponding solution standards.

To do this, the following experiments were carried out. Stock solutions were prepared containing the fructose and glucose combinations shown in Table II.1. The concentrations of glucose and fructose ranged between 0 and 2.2 mM. Each combination was prepared in triplicate; 50 µl was removed from each, 1.5 ml anthrone reagent was added and the mixtures were then treated as described in the previous section. The results of a typical experiment are shown in Fig. 2.3.

The optical densities both at 25°C and 95°C showed a linear relationship with sugar concentration for all concentration conditions (Figs. 2.3a and 2.3b).

Glucose, even though present at concentrations between 0 and 2.2 mM did not produce colour with anthrone or interfere with the fructose

TABLE II.1

COMPOSITION OF GLUCOSE AND FRUCTOSE SOLUTIONS FOR DETERMINING OD_1 AND OD_2 AT DIFFERENT GLUCOSE/FRUCTOSE RATIOS

nMoles Fructose in 50 µl of Solution	nMoles Glucose in 50 µl of Solution			
	0	27.8	55.6	111.2
0	0	0	0	0
27.8	0	27.8	55.6	111.2
	27.8	27.8	27.8	27.8
55.6	0	27.8	55.6	111.2
	55.6	55.6	55.6	55.6
111.2	0	27.8	55.6	111.2
	111.2	111.2	111.2	111.2

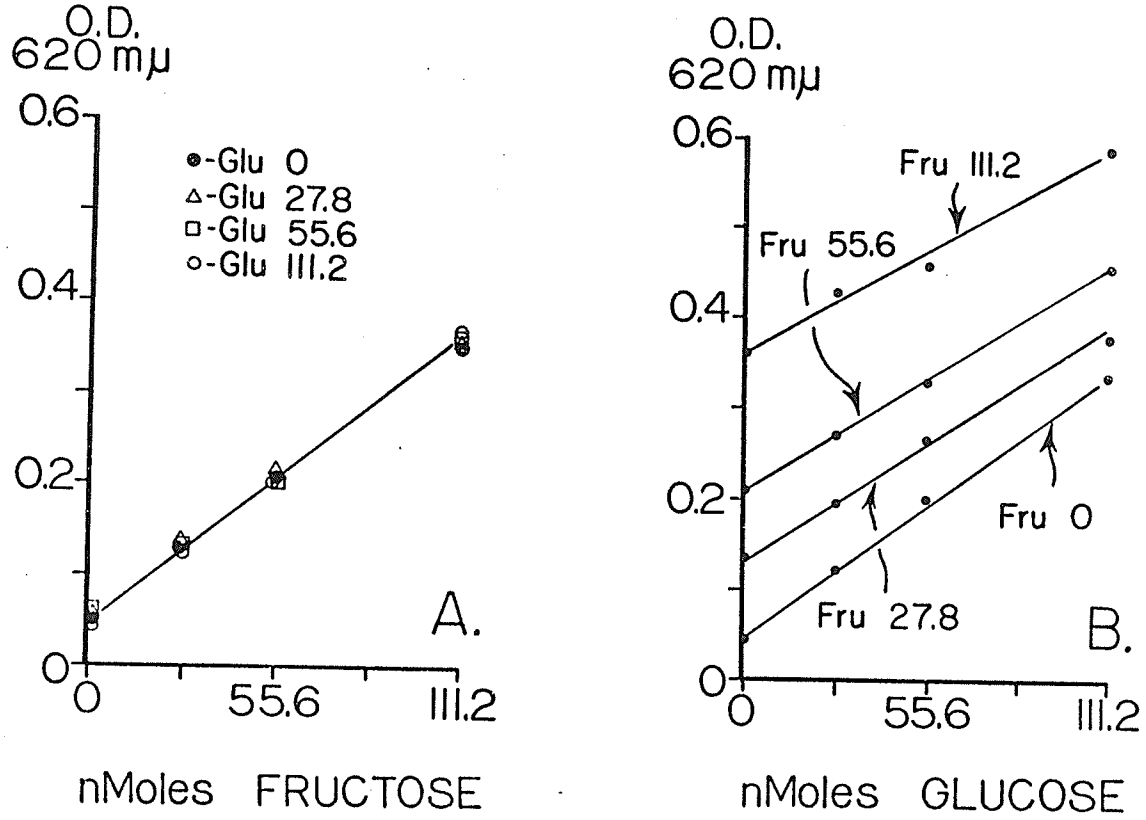

Figure 2.3. Optical dentity of solutions containing anthrone reagent and varying amounts of glucose and fructose; (A) after incubation at 25°C for 2 hours, and; (B) after heating at 95°C for 6 minutes. The composition of a mixture corresponding to each point is the amount of glucose (0, 27.8, 55.6 or 111.2 nmoles) and/or fructose (0, 27.8, 55.6 or 111.2 nmoles) shown on the abcissa plus the glucose or fructose indicated by the line through the point.

analyses (Fig. 2.3a). Upon heating at 95°C, the solutions containing glucose increased in colour intensity while those containing only fructose showed a decrease (cf. Figs. 2.3a and 2.3b).

A linear regression of the fructose standards on the optical density readings at 25°C was fitted to an equation of the type:

$$F = F_0 + F_1.OD_1 \qquad (1)$$

where F equals µg of fructose, F_0 is the value of F for an OD_1 equal to 0, and F_1 is the increase in F per unit increase in OD_1.

A multilinear regression of the glucose standards on both OD_1 and OD_2 was then fitted to an equation of the type:

$$G = G_0 - G_1.OD_1 + G_2.OD_2 \qquad (2)$$

where G equals µg of glucose, G_0 is the value of G for an OD_2 equal to 0, G_1 is the increase in G per unit increase in OD_1 and G_2 is the increase in G per unit increase in OD_2.

Predictability of equation 1 computed from 18 standards as the square of the correlation coefficient averaged 94 per cent. That of equation 2 computed as the square of the multiple correlation coefficient averaged 96 per cent. Therefore, the error of the method was considered acceptable.

Since the number of standards required to obtain the curves in Fig. 2.3 are too many to be practical, the much smaller number of standards shown in Table II.2 were tested for suitability to compute the

TABLE II.2

COMPOSITION OF PARTIAL SET OF STANDARD SOLUTIONS

FOR DETERMINING OD_1 AND OD_2

nMoles Glucose in 50 µl of Solution

nMoles Fructose in 50 µl of Solution

0 / 0			
0 / 27.8	27.8 / 27.8		
0 / 55.6		55.6 / 55.6	
0 / 111.2			111.2 / 111.2

coefficients in equations 1 and 2. The computed coefficients for both sets of standards and the multiple correlation coefficient between the two methods are compared in Table II.3. As can be seen from the table, the much smaller number of standard fructose and glucose solutions gave essentially the same results as the set containing the much larger number of standards.

D. Analysis of a Number of Levans and Dextrans for Glucose and Fructose

The reactions with anthrone of the dextrans and levans listed in Table II.4, were examined. One hundred mg of each was weighed out and made up in distilled water at a concentration of 100 µg/ml. Glucose and fructose standards were prepared as in Table II.1. Three aliquots (50 µl) were removed from each solution and transferred to 10 x 75 mm test tubes and analyzed for glucose and fructose as described in section C.

Dried samples of the dextrans and levans listed in Table II.5 were also analyzed following solubilization by a procedure used previously to solubilize tissue or dental plaque (Kleinberg et al, 1971). Aliquots (50 µl) from 100 µg/ml solutions of each carbohydrate polymer were transferred to 10 x 75 mm test tubes and taken to dryness by heating in a boiling water bath. The dried samples were solubilized by adding 50 µl of 50 per cent H_2SO_4 (V/V) and heating at 70°C for 5 minutes. Anthrone reagent was then added and samples then analyzed. The results are shown in Tables II.4 and II.5. From the tables it is evident that all the dextrans tested do not react with anthrone at 25°C. On the other

TABLE II.3

COMPARISON OF COEFFICIENTS COMPUTED FROM COMPLETE (I) AND PARTIAL (II) SETS OF STANDARDS

	I	II
F_0	-2.66	-2.79
F_1	81.70	82.60
G_0	-4.27	-4.46
G_1	71.20	74.80
G_2	81.80	84.20
Multiple Correlation Coefficient equation (1)	0.99(8)	0.99(8)
Multiple Correlation Coefficient equation (2)	0.99(8)	0.99(8)

TABLE II.4

ANALYSIS OF 5 µg SAMPLES OF A NUMBER OF DEXTRANS AND LEVANS WITH ANTHRONE

Carbohydrate Analyzed	Amounts Found	
	µg Fructose	µg Glucose
Water control	0	0
Dextran 512	0	4.8
Dextran 742	0	5.2
Dextran 1355	0	4.7
Dextran 1196	0	5.7
Dextran 523	0	5.6
Dextran 1191	0	5.1
Levan 523	5.1	-
Levan 512	4.7	-
Levan 1662	4.8	-
Fructose	5.3	-
Glucose	-	5.1

The above and other dextrans and levans used in this study were obtained from Northern Utilization Research Branch, Agricultural Research Service, United States Department of Agriculture, Peoria, Illinois, U.S.A.

TABLE II.5

COMPARISON OF ANALYSIS OF 5 µg SAMPLES OF SEVERAL DEXTRANS AND LEVANS IN AQUEOUS SOLUTION AND IN A DRIED STATE

Carbohydrate Analyzed	Aqueous Solution		Dried	
	µg Fructose	µg Glucose	µg Fructose	µg Glucose
Levan 523	4.6	0	4.6	0
Levan 512	4.8	0	4.5	0
Dextran 512	0	4.9	0	5.5
Dextran 1196	0	5.3	0	5.2
Levan 523 + Dextran 512 (2.5 µg of each)	2.3	2.7	2.4	2.4
Levan 512 + Dextran 1196 (2.5 µg of each)	2.4	2.6	2.4	2.4
Fructose	4.8	0	4.4	0
Glucose	0	4.9	0	4.9
Blank	0	0	0	0

hand, levans do react at that temperature. Also levans and dextrans in a mixture can be differentiated just as mixtures of their constituent monosaccharides can be differentiated. It is also evident (Table II.5), that essentially the same recovery of the samples is obtained whether in solution or in a dried state and that solubilization with H_2SO_4 and brief heating has no deleterious effect on the polymers analyzed.

DISCUSSION

The results confirm the findings of Bonting (1954) and Van Handel (1967) that only fructose residues react with anthrone at room temperature while glucose residues require a higher temperature.

Based on the difference in reactivity between glucose and fructose, the procedure arrived at for their analysis permits estimation of both sugars when present either alone or together in solution or solid form and either free or as constituents of glucose and fructose polymers.

As will be seen in the subsequent chapters, the method in conjunction with the preparative techniques such as centrifugation, alcohol precipitation is particularly suited for studies on sucrose metabolism where this molecule is hydrolyzed and/or provides the residues for the synthesis of glucose and fructose polymers.

CHAPTER III

METABOLISM OF SUCROSE BY THE BACTERIA IN SALIVARY SEDIMENT IN COMPARISON TO THE METABOLISM OF GLUCOSE AND FRUCTOSE

Studies on dental plaque *in situ* have established that the extent and duration of the pH fall following exposure to glucose is depent upon the availability of glucose to the plaque bacteria (Kleinberg, 1961). A similar relationship between glucose availability and pH was demonstrated in salivary sediment and plaque-saliva mixtures incubated *in vitro* (Kleinberg, 1967; Singer and Kleinberg, 1970). In the more easily studied salivary sediment system, a number of parameters of the carbohydrate metabolism of the sediment microflora was examined and related to the pH changes seen when the availability of glucose is varied (Sandham and Kleinberg, 1969a, 1970a and b).

The major parameters found necessary to relate the carbohydrate metabolism of the sediment microflora to the pH changes arising from the metabolism of glucose were; (i) uptake of the glucose from the medium; (ii) formation and utilization of certain intermediates, polyglucose and lactic acid, and; (iii) formation of CO_2 and acid other than lactic named hetero-acid and consisting of the difference between the total and lactic acids formed, (Sandham and Kleinberg, 1970a).

Polyglucose and lactic acid both increased while there was still glucose in the medium for the bacteria to utilize. Once the glucose was used up, polyglucose and lactic acid were immediately catabolized. Carbon

dioxide and hetero-acid on the other hand accumulated until glucose utilization was complete.

A number of studies have provided evidence indicating that the polysaccharides formed from glucose and sucrose can serve as substrates for acid formation when these sugars are no longer available from the extracellular environment. Manly (1961) showed that exposure of sediment to sucrose for 20 minutes was long enough for the bacteria in salivary sediment to store substantial amounts of polysaccharide. He proposed that these polysaccharides could serve as substrates for acid formation and thus enable acid formation to continue for several hours after depletion of sucrose was complete. Gibbons and Kapsimalis (1963) showed this to be possible in experiments with glucose and a pure culture of Streptococcus mitis isolated from dental plaque. This microorganism formed an intracellular polyglucose of the glycogen-amylopectin type while growing in a medium containing glucose but when transferred to a buffer at pH 7.0, the pH decreased within 20 minutes to a pH of 5.6.

In salivary sediment, Sandham and Kleinberg (1969a) demonstrated that utilization of glucose, acid formation and formation and utilization of stored polyglucose are closely integrated processes. In experiments in which the glucose supplied to the bacteria was limited, as soon as the bacteria in the sediment had used up all the glucose, they immediately switched to the utilization of the carbohydrate that had been stored during the period that glucose was still in the medium. Thus, acid formation was able to continue without interruption and be sustained for longer

periods than might otherwise be possible.

Although hydrolysis of starchy foodstuffs by salivary and bacterial enzymes produces glucose, as pointed out in Chapter I, the main sugar of human diets is sucrose. Numerous studies have been carried out on this sugar in relation to the caries process but many aspects of its metabolism by the mixed bacterial populations found in saliva and in plaque that might account for its high cariogenicity, are still not known. As a basis for the later studies in this thesis, in the present chapter, the effect of sucrose on the main parameters of the carbohydrate metabolism of the bacteria in the suspended salivary sediment (SSS) system has been examined in the same way as previously done for glucose (Sandham and Kleinberg, 1969a; 1970a and b). The parameters examined were, pH, substrate utilization, polysaccharide formation and utilization, acid and carbon dioxide production. Because sucrose upon hydrolysis yields the two monosaccharides, glucose and fructose, the metabolism of glucose and fructose alone and glucose and fructose together in 1:1 ratio was also examined and compared to that of sucrose. The comparisons were carried out at three substrate concentrations, viz., 5.6, 27.8, and 277.8 mM for glucose, fructose and equimolar glucose and fructose (each present at half these concentrations); for sucrose the concentrations were, 2.8, 13.9, and 138.9 mM, since upon hydrolysis these would become 5.6, 27.8, and 277.8 mM, respectively. As in the earlier studies with glucose (Kleinberg, 1967; Sandham and Kleinberg, 1969a), the cell concentration in the SSS system was standardized at 16.7 per cent (V/V) and experiments

were carried out for 4 hours at 37°C.

METHODS

Preparation of Salivary Sediment Mixtures and Incubation Procedures

Wax-stimulated whole saliva from 6-8 subjects who had not eaten or cleaned their teeth for at least 12 hours was collected in 25 x 150 mm test tubes held in cracked ice. The saliva was centrifuged at 1740g for 15 minutes; the supernatant was stored while the sediment was washed three times with cold distilled water and made up in distilled water as a 50 per cent suspension (V/V).

Interrelation Between Decrease in pH, Sugar Utilization and Polyglucose and Polyfructose Storage

In this series of experiments, the aspect of the carbohydrate metabolism of the sediment microflora examined was the interrelation between the decrease in pH, disappearance of each of the sugars from the medium and total polyglucose and/or polyfructose stored. For each experiment, five incubation mixtures were prepared in 25 x 75 mm test tubes fitted with rubber stoppers containing three perforations; one to allow the insertion of a glass pH electrode (Beckman No. 39167), another for a salt bridge leading from a calomel reference electrode and a third to allow insertion of a micropipette for removal of aliquots for carbohydrate analysis (Sandham and Kleinberg, 1969a). Each mixture consisted

of 1 ml of 50 per cent sediment suspension, 1 ml of salivary supernatant and 1 ml of either distilled water, glucose, fructose, glucose plus fructose in 1:1 ratio, or sucrose. Only one concentration was tested in each experiment and all experiments were carried out at least twice. The final sugar concentrations were 2.8-5.6, 13.9-27.8 and 138.9-277.8 mM; the first of each pair was that for sucrose and the second was that for the other sugars.

Mixtures were incubated at 37°C for 4 hours. Before and at regular intervals during each incubation, the pH was measured (on a Model TT1C pH meter-titrator, Copenhagen) and aliquots were removed in triplicate with a calibrated 24 µl Lang-Levy pipette (H. E. Pedersen, Copenhagen) for centrifugation and determination of the carbohydrate contents of the supernatant and sediment.

In the experiments with the lower substrate concentrations (2.8-5.6 and 13.9-27.8 mM), each 24 µl aliquot was pipetted into a 10 x 75 mm test tube containing 0.6 ml of 1 per cent NaF and previously chilled in cracked ice. In the experiments in which the sugar concentration was 138.9-277.8 mM, the volume of NaF solution used to stop the metabolism and dilute the aliquot was 1.8 ml. After mixing the contents of each tube by vibrating on a vortex mixer, sediment and supernatant were separated by centrifuging at 1740g for 5 minutes. From the supernatant, three 50 µl aliquots were removed for determination of its carbohydrate content.

The remainder of the supernatant was carefully removed by suction

leaving a pellet of sediment. The pellet was washed either two or three times in 2 ml volumes of cold 1 per cent NaF solution. After the last washing , the sediment was dried by heating in a hot water bath. The dry sediment was then dissolved in 50 µl of 50 per cent H_2SO_4 by heating at 70°C for 5 minutes.

The glucose and fructose contents of both the supernatant and the sediment were determined by the anthrone procedure described in Chapter II.

In similar experiments, the disappearance of each of the sugars was followed by paper chromatography, particularly to determine whether sucrose is hydrolyzed extracellularly. The experiments were carried out the same way as those in the previous series except that a large number of identical sediment mixtures were prepared (42, each having a total volume of 150 µl). At regular intervals during a 4 hour incubation, 3 mixtures were removed for measurement of the pH and 3 for obtaining aliquots of supernatant for spotting on paper chromatograms - rather than removing aliquots from a single mixture.

The sample sizes applied to the origin from mixtures containing sugar concentrations of 2.8-5.6; 13.9-27.8; and 138.9-277.8 mM were 25, 15 and 10 µl, respectively. Ascending chromatograms were run in one direction in a solvent consisting of pyridine:ethylacetate:water (4:10:3). After allowing the chromatograms to dry, the sugars were located by dipping the chromatograms in p-anisidine reagent prepared by dissolving 0.5 gm p-anisidine in 25 ml of absolute ethanol and adding 25 ml H_2O and 2 ml concentrated H_3PO_4 (Mukherjee et al, 1952). The chromato-

grams were dried and developed in a hot air oven at 95°C for 15 minutes.

It was evident from these experiments that polymers formed by the sediment bacteria during incubation with sucrose are released into the medium. Exploratory experiments showed that repeated washing of sediment with water solubilizes some of the polysaccharide associated with the sediment. Attempts to solubilize the remaining carbohydrate with 1 N KOH disclosed the presence of cold KOH-soluble polysaccharides. Cold KOH has been used by numerous investigators to extract extracellular polysaccharide associated with bacterial cells (Guggenheim and Schroeder, 1967), while hot KOH has been used to extract intracellular polysaccharide, mainly glycogen (Bramstedt and Lusty, 1968).

In the following experiment, the polyglucose and polyfructose contents of the carbohydrate soluble in water (containing presumably the extracellular carbohydrate in the medium) and the carbohydrate soluble in KOH (containing extracellular carbohydrate attached to the cells) of sediment mixtures incubated with each of the above sugars was examined. Five sediment mixtures (15 ml) were prepared in 125 ml Erlenmeyer flasks by combining salivary supernatant, 50 per cent sediment suspension (V/V) and distilled water or sugar solution in 1:1:1 ratio. One mixture, the control, contained water; the other 4 mixtures contained sucrose at 138.9 mM and glucose, fructose and glucose-fructose in 1:1 ratio at 277.8 mM. After incubating the mixtures for 2 hours, 95 per cent ethanol was added to bring the ethanol concentration of each mixture to 70 per cent. The content of each flask were stirred continuously for 1 hour at 4°C after

which each mixture was centrifuged at 12,400g for 20 minutes at 4°C. The supernatant was discarded and the resulting sediment was extracted twice with distilled water. The washings were combined and ethanol added at a final concentration of 70 per cent (V/V) to precipitate the polysaccharide. The precipitate was harvested by centrifugation at 12,800g for 30 minutes, redissolved in water, dialyzed against distilled water for 18 hours and lyophylized.

The sediment remaining after water extraction was next treated with 1 N KOH at 4°C for 4 hours. The resulting suspension was centrifuged at 12,800g for 30 minutes at 4°C. The supernatant containing the KOH soluble polysaccharides was dialyzed against distilled water for 18 hours and lyophylized.

The polyglucose and the polyfructose contained in the water and KOH soluble fractions along with that in the final pellet was determined by the anthrone method described in Chapter II.

A number of investigators have reported that the polysaccharide extracted with cold KOH from the oral streptococci after incubation with sucrose is mainly dextrans and levans. Although this is likely to be so for the polysaccharide in the KOH soluble fraction in the experiments in this and in subsequent chapters it was considered advisable to test whether intracellular glycogen-amylopectin type polysaccharide was extracted by the cold-KOH procedure. This was done with the iodine test described by Gilbert and Spragg (1964; see also Van Houte and Jansen, 1968a). This test was also performed on samples of the water soluble fraction.

The nitrogen content of both water soluble and the KOH soluble fraction was also determined. Samples of both fractions were digested with H_2SO_4 and the ammonia produced estimated by Nesslerization (Hawk et al, 1954).

Comparison of Lactic, Hetero-acid and CO_2 Formation with the Different Sugars

Lactic and total acid and CO_2 formation were determined in separate experiments. For each experiment, 5 series of identical sediment mixtures (150 µl) were prepared in 10 x 75 mm test tubes with each series consisting of 21 mixtures and each series of mixtures containing one of the above sugars or distilled water. Prior to and at each of several times during a 4 hour incubation, 3 of the mixtures in each series were removed from the incubation for analysis. Mixtures for lactic acid determination were immediately centrifuged at 1740g in a clinical centrifuge for 5 minutes at 4°C. Aliquots (50 µl) were removed from each supernatant, transferred to 10 x 75 mm test tubes and assayed for lactic acid by the method of Cohen and Noell (1960) as described previously (Sandham and Kleinberg, 1970a). Total acids were determined by back titration of the whole sediment mixture with standardized NaOH (0.1 N) as described previously by Sandham and Kleinberg (1970a). Hetero-acid was calculated as the difference between the titratable acid and the lactic acid.

Analysis of CO_2 was carried out as reported by Sandham and Klein-

berg (1970b). Incubation tubes were sealed with serum stoppers to which a teflon cup was attached by a 1-1.5 inch length of stainless steel orthodontic wire. The cup contained 50 µl of 0.1 N NaOH and 5 per cent (V/V) mixed indicator. At the end of an incubation interval, 100 µl of 0.1 N HCl was injected with a hypodermic syringe into the reaction mixture. The resulting low pH in the mixture served both to inhibit the metabolism and to release CO_2 from the medium. The sodium hydroxide in the cup traps any CO_2 evolved during the incubation and thereafter the dissolved CO_2 released from solution when the sediment mixture is acidified by adding the HCl.

To ensure that diffusion was complete, all tubes were kept overnight at room temperature before the teflon cups were removed and their contents titrated with 0.1 N HCl (see Sandham and Kleinberg, 1970b for details). The amounts analyzed were between 0 and 3 µmoles and were determined by interpolation on a standard curve relating the volume titrated to the amount of carbon dioxide obtained by treating known amounts of sodium bicarbonate in the same way as the unknowns (Sandham and Kleinberg, 1970b).

Acid Formation from the Polysaccharide Synthesized from the Different Sugars

Five incubation mixtures (1.5 ml) were prepared in which the final concentration of sucrose was 138.9 mM and glucose, fructose and

glucose-fructose in 1:1 ratio were each 277.8 mM. The mixtures were incubated for one hour at 37°C to allow the bacteria in the sediment to synthesize polysaccharide in the presence of each sugar. After measuring the pH, each incubation mixture was centrifuged at 1740g for 15 minutes. The supernatant was decanted and the sediments were each washed three times with cold distilled water. Each sediment was then used along with fresh saliva to prepare incubation mixtures exactly like the originals except that no sugars were added. The mixtures were re-incubated at 37°C for a period of 4 hours and the pH was measured at regular intervals.

RESULTS

pH Changes

When the substrate concentration was 2.8-5.6 mM, all of the sugars showed Stephan curves; i.e., the pH in each case rapidly fell, reached a minimum during the first 15-30 minutes and then slowly rose during the remainder of the 4 hour incubation (Fig. 3.1a). The pH curves were almost identical except that fructose consistently fell to a slightly lower pH.

At the 13.9-27.8, and the 138.9-277.8 mM concentrations, the pH showed the initial rapid fall seen at the lower sugar concentrations with each of the sugars tested but not the subsequent rise (Figs. 3.1b

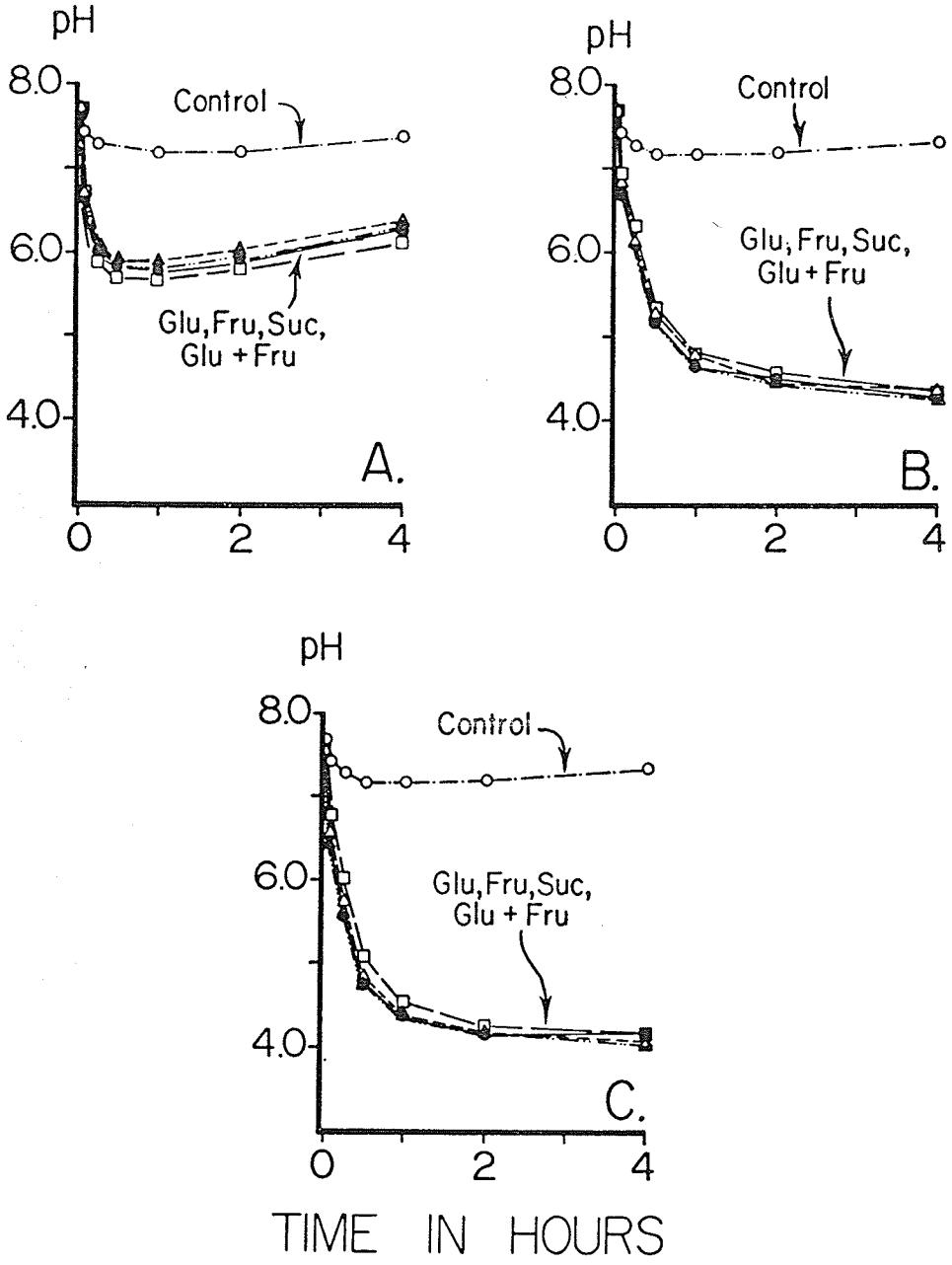

Figure 3.1. pH of salivary sediment mixtures incubated with; (i) no sugar; (ii) glucose; (iii) fructose; (iv) sucrose, and; (v) glucose-fructose in 1:1 ratio. The concentrations in (A) were 2.8 mM for sucrose and 5.6 mM for the other sugars, in (B) were 13.9 mM for sucrose and 27.8 mM for the other sugars and in (C) were 138.9 mM for sucrose and 277.9 for the other sugars. Δ - glucose; □ - fructose; ● - sucrose; ▲ - glucose+fructose (1:1); o - distilled water.

and c). Fructose again showed a small difference from the other sugars but this time the fall in pH with fructose was slightly less than that with the other substrates.

Sugar Utilization

At the lowest sugar concentration (2.8-5.6 mM) utilization occurred at the same rate for all sugars and was complete between 15 and 30 minutes (Fig. 3.2a and b). At the higher concentrations (13.9-27.8 and 138.9-277.8 mM), utilization was incomplete by the end of 4 hours (Fig. 3.3 and 3.4). At the 27.8 mM concentration, glucose and fructose in the different mixtures were utilized at approximately the same rates (Fig. 3.3a and b). However, when the two sugars were incubated together in equimolar concentration, glucose utilization was favoured slightly. This difference between the rates of uptake of glucose and fructose was also observed when sucrose was the substrate.

At a sugar concentration of 138.9-277.8 mM, only a small percentage of the available sugar was utilized (Fig. 3.4a and b). Sucrose and glucose-fructose were again utilized at the same rate.

Chromatograms showing the utilization of the different sugars are presented in Figs. 3.5, 3.6 and 3.7.

At a sugar concentration of 2.8-5.6 mM, sucrose disappeared the fastest; disappearance of fructose was the slowest. In the glucose-fructose mixtures, it seemed that glucose was utilized faster than fructose

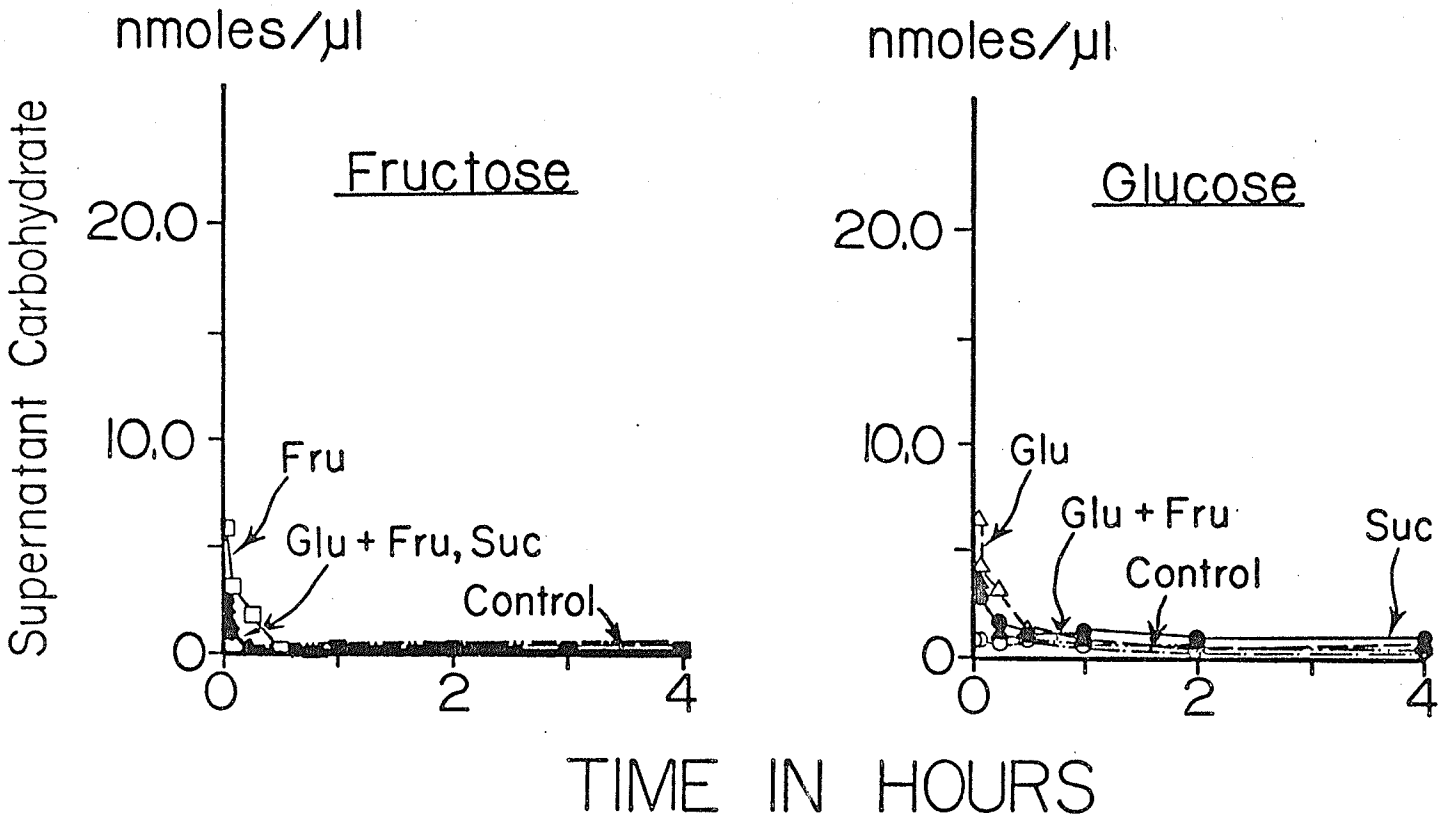

Figure 3.2. Decrease in supernatant (a) fructose and (b) glucose in salivary sediment mixtures incubated with; (i) no sugar; (ii) glucose; (iii) fructose; (iv) sucrose, and; (v) glucose-fructose in 1:1 ratio. Sucrose concentration was 2.8 mM; concentration of other sugars were 5.6 mM. Concentrations are expressed as nmoles/µl of sediment mixture. Δ - glucose; □ - fructose; ● - sucrose; ▲ - glucose+fructose (1:1); o - distilled water.

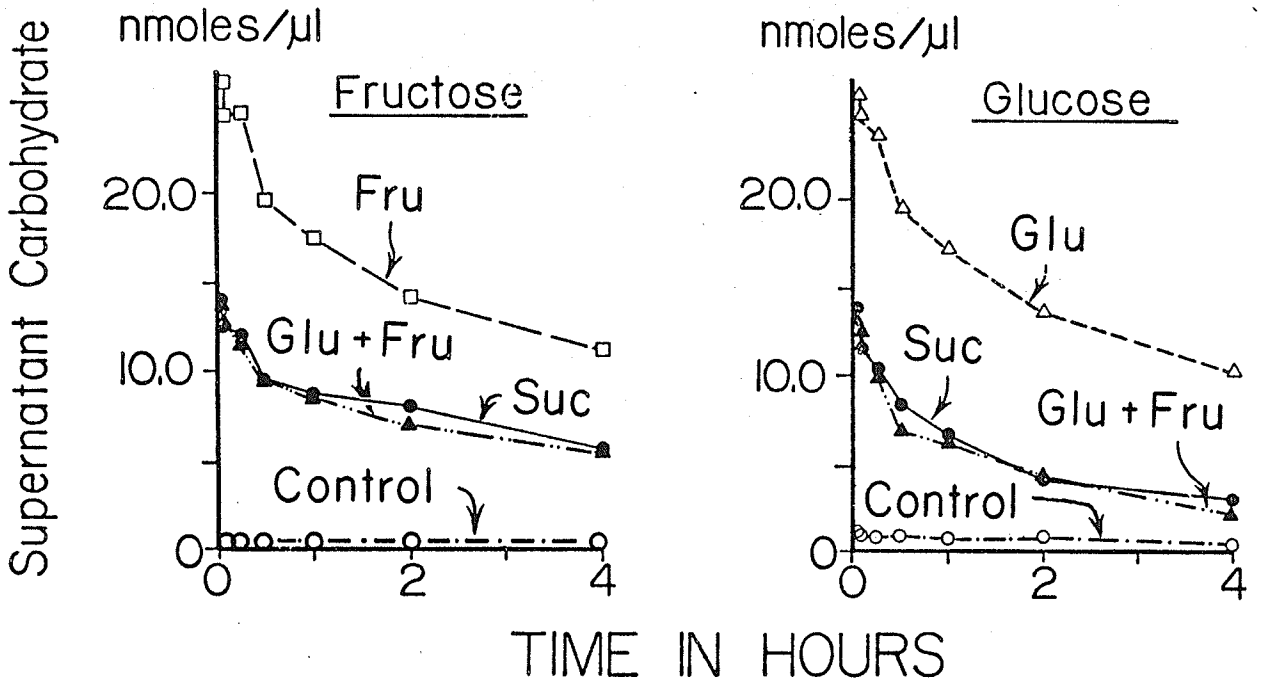

Figure 3.3. Decrease in supernatant (a) fructose and (b) glucose in salivary sediment mixtures incubated with; (i) no sugar; (ii) glucose; (iii) fructose; (iv) sucrose, and; (v) glucose-fructose in 1:1 ratio. Sucrose concentration was 13.9 mM; concentration of other sugars were 27.8 mM. Concentrations are expressed as nmoles/µl of sediment mixture. Δ - glucose; □ - fructose; ● - sucrose; ▲ - glucose+fructose (1:1); o - distilled water.

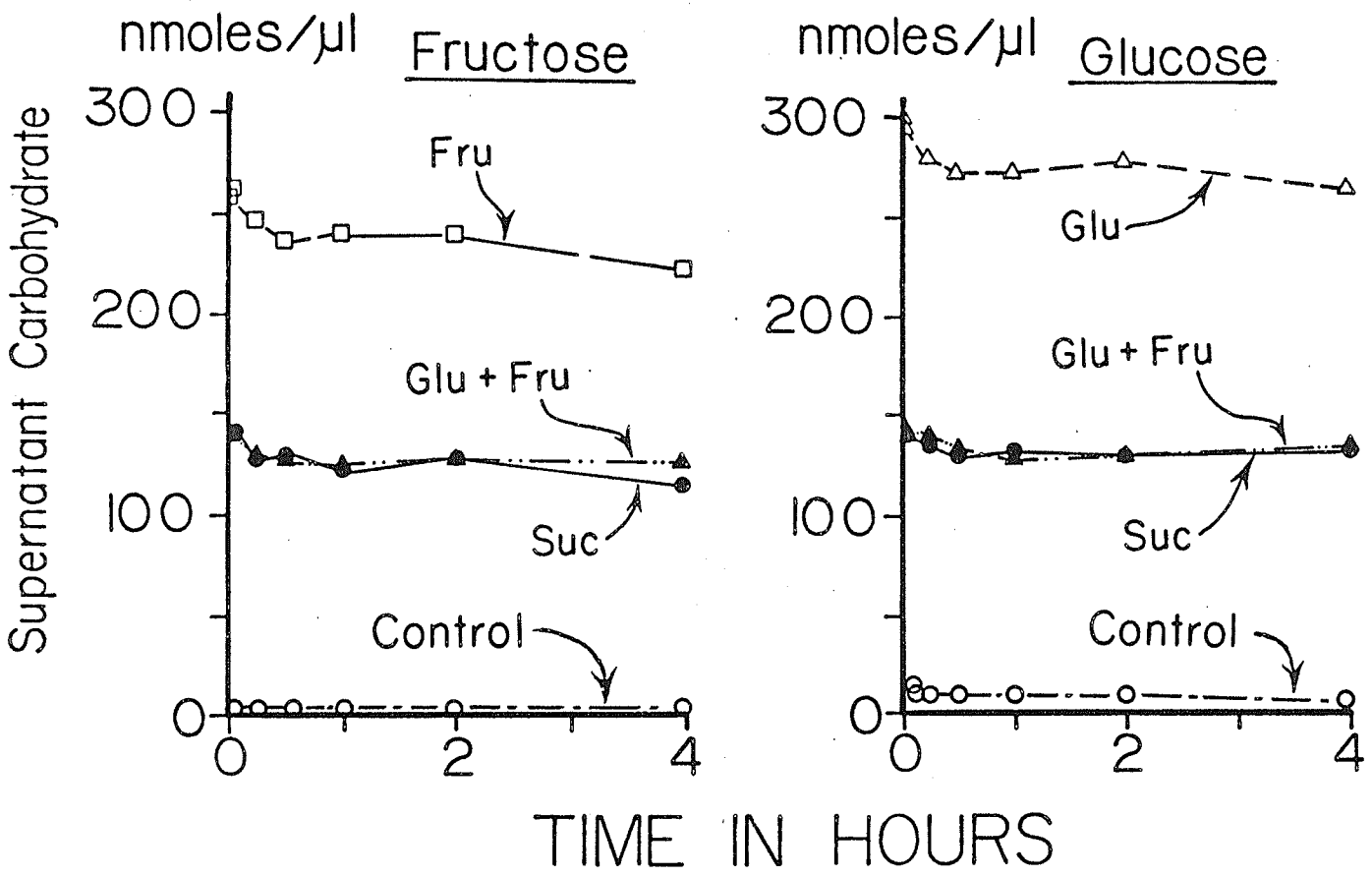

Figure 3.4. Decrease in supernatant (a) fructose and (b) glucose in salivary sediment mixtures incubated with; (i) no sugar; (ii) glucose; (iii) fructose; (iv) sucrose, and; (v) glucose-fructose in 1:1 ratio. Sucrose concentration was 138.9 mM; concentration of other sugars was 277.8 mM. Concentrations are expressed as nmoles/µl of sediment mixture. Δ - glucose; □ - fructose; ● - sucrose; ▲ - glucose+fructose (1:1); o - distilled water.

(Fig. 3.5).

The early appearance of both glucose and fructose spots of equal intensity on the sucrose chromatograms clearly indicates that hydrolysis of sucrose occurs with little difficulty. With the exception of very small immobile spots where the 5 and 15 minute samples were spotted on the sucrose chromatograms, no other carbohydrate spots were evident at other time points or at any time points with the other substrates.

At a sugar concentration of 13.9-27.8 mM (Fig. 3.6), the results were essentially the same as at 2.8-5.6 mM except that the differences were more obvious. In the mixtures containing glucose, fructose and an equimolar mixture of these two sugars, both glucose and fructose decreased continuously and were still present in the medium at the end of the 4 hour incubation. No polysaccharide spots were observed with any of these substrates. On the other hand, with sucrose, the sucrose almost disappeared within 1 hour and glucose and fructose spots appeared at 5 minutes. As in the mixtures with the other sugars these decreased and were still present in the medium until the end of the incubation. Clearly, extracellular hydrolysis of sucrose occurred and at a very fast rate. The polysaccharide spot was again evident. The intensity of the spot increased up to 1 hour and then decreased, indicating that formation and utilization of a readily released extracellular polysaccharide occurs with sucrose.

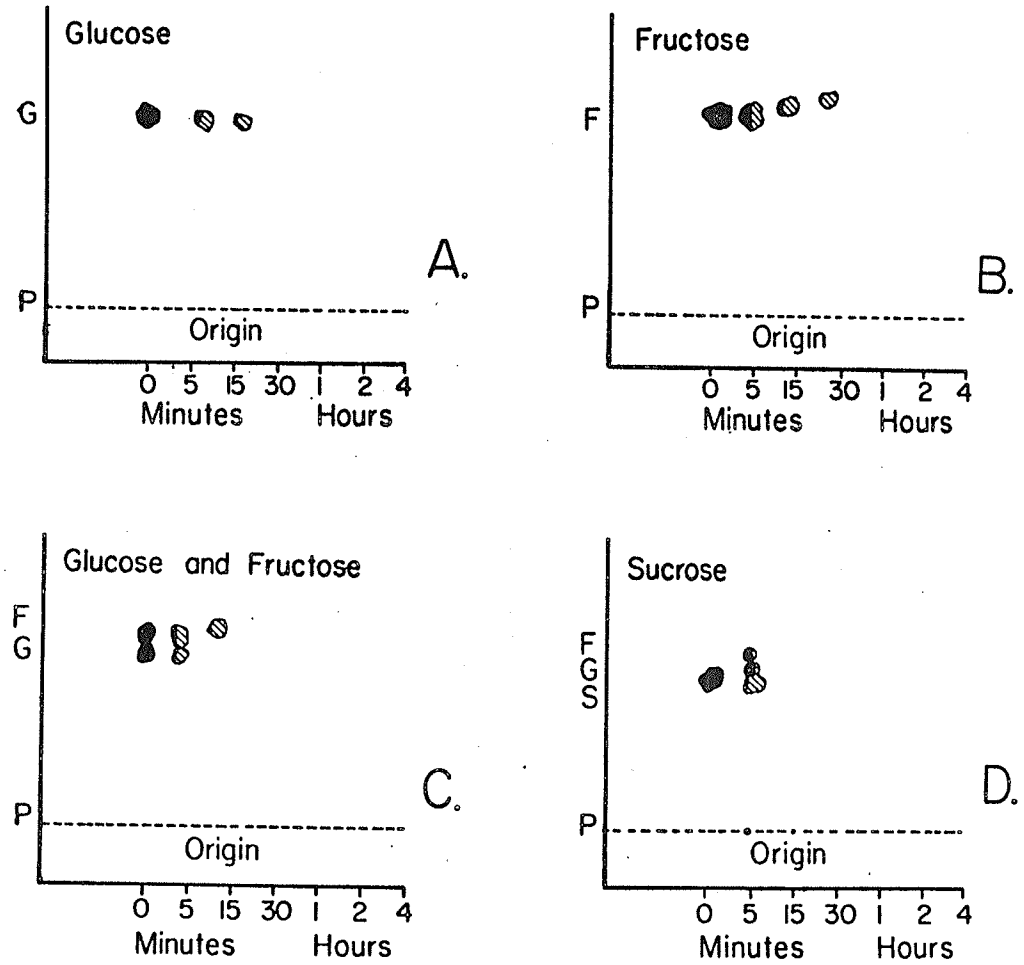

Figure 3.5. Copy of chromatograms of aliquots removed from mixtures corresponding to different time points in the incubation of salivary sediment mixtures with; (A) glucose; (B) fructose; (C) glucose-fructose in 1:1 ratio, and; (D) sucrose. The concentrations in the incubation mixtures were, sucrose at 2.8 mM and the other sugars at 5.6 mM. Each incubation mixture had a total volume of 150 µl; 25 µl of the supernatant was spotted at each point shown. The figures on the abcissa show the sampling intervals. S-sucrose; G-glucose; F-fructose; P-polysaccharide; ● - high intensity; ◍ - low intensity.

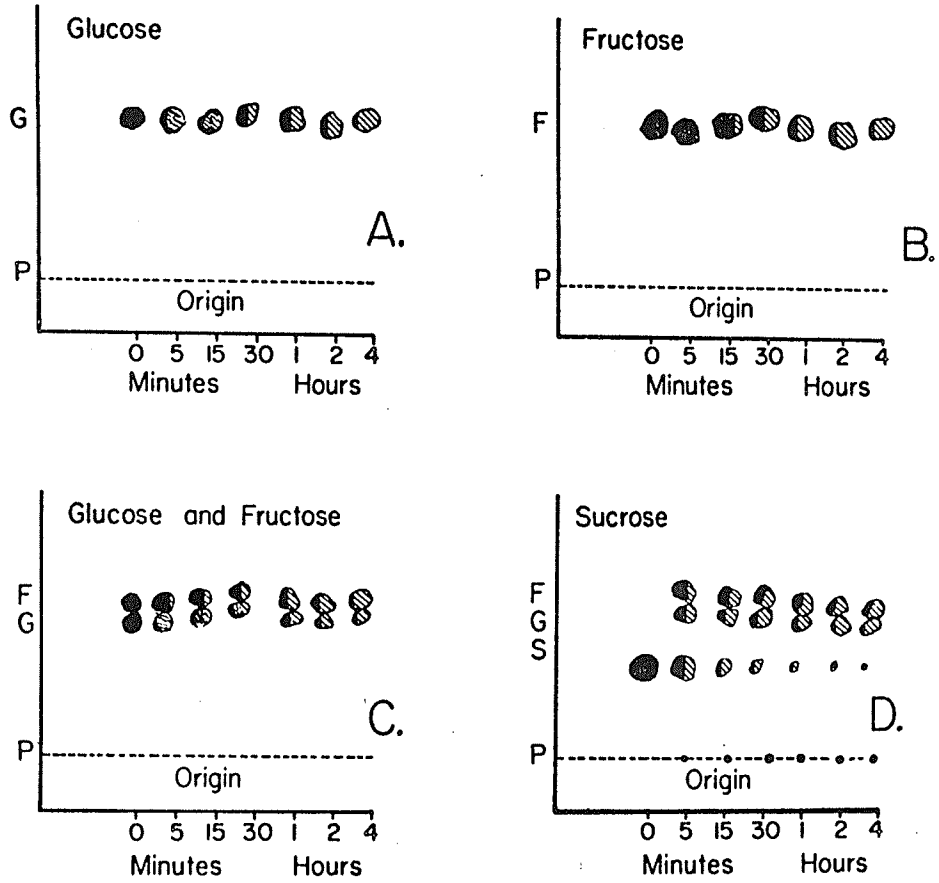

Figure 3.6. Copy of chromatograms of aliquots removed from mixtures corresponding to different time points in the incubation of salivary sediment mixtures with; (A) glucose; (B) fructose; (C) glucose-fructose in 1:1 ratio, and; (D) sucrose. The concentrations in the incubation mixtures were, sucrose at 13.9 mM and the other sugars at 27.8 mM. Each incubation mixture had a total volume of 150 µl; 15 µl of the supernatant was spotted at each point shown. The figures on the abcissa show the sampling intervals. S-sucrose; G-glucose; F-fructose; P-polysaccharide; ● - high intensity; ◍ - low intensity.

At a sugar concentration of 138.9-277.8 mM (Fig. 3.7), the glucose and fructose spots in the mixtures containing glucose, fructose or a mixture of the two, decreased slightly and remained in the medium until the end of the incubation. With sucrose, the sucrose spot decreased slightly and together with the glucose and fructose spots that appeared at the 5 minute interval, persisted until the end of the experiment. In contrast to the results obtained at 13.9 mM sucrose, the polysaccharide spot appeared at 5 minutes and increased up to the end of the experiment.

Storage of Polyglucose and Polyfructose

Salivary sediment from fasting subjects contains some polyglucose but no polyfructose (Fig. 3.8a and b). Absence of added substrate favours utilization of the polyglucose.

At the 2.8-5.6 mM sugar concentration, polyglucose rapidly increased during the first 15 minutes of incubation regardless of the type of substrate used (Fig. 3.8a); no synthesis of polyfructose occurred (Fig. 3.8c). A slow decrease in the sediment polyglucose followed the initial rapid increase. Sediment polyglucose increased least with fructose (0.6 µg/µl of sediment) and most with sucrose (1.3 µg/µl). An equal increase of 1.1 µg/µl occurred with glucose and the glucose-fructose mixture.

At the 13.9-27.8 mM concentration, all the sugars produced a rapid

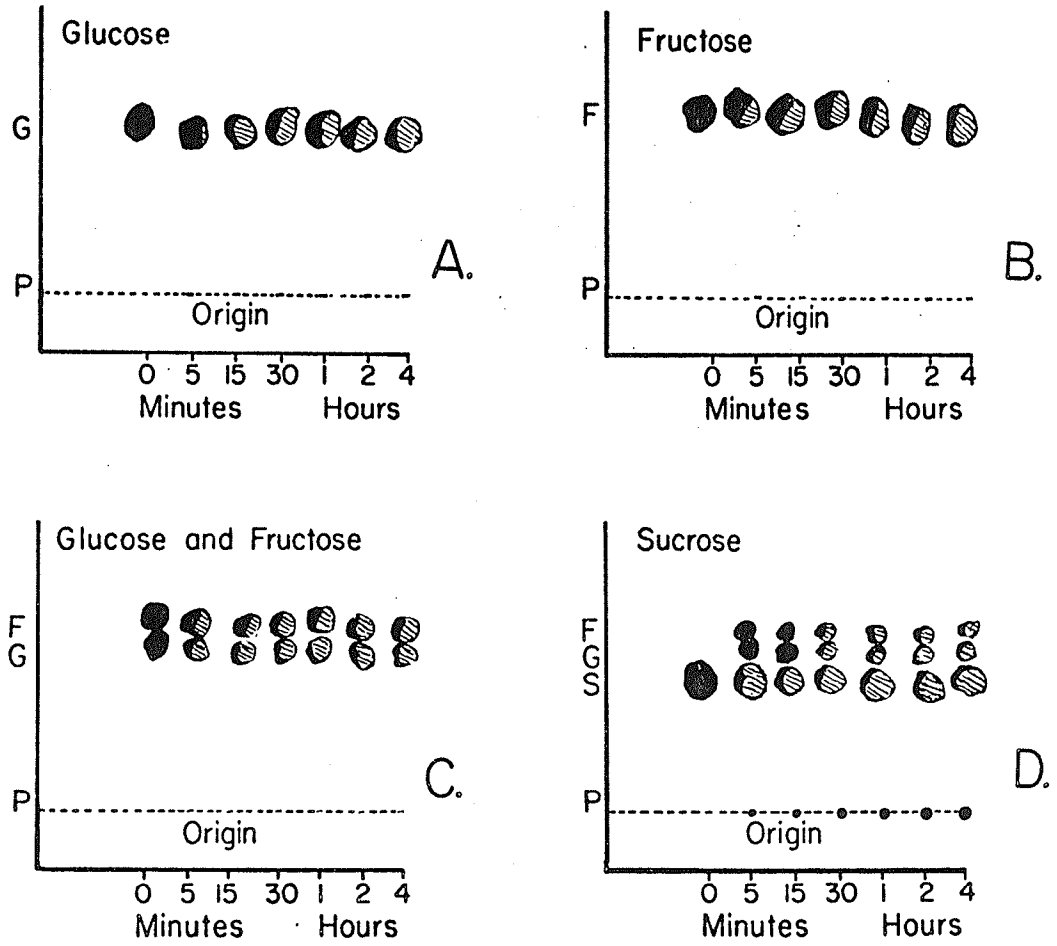

Figure 3.7. Copy of chromatograms of aliquots removed from mixtures corresponding to different time points in the incubation of salivary sediment mixtures with; (A) glucose; (B) fructose; (C) glucose-fructose in 1:1 ratio, and; (D) sucrose. The concentrations in the incubation mixtures were, sucrose at 138.9 mM and the other sugars at 277.8 mM. Each incubation mixture had a total volume of 150 µl; 10 µl of the supernatant was spotted at each point shown. The figures on the abcissa show the sampling intervals. S-sucrose; G-glucose; F-fructose; P-polysaccharide; ● - high intensity; ⌀ - low intensity.

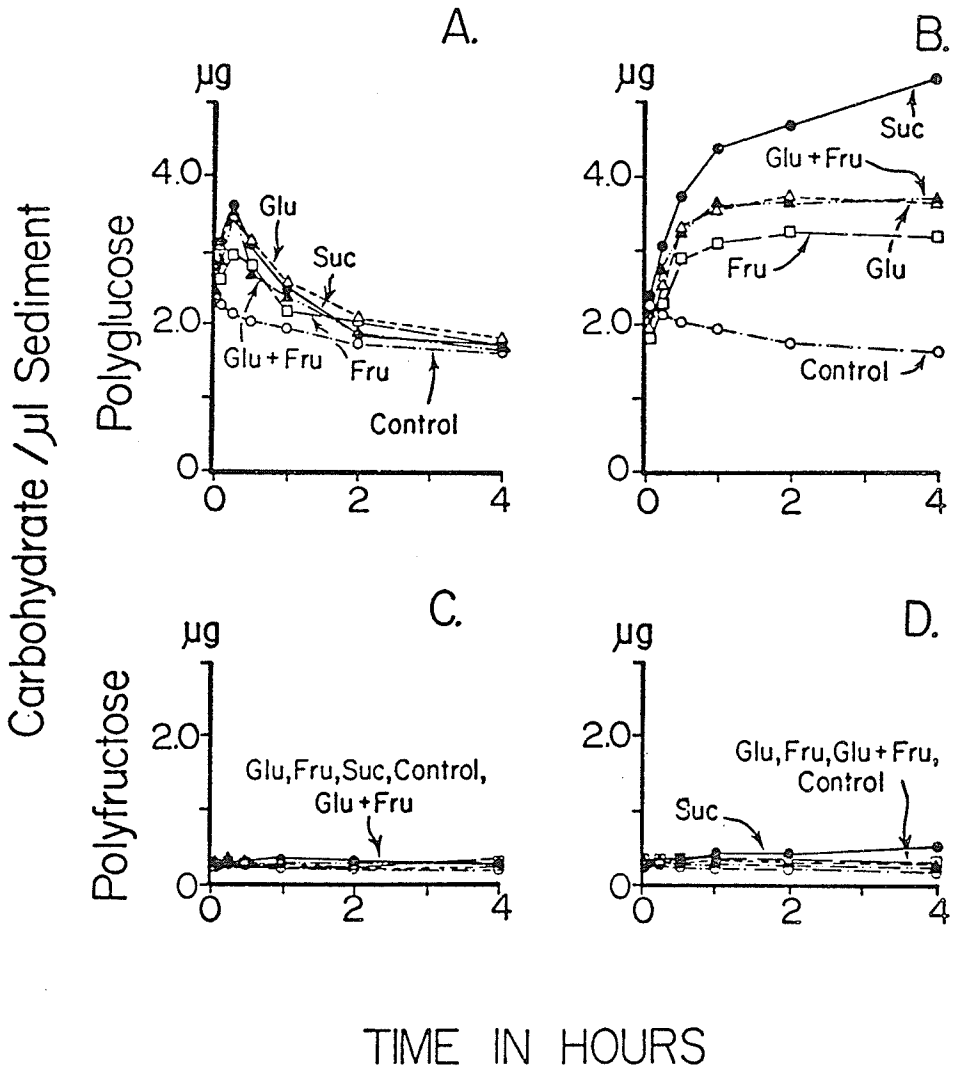

Figure 3.8. Sediment carbohydrate in salivary sediment mixtures incubated with; (i) no sugar; (ii) glucose; (iii) fructose; (iv) sucrose, and; (v) glucose-fructose in 1:1 ratio. In (A) and (C) sucrose concentration was 2.8 mM; the concentration of the other sugars was 5.6 mM. In (B) and (D) sucrose concentration was 13.9; the concentration of the other sugars was 27.8 mM.

increase in the sediment polyglucose most of which occurred during the first ½ hour of incubation (Fig. 3.8b). Fructose caused the smallest increase while sucrose caused the largest (1.5 µg/µl as compared to 3.5 µg/µl of sediment). The increase caused by glucose and its mixture with fructose was similar, 2.0 and 1.9 µg/µl, respectively. Only sucrose showed an increase in sediment polyfructose (Fig. 3.8d). The increase for the entire incubation period totalled 0.4 µg/µl of sediment. Of interest, the ratio of polyfructose to polyglucose in the sediment carbohydrate formed from sucrose was about 1:10.

At 138.9-277.8 mM, sediment polyglucose increased with all the sugars and much more was observed than at lower substrate concentrations (Fig. 3.9a). Sucrose produced much more (1.69 µg/µl) sediment polyfructose than occurred at 13.9 mM sucrose; as before, the other sugars produced little or no polyfructose (Fig. 3.9b). As for the 13.9-27.8 mM concentrations, most of the increase in polyglucose occurred during the first ½ hour of incubation. Again, sucrose gave the largest increase (5.9 µg/µl) while fructose gave the least (1.9 µg/µl). Glucose and its equivalent mixture with fructose both caused increases of 2.9 µg/µl.

Polyglucose and Polyfructose Content of the Water Soluble, KOH Soluble and Residue Fractions of Sediment Mixtures Incubated with the Different Sugars

The composition of the water soluble, KOH soluble and residue fractions of sediment mixtures incubated for 2 hours with the different sugars is shown in Fig. 3.10. Polyglucose formation was most with sucrose

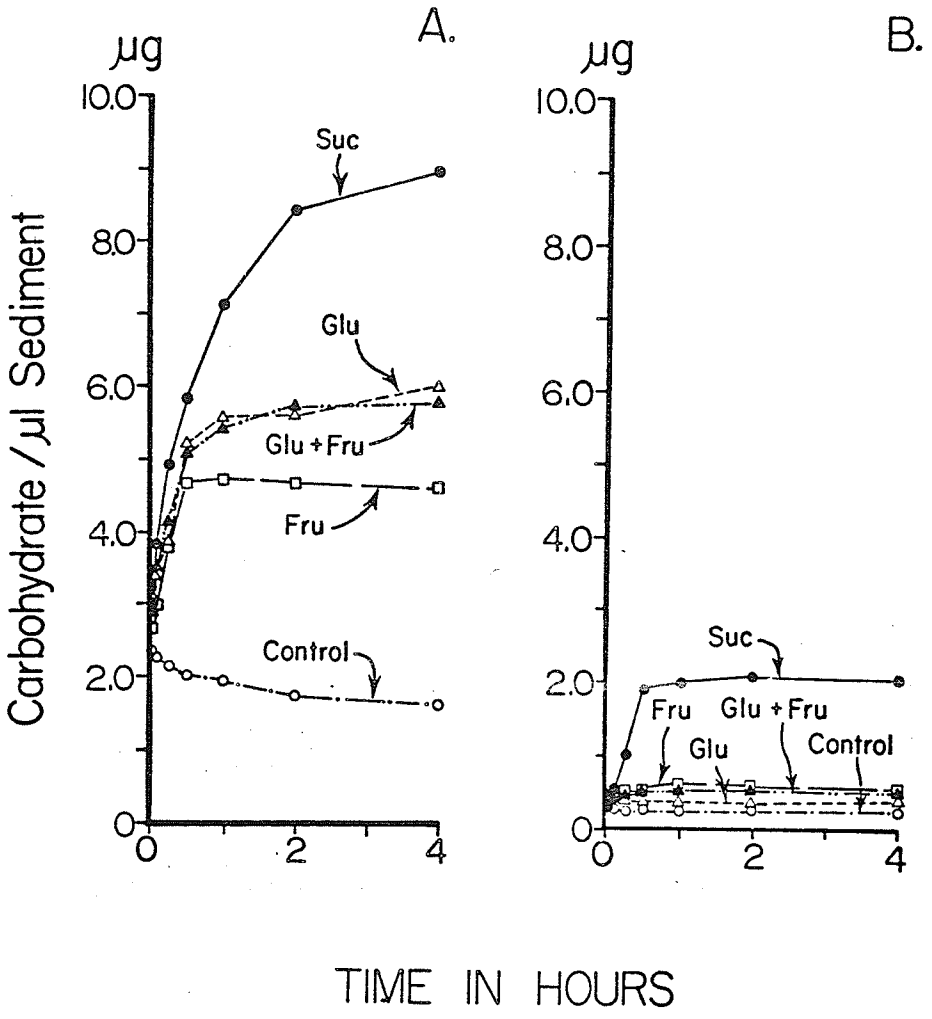

Figure 3.9. Sediment carbohydrate in salivary sediment mixtures incubated with; (i) no sugar; (ii) glucose; (iii) fructose; (iv) sucrose, and (v) glucose-fructose in 1:1 ratio. A-polyglucose; B-polyfructose. Sucrose concentration was 138.9 mM; the concentration of the other sugars was 277.8 mM.

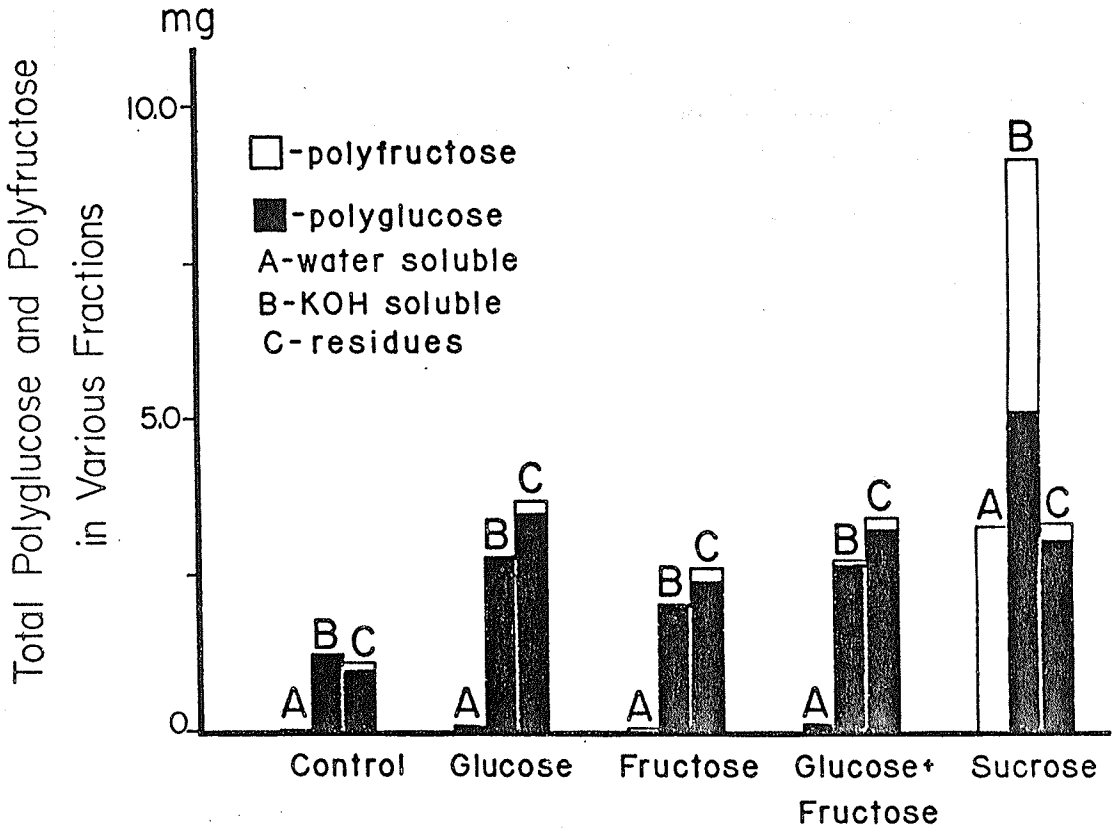

Figure 3.10. Distribution of polyglucose and polyfructose between water soluble, cold KOH-soluble and residual fractions of salivary sediment mixtures incubated with; (i) no sugar; (ii) glucose; (iii) fructose; (iv) sucrose, and; (v) glucose and fructose in 1:1 ratio. The concentration of sucrose was 138.9 mM; other sugars were 277.8 mM and the total volume of each incubation mixture was 15 ml.

and least with fructose. Large amounts of polyfructose were formed with sucrose, but only very small amounts could be detected with fructose or with its equimolar mixture with glucose.

The largest amount of water soluble fraction occurred with sucrose and this was mainly polyfructose. Sucrose also produced the most KOH soluble fraction which contained almost equal amounts of glucose and fructose. The other sugars showed mostly glucose in this fraction. The carbohydrate, mainly polyglucose in the residue fractions seemed to be similar in amounts with all the sugars except fructose; fructose showed lesser amounts, but the amounts were above those of the control.

The iodine test showed that neither the KOH soluble nor the water soluble fraction contain polysaccharide of the glycogen-amylopectin type. The amount of nitrogen in the KOH soluble and water soluble fractions was very small, the carbohydrate/N ratio was approximately 10 and 17, respectively. It was concluded therefore that the polymers in both fractions were mainly polysaccharide and mainly of extracellular origin.

Acid Formation

In the absence of added sugar, no lactic acid was found (Fig. 3.11a); only hetero-acid was formed from the small amount of sediment carbohydrate that was degraded (Fig. 3.11b). The hetero-acid was produced during the first part of the experimental period, and the concentration remained

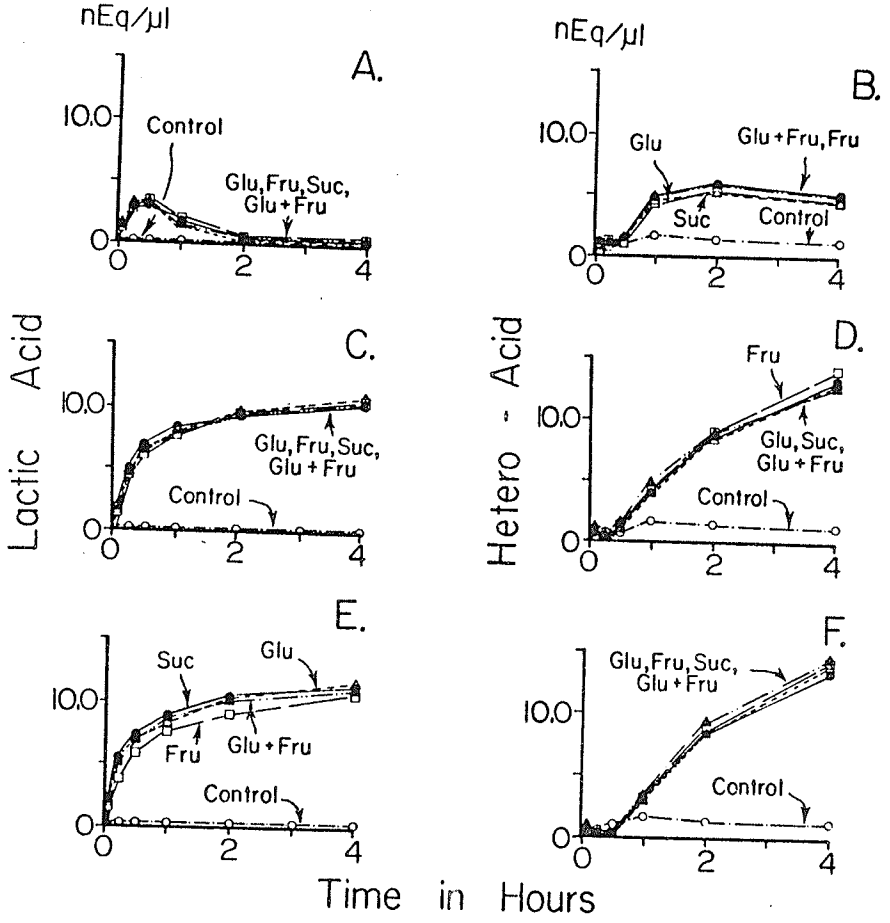

Figure 3.11. Concentration of lactic and hetero- acids in salivary sediment mixtures incubated with; (i) no sugar; (ii) glucose; (iii) fructose; (iv) sucrose, and; (v) glucose-fructose in 1:1 ratio. In (A) and (B), the sucrose concentration was 2.8 mM; the concentration of the other sugars was 5.6 mM. The sucrose concentration in (C) and (D) was 13.9 mM; other sugars were at 27.8 mM. In (E) and (F), the sucrose concentration was 138.9 mM; that of the other sugars was 277.8 mM. Concentrations are expressed in nequivalents per µl of sediment mixture.

unchanged during the remainder of the 4 hour incubation.

At the 2.8-5.6 mM sugar concentration (Fig. 3.11a and b) lactic and hetero-acid were both formed. The lactic acid concentration rapidly increased to a maximum and then slowly decreased to zero by the end of 2 hours. The hetero-acid concentration rose more slowly during the early part of the experiment, reaching a maximum before showing a slight decrease towards the end of the experimental period.

With the higher sugar concentrations (Fig. 3.11c, d, e and f), the lactic acid concentration increased rapidly initially and then more slowly during the remainder of the 4 hour incubation period. The lactic acid curves did not show the rise and fall observed at lower sugar concentrations; instead their shapes were asymptotic. In contrast, the hetero-acid concentration, after an initial lag, progressively increased during the incubation and did not reach an asymptote.

CO_2 Formation

CO_2 formation was similar for all sugars (Fig. 3.12). In all cases, CO_2 increased progressively throughout the experimental period.

Formation of CO_2 was most at the 2.8-5.6 mM concentration; at 13.9-27.8 mM, formation was less and not markedly different from that with the sugar concentration at 138.9-277.8 mM. Formation of more CO_2 at 2.8-5.6 mM than at the higher substrate concentrations was previously observed by Sandham and Kleinberg for glucose (1970b).

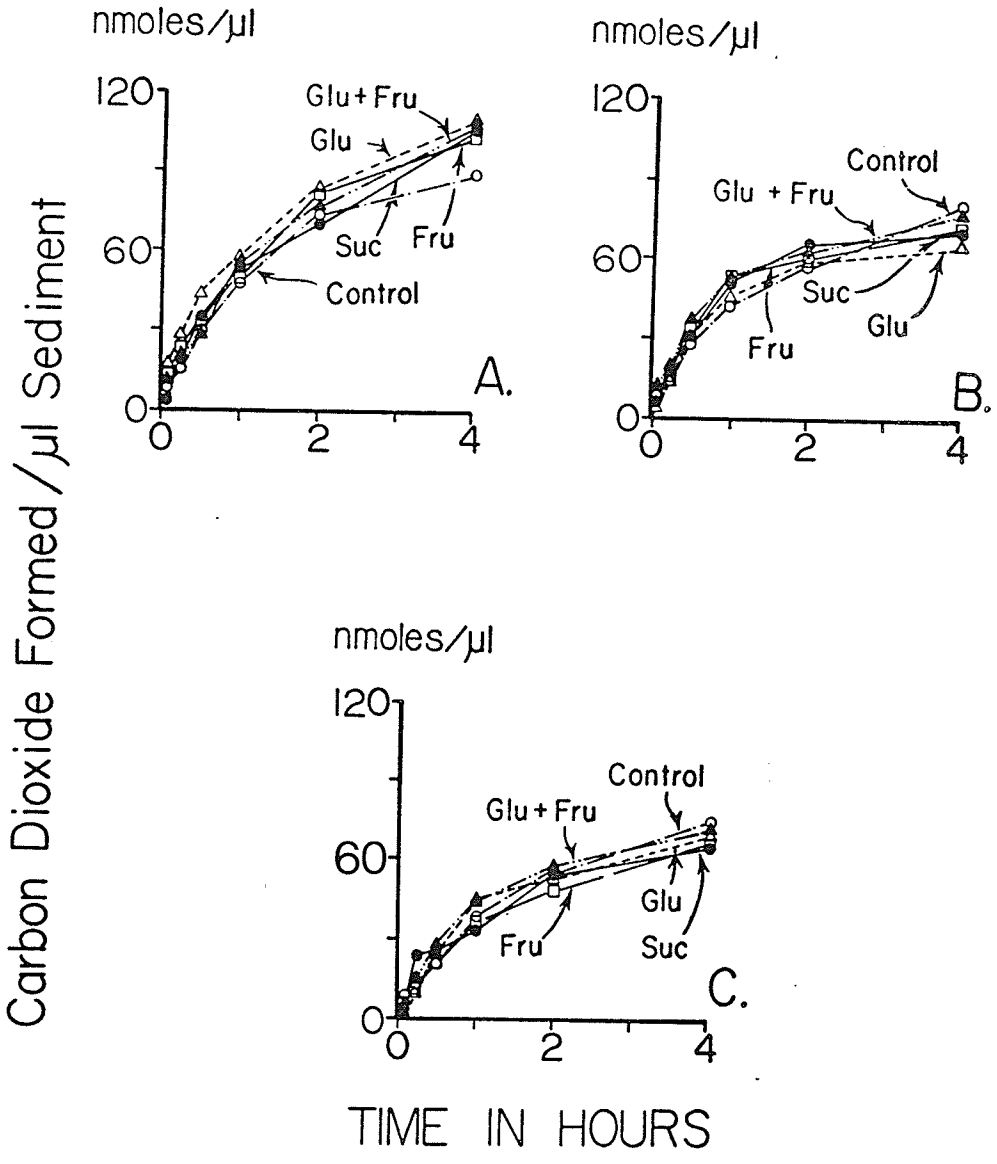

Figure 3.12. CO_2 formation in salivary sediment mixtures incubated with; (i) no sugar; (ii) glucose; (iii) fructose; (iv) sucrose, and; (v) glucose-fructose (1:1). A-sucrose at 2.8 mM; other sugars at 5.6mM; B-sucrose at 13.9 mM; other sugars at 27.8 mM; C-sucrose at 138.9 mM; other sugars at 277.8 mM.

Acid Formation from the Polysaccharide Synthesized from the Different Sugars

As shown in Fig. 3.13, during the first incubation when the sediments were permitted to store polysaccharide with each sugar, the pH fell with all sugars to about the same level. During the second incubation however, the pH fell much more with sucrose than with the other sugars. With each sugar, the pH fell to a minimum during the first hour and remained at a plateau until the end of the incubation period.

DISCUSSION

Regardless of whether sucrose, glucose or fructose was the substrate, the pH-time curves in salivary sediment mixtures at each of the substrate concentrations tested were, except for minor differences with fructose, almost identical (Fig. 3.1). At low substrate concentration (2.8-5.6 mM), the fall and rise in the pH characteristic of the Stephan curve (Stephan, 1940) occurred with all the substrates examined while at the higher concentrations, the fall but not the rise portion of the pH curve was observed. Fructose at 5.6 mM showed a slightly greater pH fall but at 27.8 and 277.8 mM, the pH fall was slightly less than with the other sugars. The slight difference between fructose and the other two sugars has been noted previously in saliva-glucose (Kleinberg, 1970a) and dental plaque mixtures (Miller et al, 1940). The difference is probably too small to be of major significance with regard to cariogenicity.

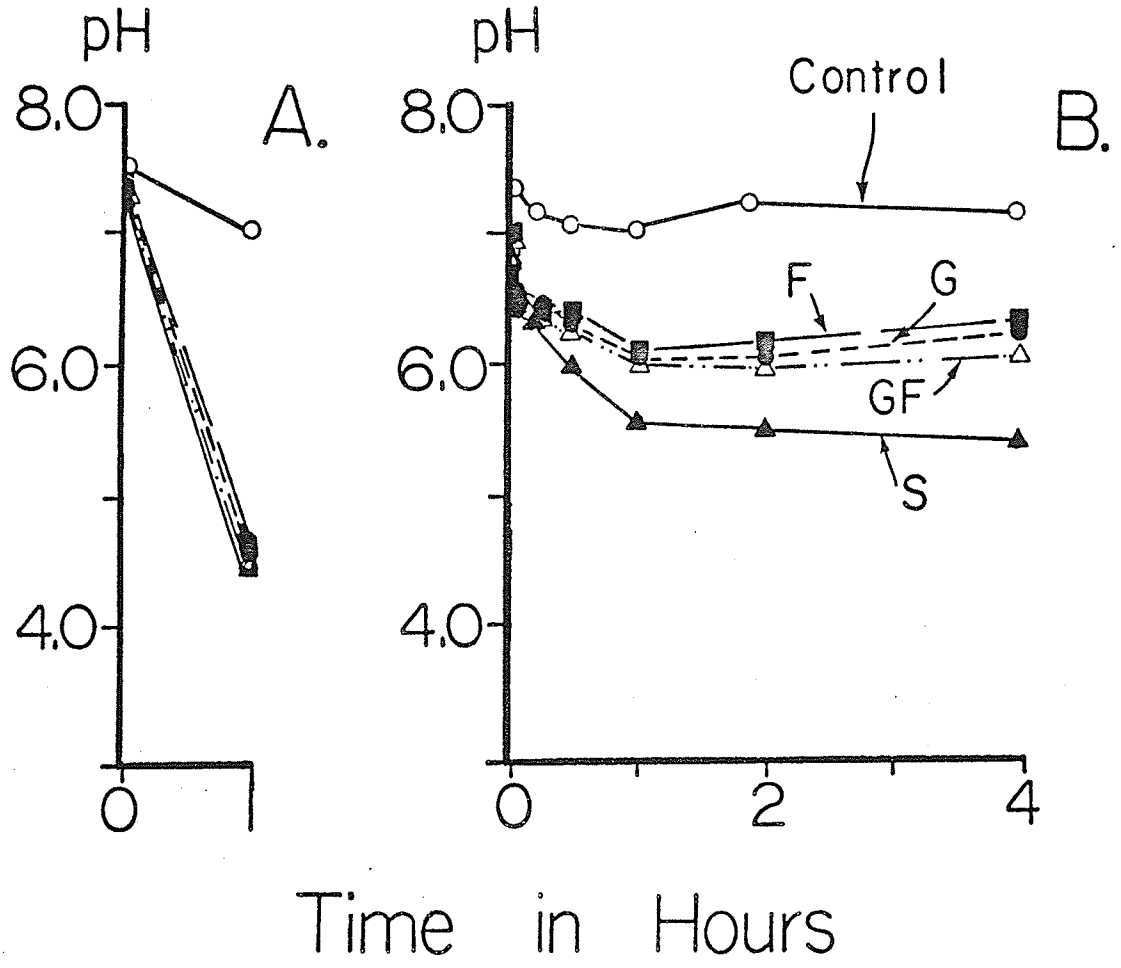

Figure 3.13. Effect of pre-incubation of salivary sediment mixtures with; (i) no sugar; (ii) glucose; (iii) fructose; (iv) sucrose, and; (v) glucose-fructose in 1:1 ratio, on the pH upon subsequent incubation without substrate. A-pH before and after the one hour pre-incubation with substrate; B-pH after discarding the supernatant and washing and resuspending the sediments in fresh salivary supernatant. The sucrose concentration was 138.9 mM; the concentration of the other sugars was 277.8 mM. G-glucose; F-fructose; S-sucrose; GF-glucose+ fructose (1:1).

Littleton, McCabe and Carter (1967) measured the pH of human plaques before and after rinsing with solutions of sucrose, glucose and fructose. Whether the subjects were previously fed normally or by tube, (the latter procedure reduces the ability of the plaque to form acid), all sugars gave the same pH curves except for fructose showing a small difference.

These pH results and the finding that CO_2 and lactic and hetero-acid formation are virtually the same with the different sugars even at very different sugar concentrations clearly indicate that any differences in the cariogenicity of these sugars does not lie in their ability to form acid. On the other hand, major differences were observed in the ability of the different sugars to synthesize glucose and fructose polymers and to subsequently form acid from these polymers.

Comparison of Uptake with the Different Sugars

Disappearance of sucrose from the medium occurred at about the same rate as the equimolar mixture of glucose and fructose but faster than glucose, which in turn occurred faster than fructose (Figs. 3.2, 3.3 and 3.5). Alone, uptake of glucose occurred at about the same rate as the uptake of fructose. However, when glucose and fructose were together either as an equimolar mixture or as parts of the sucrose molecule, disappearance of glucose from the medium occurred more rapidly than with fructose, viz., decrease in the concentration of 0.12 and 0.09 μM/μl, respectively.

The similarity of the results with sucrose and the glucose-fructose mixture is consistent with the results from the chromatography experiments where it was found that hydrolysis of sucrose into glucose and fructose, indicative of the presence of invertase, is an extremely rapid process. At 13.9 mM sucrose, almost complete hydrolysis occurred within about 1 hour.

Preferential uptake of glucose over fructose has been observed in a number of cellular systems. For example, Mateles et al (1967), in studies on Escherichia coli and Pseudomonas fluorescens growing in continuous culture found that glucose was utilized in preference to fructose when a mixed glucose-fructose feed was used. In mature erythrocytes, Bonsignore et al (1963), showed that consumption of fructose was less than that for glucose (1.8 and 2.3 µMoles/ml cells/hour, respectively) and that fructose utilization was inhibited when examined in the presence of an equimolar concentration of glucose. Moreover, the inhibition increased when the glucose concentration was increased. Similar observations were made for lamb and sheep brain (Setchell, 1963).

Polyglucose and Polyfructose Synthesis with the Different Sugars

More sediment polysaccharide was synthesized with sucrose than with the other sugars although this was dependent upon the sugar concentration. No differences were observed between the different sugars at the 2.8-5.6 mM concentration, some differences were evident at 13.9-27.8 mM and differences were clearly evident at the 138.9-277.8 mM level.

This increase in difference with increase in substrate concentration can be attributed mainly to the much greater increase in extracellular polysaccharide formation that occurs with sucrose than with its constituent monosaccharides alone or in combination. This conclusion is based upon the fact that; (i) fraction B when sucrose was the substrate contained much more polysaccharide than the same fraction with the other sugars, and; (ii) except for a slightly lower level with fructose, the polysaccharide in the C fractions was about the same (Fig. 3.10).

The slightly lower level of fraction C observed with fructose than with the other sugars is consistent with the results shown in Fig. 3.9 in that polyglucose formation was least with fructose (see Fructose section below). The results for glucose were almost identical to those reported earlier by Sandham and Kleinberg for the same sugar (1969a).

Very little of the polyglucose or polyfructose formed with glucose and fructose appeared in fraction A. With sucrose, on the other hand, this fraction contained substantial amounts of polyfructose. Since both fractions A and B are mainly of extracellular origin (see results above), it is evident and consistent with a large number of earlier studies (Sexton, 1969; Gibbons and Banghart, 1967) showing that extracellular polysaccharide formation occurs mainly with sucrose.

Possible Explanation for the Effect of Sucrose Concentration on Polyglucose and Polyfructose Synthesis

The effect of sucrose concentration on the formation of polyglucose

and polyfructose assuming these to be mainly dextrans and levans can be explained as follows.

At low sucrose concentration (2.8 mM), the added sucrose is rapidly hydrolyzed to glucose and fructose both of which appear and then rapidly disappear from the medium; the disappearance occurs within the first few minutes of incubation. Since the formation of dextrans and levans by dextransucrase and levansucrase require unhydrolyzed sucrose as substrate, one would expect little of these polymers to be formed at 2.8 mM sucrose. The small increase in sediment polysaccharide observed at this concentration (Fig. 3.8a) is probably an increase in intracellular polyglucose.

On the other hand, at very high sucrose concentration (138.9 mM), although rapid hydrolysis of sucrose occurs (Fig. 3.7), the sucrose concentration is too high for all of the sucrose to be hydrolyzed. Unhydrolyzed sucrose is therefore still present in the incubation medium, permitting extracellular polysaccharide formation to occur. The much smaller amounts of polysaccharide formed with the glucose-fructose mixture is consistent with the presence of intact sucrose molecules being necessary for levan and dextran formation.

The appearance of fructose and glucose in the medium during the metabolism of sucrose indicates that the rate of transport of these sugars into the cells is less than their rate of formation from sucrose. Since in the presence of fructose and glucose, sucrose breakdown by a sucrose hydrolyzing enzyme is inhibited (Edelman, 1954), it follows that accumulation of these sugars in the medium will inhibit sucrose break-

down and ensure that more sucrose will be available for synthesis of extracellular polysaccharide.

It has been observed that the rate of glucose utilization by salivary sediment and dental plaque decreases with decrease in the pH (Korayem and Kleinberg, 1970 and 1971). Applying this observation to the present study, decrease in the uptake of glucose and fructose with decrease in the pH, may lead to their accumulation in the medium and if sucrose breakdown is inhibited, more polysaccharide would be formed.

Polysaccharide Synthesis with Fructose

Less polysaccharide was formed from fructose than the other sugars and being polyglucose (Figs. 3.8 and 3.9), it was probably of a glycogen-amylopectin type. If so, one possible explanation for the formation of less polysaccharide with fructose may be that as in some microorganisms, the initial step in the metabolism of fructose is the formation of fructose-1-phosphate. This has been shown to occur through a phosphenol-pyruvate dependent phosphorylation of fructose in Aerobacter aerogenes (Hansen and Anderson, 1968) and Escherichia coli (Frankell, 1968). Should the findings of Zalitis and Oliver (1967) that fructose-1-phosphate inhibits glucose phosphate isomerase (which is involved in the transformation of fructose-6-phosphate to glucose-6-phosphate in liver and muscle) be true for bacteria, one would expect a decrease in the amount of glucose-6-phosphate and as a result, less storage of intracellular glycogen.

Comparison to Findings in Other Studies

The results in the present study with salivary sediment show many similarities to those observed in other bacterial systems. For example, exposure of dental plaque *in vivo* to different sugars clearly indicated that polysaccharide formation was least with fructose and most with glucose and sucrose (Saxton, 1969). Greater synthesis of polyglucose and polyfructose from sucrose observed in the present study with salivary sediment was also observed in studies with bacterial isolates (Gibbons and Banghart, 1967; Gibbons and Nygaard, 1968) and in studies on dental plaque *in vitro* and *in vivo* (Manly *et al*, 1966; Critchley *et al*, 1967).

Since direct acid formation from sucrose was similar to that from the other sugars tested, one must conclude that any difference in cariogenicity between sucrose and its monosaccharide derivatives is more likely to arise from the capacity of the oral microorganisms to store larger amounts of polysaccharide from sucrose. As suggested by Manly (1961) and Gibbons and Socransky (1962) polysaccharides synthesized during periods of substrate excess would provide substrate for the formation of acid when the external environment is free of utilizable substrate. That sucrose is more suited for this purpose than the other sugars is evident from Fig. 3.13, where exposure of salivary sediment to different sugars for one hour to allow cellular accumulation of carbohydrate then resuspending the washed sediment in fresh saliva, results in the pH falling with sucrose to a greater extent and for a longer time period than with the other sugars. This particularly demonstrated major difference between

sucrose and the other sugars which may be significant in their roles in the caries process.

CHAPTER IV

EFFECT OF pH AND SUCROSE CONCENTRATION ON THE FORMATION OF EXTRACELLULAR GLUCOSE AND FRUCTOSE POLYMERS BY THE BACTERIA IN SALIVARY SEDIMENT

The formation of extracellular glucose and fructose polymers from sucrose has been demonstrated in numerous studies with pure and mixed cultures of the microorganisms from saliva and dental plaque.

Formation of dextran from sucrose has been demonstrated in dental plaque (Wood, 1967b; Gibbons and Banghart, 1967) and in several strains of streptococci (Guggenheim and Schroeder, 1967; Wood and Critchley, 1966; De Stoppelaar et al, 1967). Levan formation has been observed in bacterial systems such as salivary sediment (Manly, 1961), dental plaque (McDougall, 1964; Manly et al, 1966) and a variety of oral bacteria (Snyder et al, 1955; Howell and Jordan, 1967; Gibbons and Nygaard, 1968).

Several studies have shown that the extracellular polymers synthesized are quite variable in composition. For example, glucose polymers containing about 2-5 per cent ketohexose have been found in polysaccharide isolated from human streptococcus strains PK1, GS5 and 120 (Gibbons and Banghart, 1967), mixtures of levan and dextran in streptococcus strain GS5 and LM7 (Gibbons and Nygaard, 1968), structurally different dextrans in Streptococcus mutans OMZ 61 and OMZ 176 (Guggenheim and Schroeder, 1967), a glucan plus heteropolysaccharides of various composition have been demonstrated in several strains of lactobacillia (Dunican and Seeley, 1965) and in Leuconostoc mesenteroides, polymers containing both

glucose and fructose were found amidst polymers containing only glucose or fructose (Wilham et al, 1955).

Despite the numerous studies that have been carried out, there is little information about the factors determining the amounts and relative quantities of extracellular glucose and fructose polymers synthesized from sucrose under conditions that normally occur in the oral cavity. For example, sucrose concentration can vary considerably during eating (Lanke, 1957) and from the studies in Chapter III, can have a considerable effect on the formation of such polymers. There it was shown in salivary sediment mixtures incubated with sucrose that a high sucrose concentration was necessary for substantial amounts of both polyglucose and polyfructose to be formed. Much of the polysaccharide formed was extracellular since part was released into the medium during the incubation and part remained with the sediment. This latter could be extracted with cold KOH (Guggenheim and Schroeder, 1967).

In the oral cavity, pH, an important regulator of many metabolic processes, can also vary considerably. For example, after the ingestion of sucrose the pH can rapidly decrease from approximately 7.0 to a pH below 5.0 (Stephan, 1940 and 1944; Stralfors, 1950).

Because of their probable importance, the variables of pH and sucrose concentration have been examined in the experiments in the present chapter for their effects on the glucose and fructose polymers formed in incubated salivary sediment mixtures. The homogeneity and solubility of the glucose and fructose polymers in the supernatant and KOH-sediment

components of the extracellular carbohydrate were examined by differential precipitation in alcohol solutions of different concentration (Wilham et al, 1955). Experiments are also reported in this chapter which show that release of polyglucose and polyfructose into the medium also occurs during incubation of plaque-saliva mixtures with sucrose.

METHODS

Preparation of Sediment Mixtures and Incubation Procedures

Sediment mixtures were prepared from wax-stimulated whole saliva as described in the previous chapter. Saliva from 8-10 donors was collected between 9:00 and 10:00 a.m. in 25 x 150 mm test tubes chilled in cracked ice. The saliva was centrifuged at 1740g; supernatant was decanted and the sediment then washed 3 times and suspended in cold distilled water at a concentration of 50 per cent (V/V). Sediment, supernatant and sucrose solution were then combined in the ratio 1:1:1 so that the final sediment concentration was 16.7 per cent (V/V) and the sucrose concentration was either 2.8, 13.9 or 138.9 mM. The total volume of each sediment mixture was 3 ml. Control mixtures contained distilled water instead of sucrose.

a. Effect of sucrose concentration on extracellular polysaccharide formation

For each experiment, 4 series of 6 sediment mixtures were prepared; the mixtures in each series contained sucrose at 0, 2.8, 13.9 and 138.9

mM. At each of 0, 15, 30 minutes and 1, 2 and 4 hours of a 4 hour incubation at 37°C a mixture was removed from each series and supernatant and sediment immediately separated by centrifugation at 4°C and 12,400g for 20 minutes (Lourdes Centrifuge, Lourdes Instrument Corp).

To each sediment, 1 ml of cold KOH (1.0 N) was added to release sediment carbohydrate. After standing for 4 hours the extract was centrifuged at 4°C and 12,800g for 30 minutes and the sediment discarded. The KOH extract of the sediment was viscous and turbid in contrast to the supernatant of the sediment mixture which was clear.

Both solutions were then subjected to differential ethanol precipitation. Three aliquots (70 µl) were removed from each supernatant of a sediment mixture and transferred to 10 x 75 mm test tubes containing 630 µl of ethanol to give a final concentration of 30, 50 or 70 per cent.

Three aliquots (30 µl) were then removed from the KOH extract and added to 270 µl of ethanol to give the same final concentrations. To ensure complete precipitation, each tube was kept at 4°C for 12-16 hours. After centrifugation at 1740g for 5 minutes, the precipitate was washed twice with 90 per cent ethanol and dried by heating in a boiling water bath. The precipitate was then solubilized by adding 50 µl of 50 per cent sulfuric acid and heating at 70°C for 5 minutes. The fructose and glucose residues in each precipitate were determined using the anthrone procedure described in Chapter II.

b. Effect of pH on extracellular polysaccharide formation

In the experiments carried out to determine the effect of pH on the

formation of polyglucose and polyfructose from sucrose, five sediment mixtures were prepared as before except that the volumes were 6 ml rather than 3 ml and examination was restricted to sucrose at 138.9 mM. Preliminary experiments established that at this substrate concentration, the polyglucose and polyfructose yields were sufficiently high for clear comparisons to be made. The details are as follows.

Each mixture was prepared by transferring 2 ml of 50 per cent sediment suspension to a 20 ml beaker containing 2 ml supernatant and 2 ml of 416.7 mM sucrose. The pH of each mixture was then adjusted with either HCl or NaOH to 4.0, 5.0, 6.0, 7.0 or 8.0. The mixtures were then incubated for 4 hours in a water bath at 37°C. The pH of each was continuously monitored with a glass pH electrode (Fig. 4.1) and kept constant by delivery of NaOH (0.5 N) from a pH-stat (Radiometer Titrator 11, Copenhagen). The salt bridge from each of the 5 incubation mixtures was connected to a single calomel electrode. The glass electrodes immersed in the mixtures and the calomel electrode were connected through a six channel automatic switch (Bach-Simpson Ltd., London, Canada) to the pH-stat. The NaOH (0.5 N) required to keep the pH constant was delivered from microburettes constructed from 1 ml pipettes (Fig. 4.1). Each reaction mixture was stirred magnetically by a teflon coated bar immersed in each mixture. At the end of the incubation, 3 ml of each mixture was removed and the carbohydrates in the supernatant and the KOH sediment were treated and analyzed for polyglucose and polyfructose as described in the previous section.

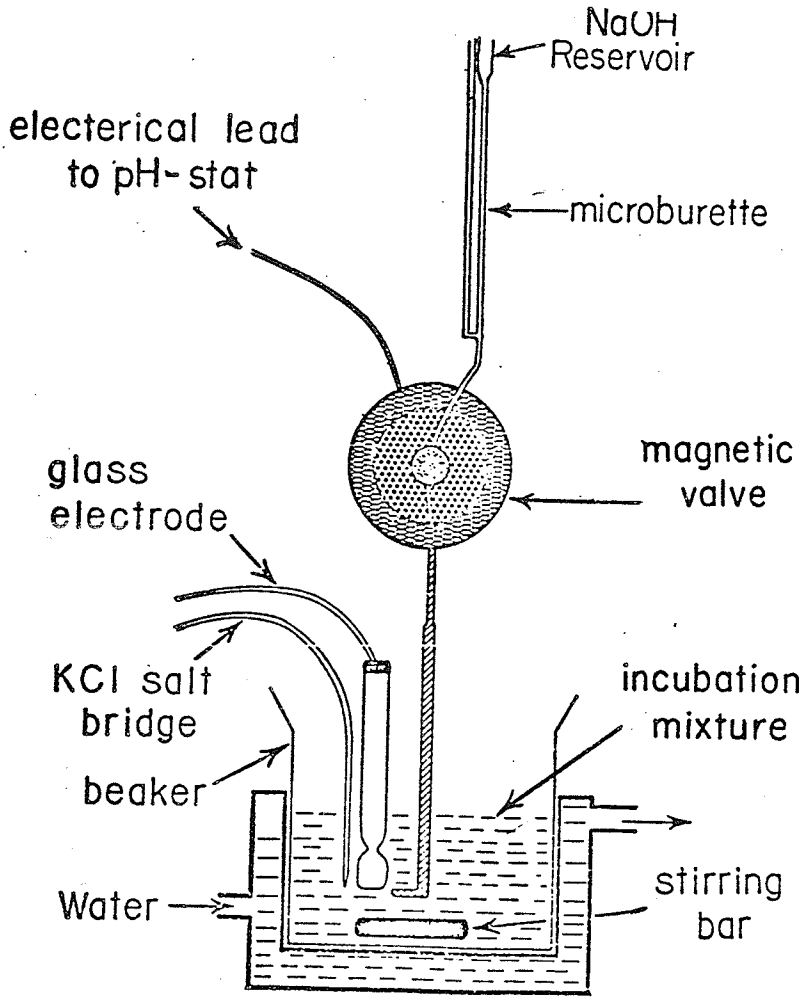

Figure 4.1. Schematic diagram showing the experimental set-up for the pH constant experiments.

c. Effect of pH on sucrose hydrolysis and utilization

To determine the effect of pH on sucrose hydrolysis and utilization by the bacteria in salivary sediment, the following type of experiment was carried out. Sediment mixtures (3 ml) were prepared and incubated for 4 hours at either pH 4.0, 5.0, 6.0, 7.0 or 8.0 using a pH-stat as in the experiments in the previous section. The sucrose concentration was either 13.9 or 138.9 mM. Before and at several intervals during an experiment, 50 µl aliquots were removed from each mixture, transferred to 6 x 50 mm Kimax test tubes and immediately centrifuged at 1740g and 4°C for 5 minutes. From each supernatant, two 15 µl aliquots (when the sucrose concentration was 13.9 mM) or two 10 µl aliquots (when the sucrose concentration was 138.9 mM) were spotted on paper chromatograms (8 x 8; Whatman No. 3) and treated as described in the methods section of Chapter III.

Preparation and Incubation of Plaque Mixtures

Dental plaque was removed with a stainless steel spatula from the buccal surfaces of the teeth of 4 subjects. The subjects were instructed to stop cleaning their teeth for three days prior to and on the morning of the fourth day when the plaque was harvested. Subjects were instructed to avoid food or liquids for at least 12 hours prior to the time of plaque collection. The plaque material was immediately transferred to a 10 x 75 mm test tube and dispersed in 1 ml cold distilled water. After centrifugation at 1740g for 15 minutes at 4°C, the supernatant was dis-

carded, the plaque was washed twice with and made up in distilled water as a 25 per cent suspension. The plaque mixture consisted of 200 µl of 25 per cent (V/V) plaque suspension, 200 µl of salivary supernatant and 200 µl sucrose at 138.9 mM. At the end of a 2 hour incubation at 37°C, the mixtures were centrifuged, 3 aliquots (70 µl) were removed from the supernatant, these were then precipitated at 70 per cent ethanol and the precipitates analyzed for carbohydrate as above for sediment mixtures.

RESULTS

Effect of Sucrose Concentration on Extracellular Polysaccharide Formation

The total quantity of extracellular polysaccharide (i.e., supernatant plus KOH-sediment polysaccharide) formed in the salivary sediment mixtures at the different concentrations of sucrose is shown in Fig. 4.2. The supernatant and KOH-sediment polysaccharide formed are shown in Fig. 4.3.

In the absence of added sucrose, the small quantity of KOH-sediment carbohydrate initially associated with the sediment (Table 4.1) was utilized during the first 5 minutes. At 2.8 mM sucrose, the small amount of KOH-sediment carbohydrate that was formed in the first 15 minutes was utilized upon further incubation. No carbohydrate could be detected in the supernatant at this sucrose concentration. At 13.9 and 138.9 mM sucrose, both supernatant and KOH-sediment polysaccharide accumul-

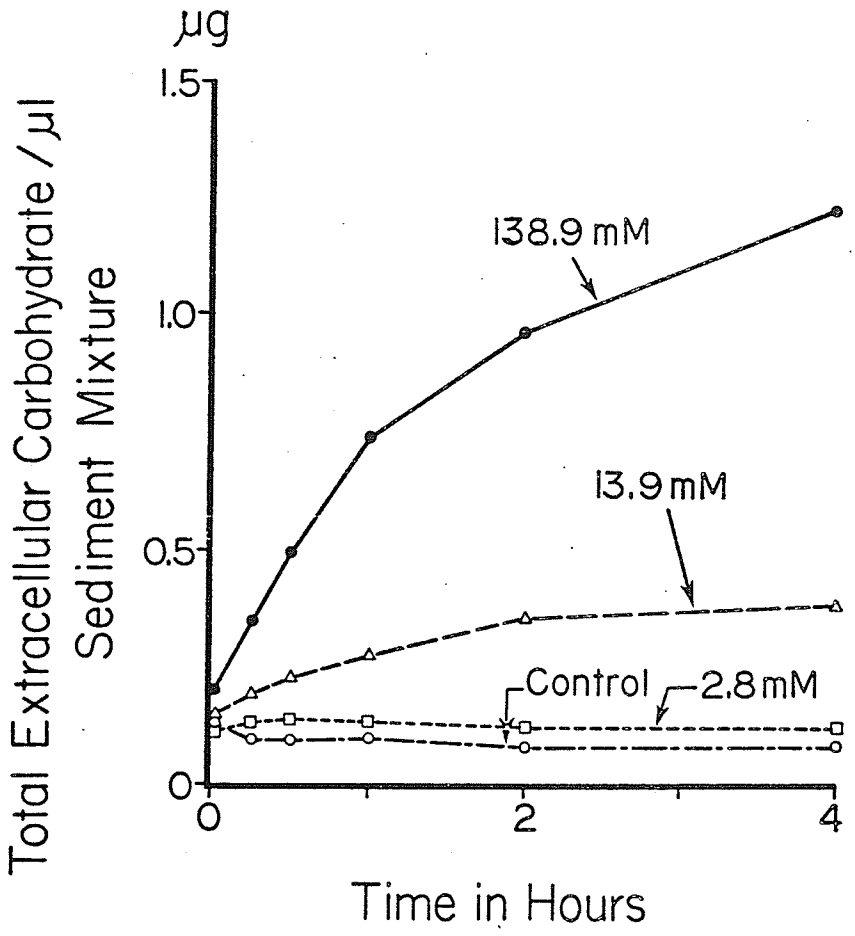

Figure 4.2. Effect of sucrose concentration on the formation of extracellular polysaccharide in salivary sediment mixtures incubated for 4 hours at 37°C. Extracellular here consists of the sum of the polyfructose and polyglucose in both the supernatant of the sediment mixture and the cold KOH-extract of the sediment.

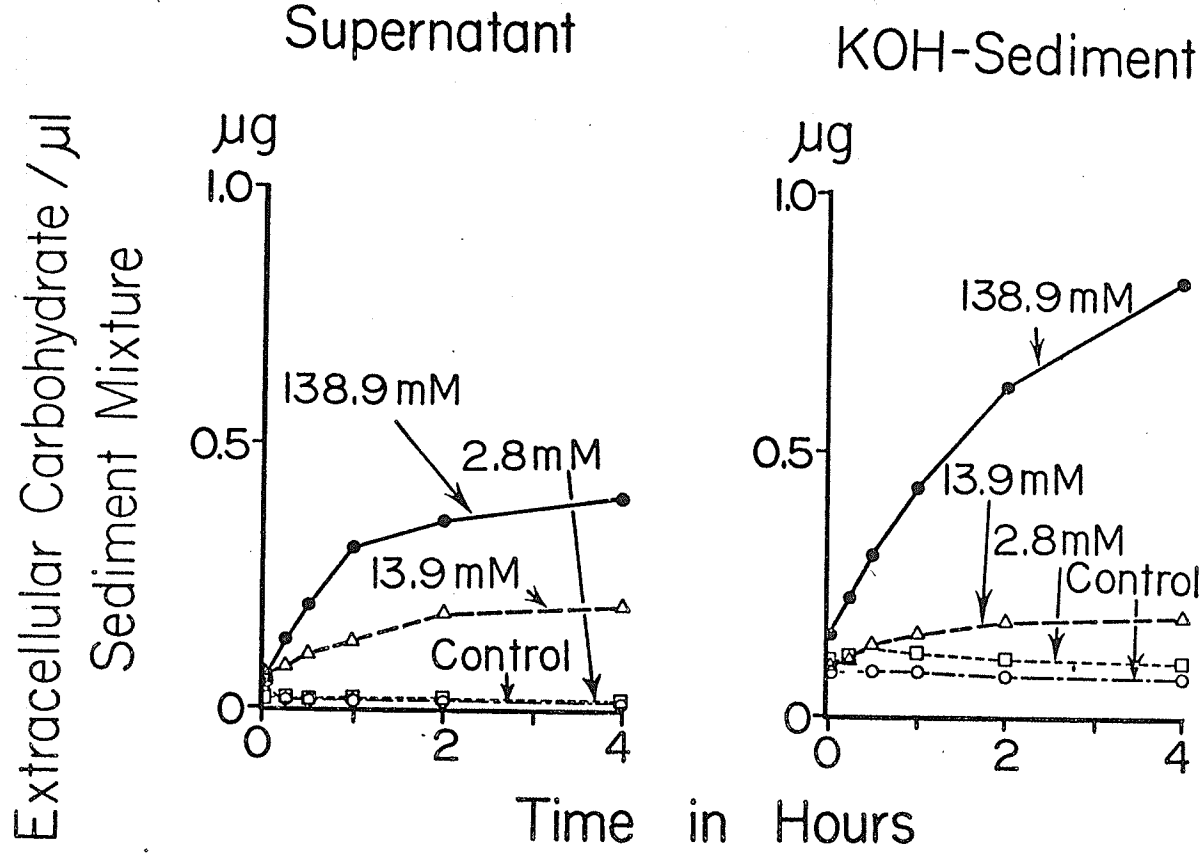

Figure 4.3. Effect of sucrose concentration on the supernatant and KOH-sediment components of the extracellular carbohydrate formed in salivary sediment mixtures incubated for 4 hours at 37°C. Each component consists of the sum of their polyfructose and polyglucose contents.

TABLE IV.1

EFFECT OF SUCROSE CONCENTRATION ON THE FORMATION OF KOH-SEDIMENT CARBOHYDRATE IN INCUBATED SALIVARY SEDIMENT MIXTURES

Sucrose Concentration mM	Total Sediment Carbohydrate (*) (µg/µl)	KOH-Sediment Carbohydrate (*) (µg/µl)	Ratio $\frac{\text{KOH-Sediment}}{\text{Total Sediment Carbohydrate}}$
2.8	3.84	0.72	0.18
13.9	5.28	1.08	0.20
138.9	10.56	4.80	0.45

* Values are the maximum amounts formed during incubation with 2.8, 13.9 and 138.9 mM sucrose. The values for total sediment carbohydrate were obtained from data in Chapter III and those for the KOH-sediment carbohydrate were obtained from Fig. 4.3b. Maximum values occurred at the 30 minute interval when the sucrose concentration was 2.8 mM and at 4 hours when the concentration was either 13.9 or 138.9 mM. The amounts are expressed in µg/µl of sediment. Unincubated sediment contains 2.58 µg carbohydrate per µl, of which 0.42 µg is KOH-sediment carbohydrate. Ratio of KOH-sediment to total sediment carbohydrate is therefore 0.16.

ated at a progressively decreasing rate throughout the 4 hour incubation. Increase in KOH-sediment polysaccharide was more than the increase in supernatant polysaccharide.

The percentage of the total sediment carbohydrate that is KOH-sediment carbohydrate was calculated from data in Chapter III and that in Fig. 4.3b (Table 4.1). With increase in sucrose concentration, the percentage of the total sediment carbohydrate that is soluble in cold KOH, increases.

The polyglucose and polyfructose composition of the KOH-sediment and supernatant polysaccharide is shown in Fig. 4.4. At 2.8 mM sucrose, no polyfructose or polyglucose could be detected in the supernatant. However, at 13.9 and 138.9 mM sucrose, both types of polysaccharide appeared in the supernatant, with polyfructose more than polyglucose especially at 138.9 mM sucrose.

The KOH-sediment fraction contained mainly polyglucose and only at 138.9 mM sucrose did that fraction contain even small amounts of polyfructose.

The results for the differential ethanol fractionation of the supernatant and KOH-sediment polysaccharides are shown in Figs. 4.5 and 4.6.

At both 13.9 and 138.9 mM sucrose, polyfructose constituted most of the supernatant polysaccharide and precipitated mainly at 70 per cent ethanol. On the other hand, most of the polyglucose in the supernatant precipitated at 30 per cent ethanol and was more heterogeneous than the

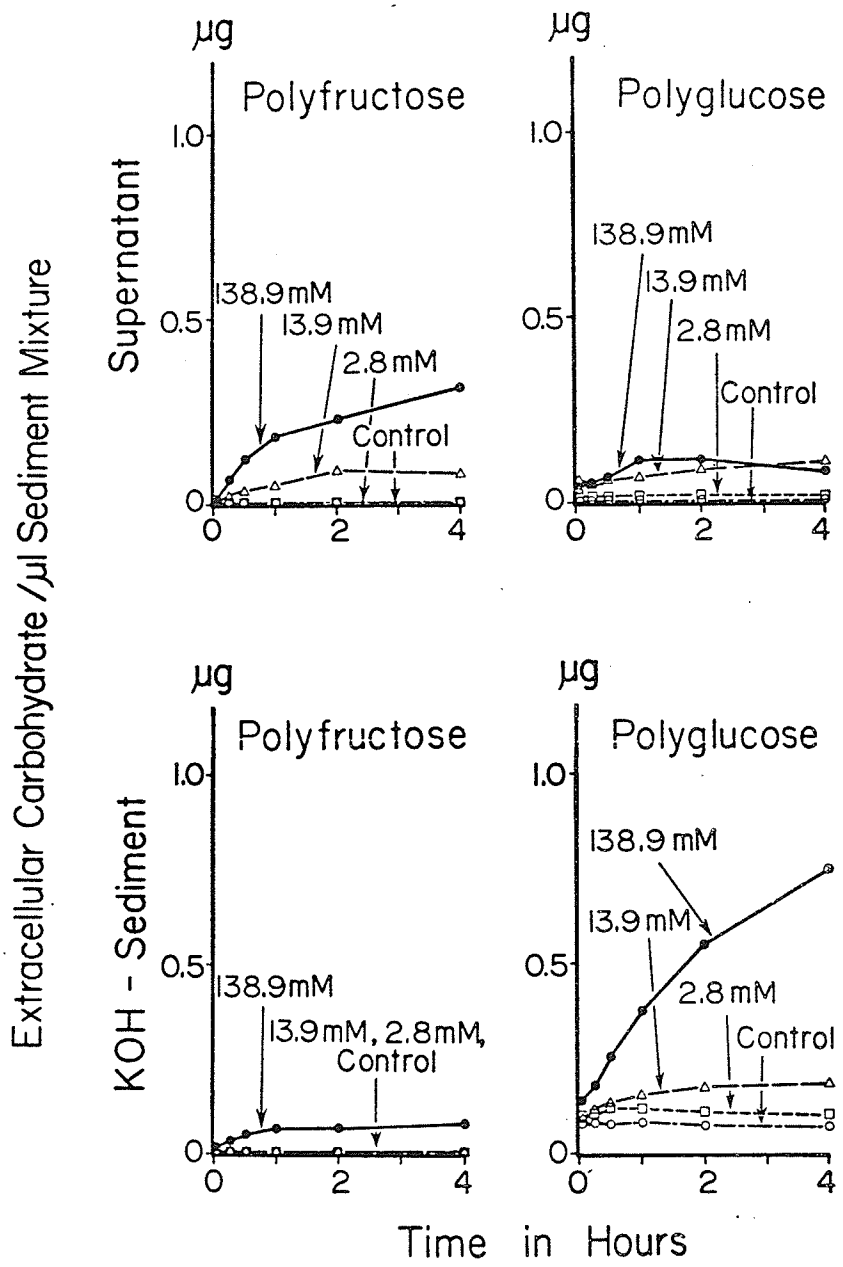

Figure 4.4. Effect of sucrose concentration on the distribution of polyfructose and polyglucose between the supernatant and KOH-sediment components of the extracellular carbohydrate formed during incubation of salivary sediment mixtures for 4 hours.

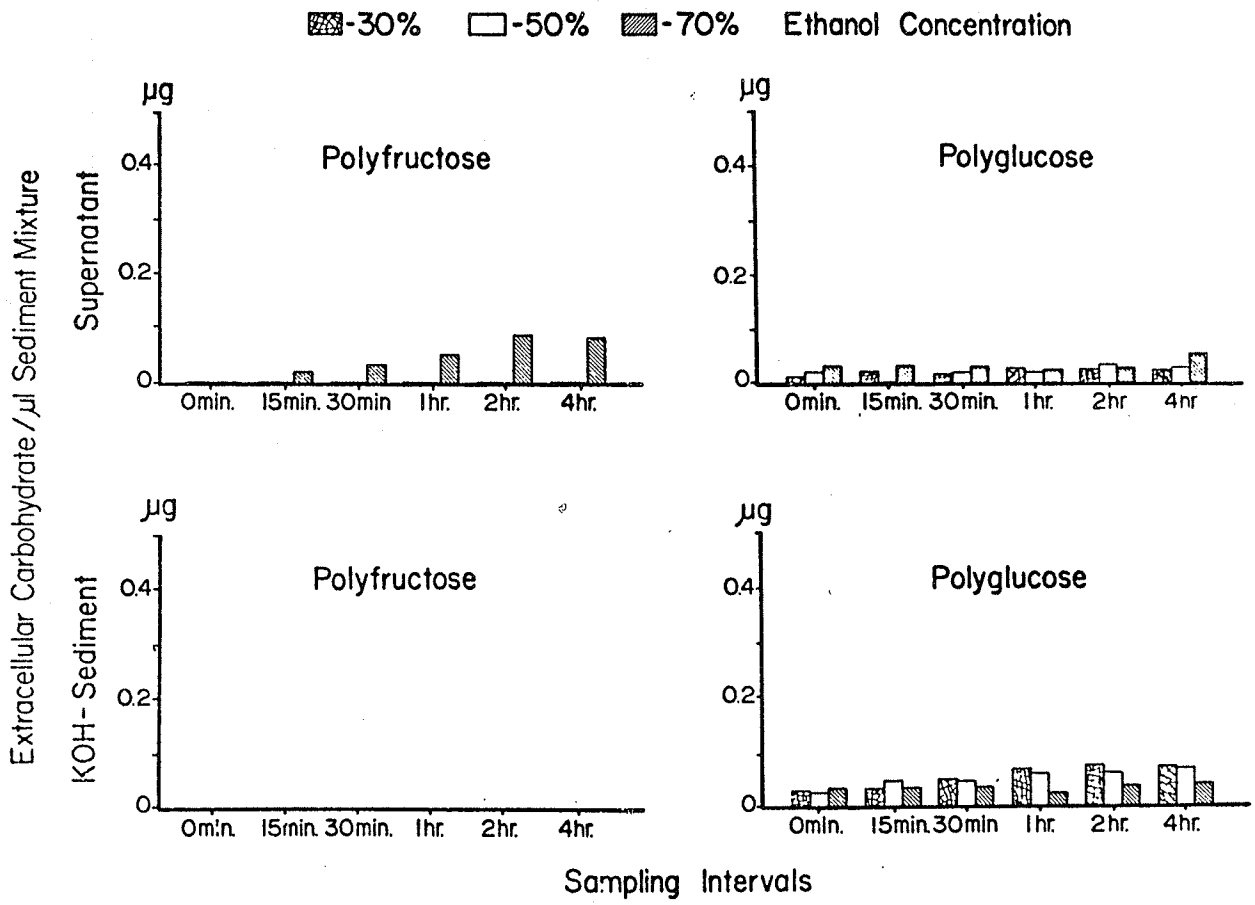

Figure 4.5. Solubility in ethanol of the glucose and fructose polymers in the supernatant and KOH-sediment components of the extracellular carbohydrate formed during incubation of salivary sediment mixtures for 4 hours. The sucrose concentration in the incubation mixture was 13.9 mM.

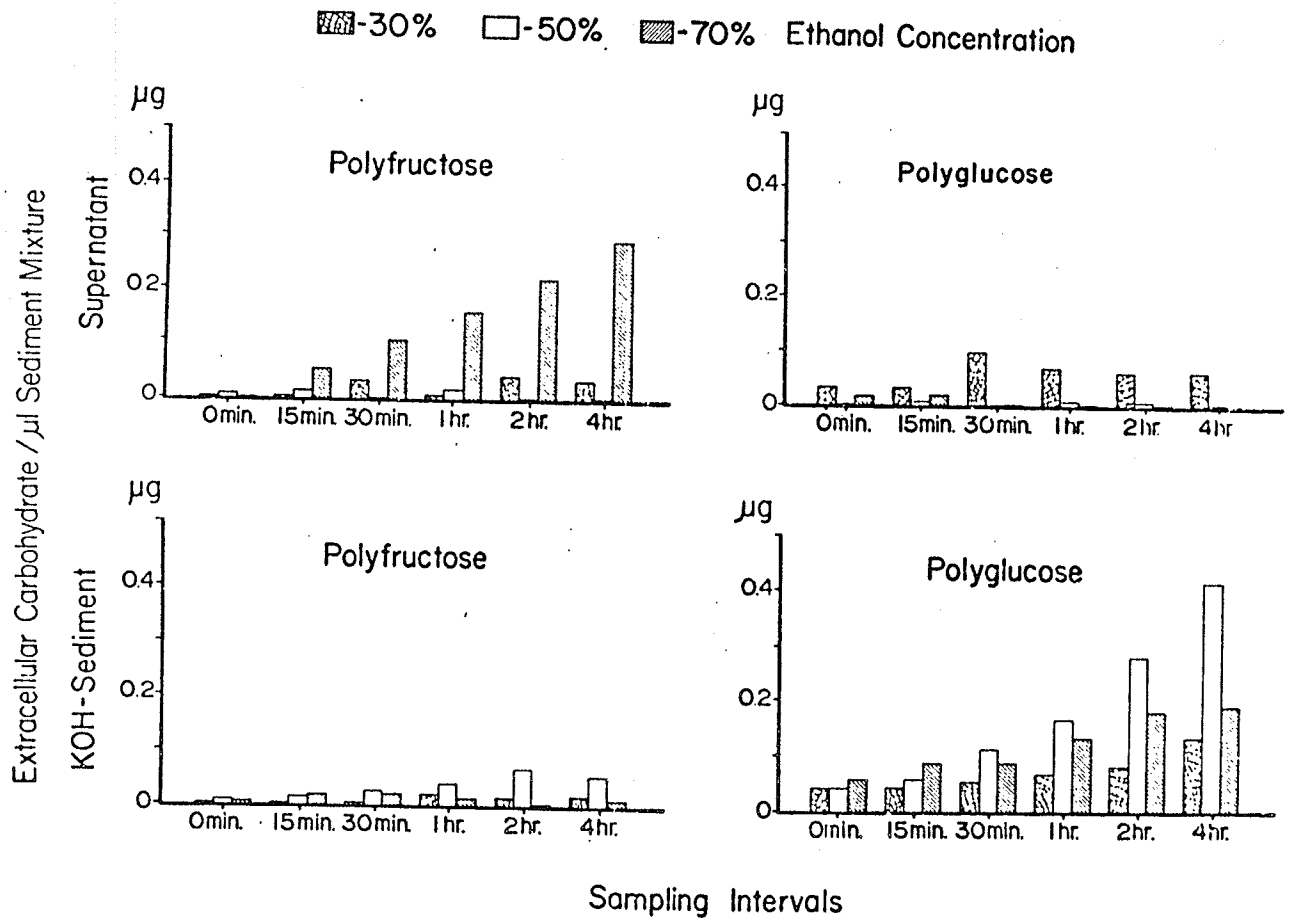

Figure 4.6. Solubility in ethanol of the glucose and fructose polymers in the supernatant and KOH-sediment components of the extracellular carbohydrate formed during incubation of salivary sediment mixtures for 4 hours. The sucrose concentration was 138.9 mM.

polyfructose. These effects were more obvious at 138.9 than 13.9 mM sucrose (Fig. 4.6).

In contrast to supernatant polysaccharide, KOH-sediment polysaccharide contained considerably more polyglucose than polyfructose and the polyglucose was more heterogeneous in that large amounts were precipitated at each of the different ethanol concentrations used rather than most of it at one ethanol concentration.

Effect of the pH on Extracellular Polysaccharide Formation

The effect of pH on the polyglucose and polyfructose formed in both supernatant and KOH-sediment during incubation with sucrose is shown in Fig. 4.7. Maximum formation of supernatant and KOH-sediment polysaccharide occurred around pH 6. A higher pH favoured more supernatant carbohydrate while a lower pH favoured more KOH-sediment carbohydrate. Comparing the amounts of polyglucose to polyfructose in both fractions, more polyfructose than polyglucose was present in the supernatant at all levels of pH, whereas their relative amounts were reversed in the KOH-sediment.

Differential ethanol precipitation (Fig. 4.8) indicated that regardless of the pH, polyfructose precipitated mainly at higher ethanol concentrations (70 per cent), whereas polyglucose precipitated mainly at a lower ethanol concentration (30 per cent).

Effect of pH on Sucrose Hydrolysis and Utilization

Both at 13.9 and 138.9 mM sucrose, hydrolysis of sucrose occurred at all pH levels tested except pH 4.0 (Figs. 4.9 and 4.10). Hydrolysis

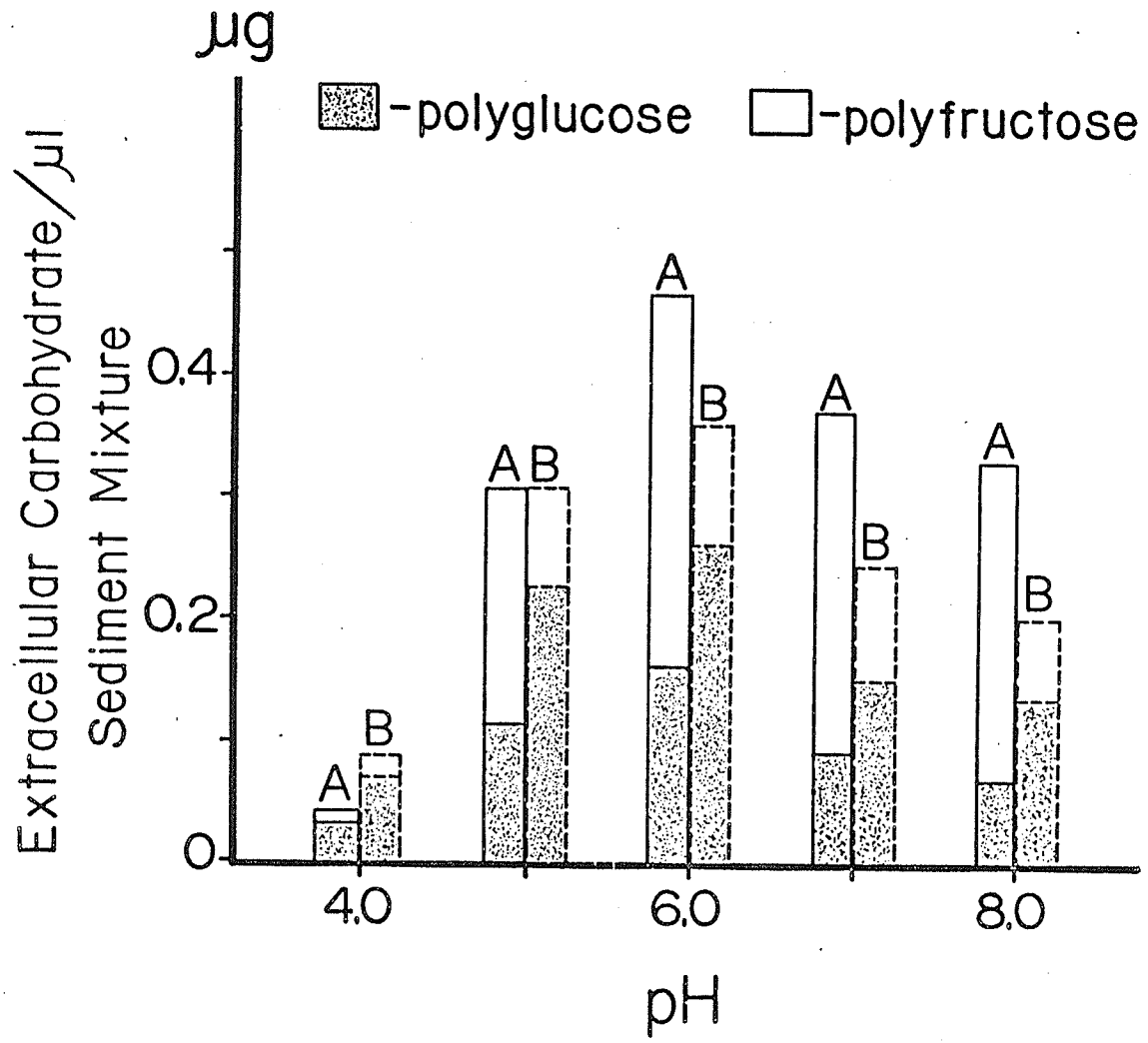

Figure 4.7. Effect of pH on the distribution of polyglucose and polyfructose between the supernatant and KOH-sediment components of the extracellular carbohydrate formed in salivary sediment mixtures incubated for 4 hours with 138.9 mM sucrose. A-supernatant carbohydrate; B-KOH-sediment carbohydrate.

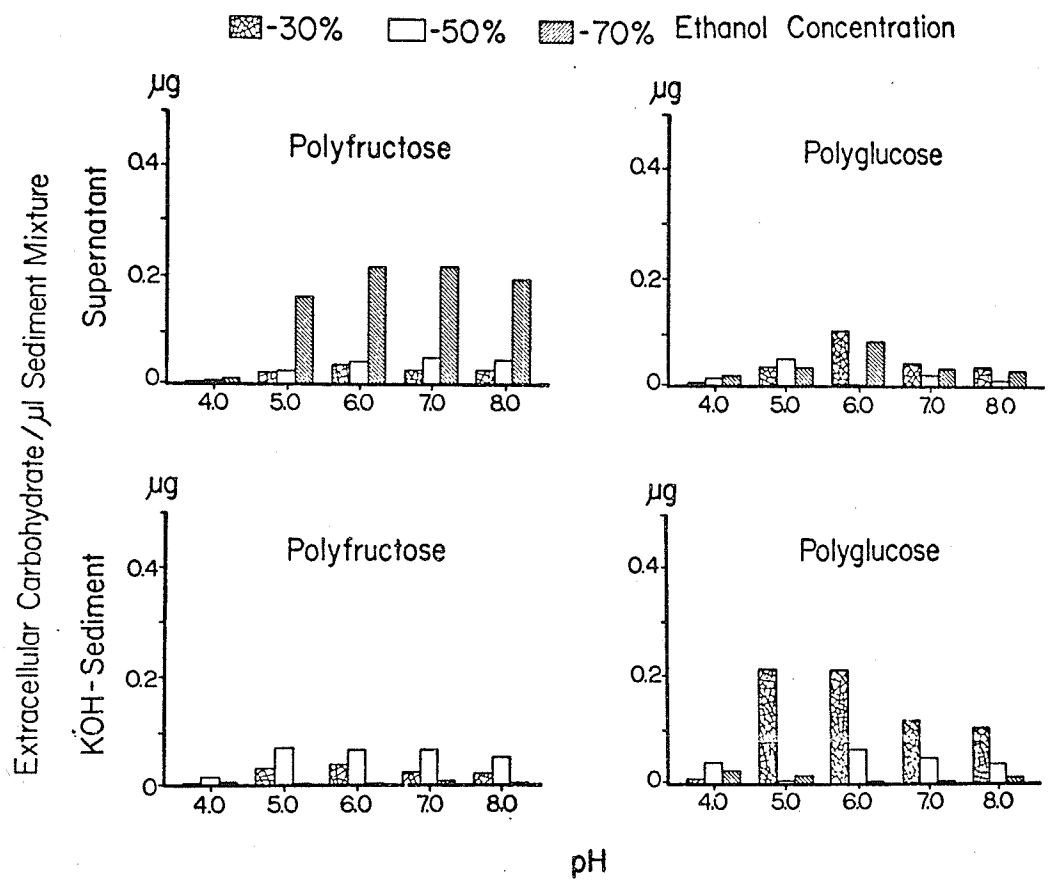

Figure 4.8. Solubility in ethanol of the glucose and fructose polymers in the supernatant and KOH-sediment components of the extracellular carbohydrate formed in salivary sediment mixtures incubated for 4 hours at different pH. Sucrose concentration was 138.9 mM.

was evident by the appearance of glucose and fructose spots on the chromatograms of the samples removed from the mixtures incubated at pH levels above 4.0. At 13.9 mM, very little sucrose disappearance occurred at pH 4.0 whereas, sucrose disappearance from the medium occurred at the other pH levels within two hours (Fig. 4.9). When the sucrose concentration was 138.9 mM, sucrose was present in sufficient quantity that sucrose and its constituent monosaccharides were still present in the medium at the end of the 4 hour incubation at all pH levels (Fig. 4.10).

Polysaccharide spots were visible at the origin of the chromatograms at all pH levels examined except for no or only slight spots at pH 4.0. Noteworthy was the observation that at 138.9 mM sucrose, the intensity of the spots increased with time of incubation. On the other hand, at 13.9 mM sucrose, the intensity of the polysaccharide spots appearing at the origin increased during the early part and then decreased or disappeared by the end of the incubation. This was not seen at pH 4.0 but was clearly visible between 5.0 and 8.0.

Plaque Experiments

When plaque suspensions were incubated with sucrose, as with salivary sediment, both polyglucose and polyfructose were synthesized and released into the medium (Fig. 4.11).

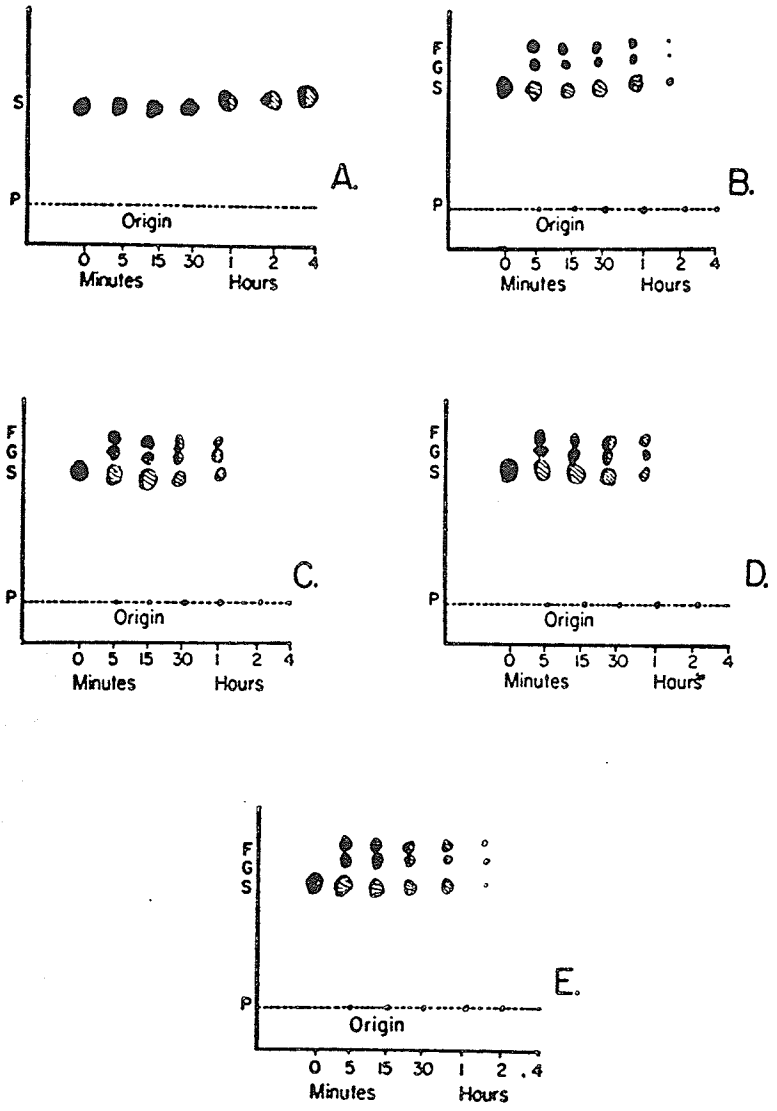

Figure 4.9. Copy of chromatograms of aliquots removed from mixtures corresponding to different time points in the incubation of salivary sediment at different pH; (A) 4.0; (B) 5.0; (C) 6.0; (D) 7.0, and; (E) 8.0. The concentration of sucrose was 13.9 mM. Each incubation mixture had a total volume of 3 ml; 15 µl of the supernatant was spotted at each point shown. The figures on the abcissa show the sampling intervals. S-sucrose; G-glucose; F-fructose; P-polysaccharide.

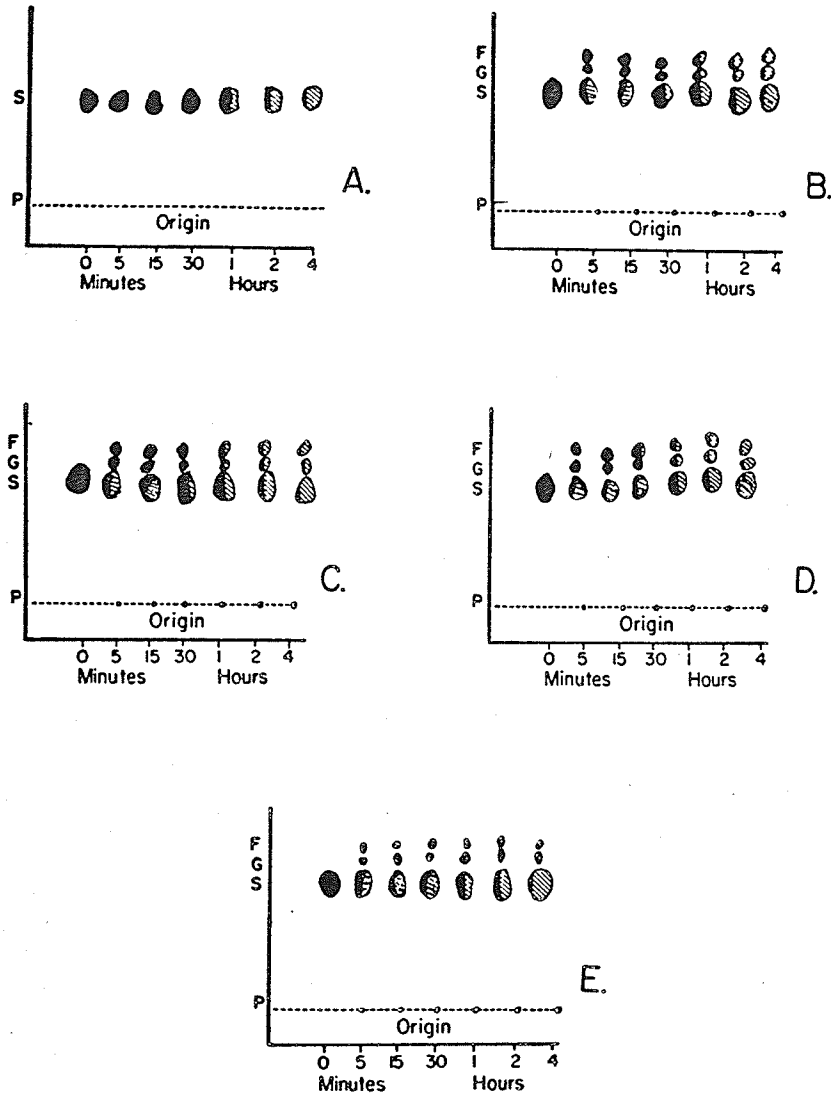

Figure 4.10. Copy of chromatograms of aliquots removed from mixtures corresponding to different time points in the incubation of salivary sediment at different pH; (A) 4.0; (B) 5.0; (C) 6.0; (D) 7.0, and; (E) 8.0. The concentration of sucrose was 138.9 mM. Each incubation mixture had a total volume of 3 ml; 10 µl of the supernatant was spotted at each point shown. The figures on the abcissa show the sampling intervals. S-sucrose; G-glucose; F-fructose; P-polysaccharide.

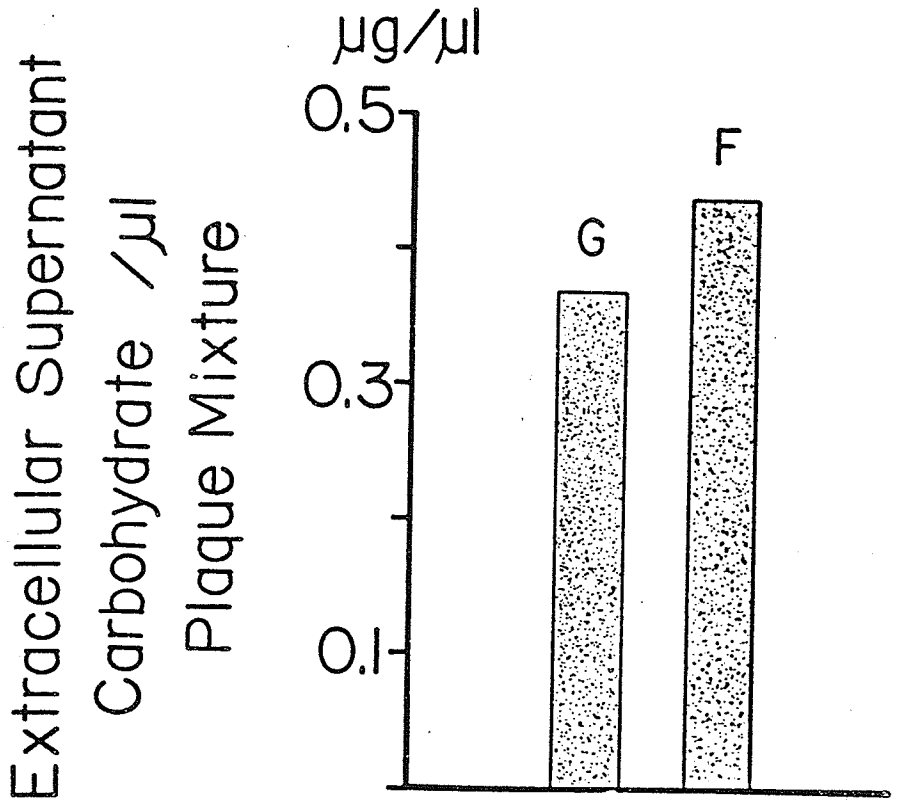

Figure 4.11. Extracellular supernatant polysaccharide formed by plaque saliva mixture incubated with sucrose at 138.9 mM. F-polyfructose; G-polyglucose.

DISCUSSION

The findings in this study confirm the observations made with pure cultures (Gibbons and Banghart, 1967; Gibbons and Nygaard, 1968; De Stoppelaar et al, 1967), that extracellular polyglucose and polyfructose are formed from sucrose and that the polyfructose formed is more soluble than the polyglucose. The observation that polyfructose is found mostly free in the medium, both in incubated sediment and plaque mixtures, indicates that once formed, polyfructose does not tend to remain with the cells. On the other hand, the necessity of using KOH in the sediment experiments to extract most of the polyglucose clearly shows that polyglucose does.

Several investigators have found much smaller amounts of extracellular polyfructose than polyglucose in samples of dental plaque (Wood, 1967b; Critchley et al, 1967). Although some investigators have interpreted this to mean that levan formation is much less than dextran formation, Leach et al (1969), have pointed out, that lower levan levels may, because of the higher lability of levan than dextran to bacterial attack, arise because of more rapid levan breakdown. The results in the present study showing that polyfructose formation is comparable in amount to the formation of polyglucose (assuming the former consists mainly of levans and the latter mainly of dextrans) supports this conclusion, but loss of polyfructose into the medium both in the sediment and plaque experiments suggests that in the oral cavity, a low plaque levan level may also arise

because of some loss of levan from the plaque into the saliva.

Most significant in this study was the finding that in addition to the total amounts, the relative amounts of polyfructose and polyglucose formed were dependent upon the sucrose concentration. At 2.8 mM sucrose, small amount of KOH-sediment polyglucose was synthesized, no synthesis of sediment polyfructose occurred. Neither supernatant polyglucose nor supernatant polyfructose were observed. With increase in the sucrose concentration, the increase in KOH-sediment carbohydrate was more than that of supernatant carbohydrate with polyglucose dominating in the sediment fraction and polyfructose dominating in the supernatant. Moreover with increase in sucrose concentration, the percentage of the total sediment carbohydrate that was extracellular increased (Table 4.1).

The chromatographic data adds convincing support for the view that extracellular polysaccharide formation requires the presence of unhydrolyzed sucrose molecules. When the sucrose concentration was 13.9 mM, polysaccharide spots appeared and increased in intensity so long as there was sucrose still present in the incubation mixture. However, once the sucrose was used up, the intensity of the spots immediately decreased indicative of their utilization (Fig. 4.9). At 138.9 mM, the sucrose was not used up before the experiment was over and the polysaccharide spots remained and increased in intensity until the end of the 4 hour incubation (Fig. 4.10).

The results also showed that the pH has a deciding effect on the polyfructose and polyglucose synthesized. The optimum for both types of

polymers in both the supernatant and KOH-sediment carbohydrate fractions was between pH 5.0 and 7.0, but it appeared that formation of polyglucose was favoured at a more acidic pH.

Several observations in the SSS system on the effect of pH on extracellular polyglucose and polyfructose formation are in agreement with those obtained with pure cultures. With regard to polyfructose, the optimum pH for its formation in the SSS system (assuming it to be mainly levans; Manly et al, 1966) was similar to that reported for levansucrase from Streptococcus mutans (Carlsson, 1970) and from Bacillus subtilis (Anderson, 1963). Also, both supernatant and KOH-sediment polyfructose formation occurred over the pH range 5-8, in agreement with the observations of Gibbons and Nygaard (1968), which showed a similar broad pH range for levan synthesis from sucrose by Streptococcus strain GS5.

While maximum formation of supernatant polyglucose occurred around pH 6.0, it was found in the present study that formation of KOH-sediment polyglucose especially those fractions precipitated at lower ethanol concentration (i.e., larger molecular size and/or lower solubility) was enhanced at a more acidic pH. Similar results were reported for other systems. For example, dextran formation from Leuconostoc mesenteroides occurred between pH 4.0-8.0 with the optimum around 5.6 (Taylor and Hibbert, 1946). In several Lactobacillus strains, Dunican and Seeley (1965) have shown that dextransucrase, the enzyme involved in dextran synthesis from sucrose, has optimum activity between approximately pH 5.0-7.0.

That the polysaccharides formed in the SSS system are not homogeneous, is evident from the experiments shown in Figs. 4.5, 5.6 and 4.8. Besides being more soluble, polyfructose appeared to be less heterogeneous than polyglucose. Since polyfructose precipitated mainly at 70 per cent ethanol whereas polyglucose precipitated mainly at 30 per cent ethanol, the bulk of the polyglucose is not only less soluble, but is likely to be of larger molecular weight.

Molecular size heterogeneity of dextrans has been observed by Jeanes et al (1954) in ninety-six strains of bacteria, which included Leuconostoc, Acetobacter and Streptococcus species. Heterogeneity has also been observed by Wilham et al (1955) in Leuconostoc mesenteroides and by Gibbons and Nygaard (1968) in plaque forming streptococci.

One factor that might affect the molecular size of dextrans is the substrate concentration (Tsuchiya et al, 1953; Anderson, 1963; Ebert and Schenk, 1968). At high sucrose concentration, smaller molecular weight polymers are formed, while at low concentrations, larger dextran molecules are obtained. Hestrin (1962) showed that when synthesis of dextran was conducted in the presence of degraded dextran, formation of high molecular weight dextrans was reduced, whereas the formation of low molecular weight products was markedly increased.

A second factor that might contribute to variation in molecular size is some spontaneous breakdown of large molecular weight dextrans, (Jeanes et al, 1957). Another is the type of acceptor molecule available in the medium during polysaccharide synthesis. Acceptor molecules ini-

tiate the growth of the carbohydrate polymer molecule and have a determinant effect on their molecular size (Ebert and Schenk, 1968). In their studies on the action of acceptor molecules on the molecular weight of dextran formed from sucrose and dextransucrase, Ebert and Schenk found that addition of acceptors such as glucose and fructose to the reacting system gives rise to small molecular weight dextran. On the other hand, when sucrose is the only acceptor molecule available, large molecular weight dextrans are formed.

It is conceivable then, that during sucrose metabolism by the mixed oral flora, a variety of such acceptor molecules (i.e., glucose, fructose and sucrose) would be available in the environment. This might contribute to the formation of carbohydrate polymers of various molecular weight.

Perhaps the most important facts that come out of the experiments in this chapter is that synthesis of extracellular glucose and fructose polymers would increase with the increase in substrate concentration and decrease with decrease in the pH. Thus, in the oral cavity during the first few minutes after ingestion of sucrose, when the sucrose concentration would be high and the plaque pH above 6.0, rapid formation of extracellular polymers would be favoured. As hydrolysis of sucrose continues and utilization of the resulting monosaccharides occurs, synthesis would decrease due to further decrease in the pH. In the meantime sucrose disappearance would be inhibited (cf. Korayem and Kleinberg, 1971) and synthesis of the polymers would continue though at a very slow rate.

This suggests that continual availability of sucrose and an acidic rather than a neutral pH in the dental plaque would favour the accumulation of plaque dextrans and levans.

CHAPTER V

UTILIZATION OF GLUCOSE AND FRUCTOSE POLYMERS BY THE BACTERIA IN SALIVARY SEDIMENT AND DENTAL PLAQUE

In the previous chapter, it was demonstrated that the bacteria in salivary sediment synthesize substantial amounts of extracellular polyglucose and polyfructose at high but very little or none at low concentrations of sucrose. The glucose polymers synthesized remained mostly with the sediment, whereas the more soluble fructose polymers were found free in the medium.

Based on studies with mixed oral bacterial growth, Gibbons and Banghart (1967) concluded that levans are labile to attack by the bacteria found in human saliva and dental plaque whereas dextrans are more or less inert. They concluded, that the low solubility and lability of dextrans would favour their accumulation amongst the bacteria in the dental plaque and thereby would contribute to the building up of plaque matrix. On the other hand, because levans are quite labile, they may by functioning as extracellular carbohydrate reserves for acid production play a more significant role than dextrans in the caries process (Leach, 1969).

The experiments in the present study are an extension of those in the previous two chapters and were initiated to examine some of the above

conclusions in the SSS system. Some experiments were carried out with suspensions of dental plaque; but, because the amounts of plaque available are necessarily much less than salivary sediment, experiments with plaque were less extensive.

The polymers synthesized from sucrose by the bacteria and released into the medium of the salivary sediment mixtures (mainly polyfructose), were tested for suitability as substrate for acid formation by the bacteria in the same system. The polymers, synthesized in sediment mixtures and extractable from the sediment with cold KOH (mainly polyglucose), were tested in the same way. Since the glucose and fructose polymers are most probably levans and dextrans (Gibbons and Nygaard, 1968), the breakdown of a variety of known bacterial levans and dextrans was also examined.

The effect of several factors that might be significant when sucrose is catabolized in the mouth by the microflora of dental plaque was investigated for their effects on the breakdown process. These were the pH, presence of sucrose and the effect of salivary supernatant.

METHODS

Preparation of Salivary Sediment Mixtures

Salivary sediment mixtures were prepared from wax-stimulated whole saliva as described in Chapter III. In the various experiments in this chapter, the total volume of each mixture was 150 µl, 300 µl, 1.5 ml or

3 ml. In all cases, sediment mixtures were prepared by combining in the ratio 1:1:1; (i) a 50 per cent sediment suspension (V/V); (ii) either salivary supernatant or distilled water, and; (iii) either a solution containing the glucose and fructose polymers synthesized by the sediment bacteria from sucrose or one of the several dextrans and levans shown in Table V.1. As in earlier studies with the SSS system (Kleinberg, 1967; Sandham and Kleinberg, 1969a), the final sediment concentration was 16.7 per cent (V/V).

Preparation of Plaque-Saliva Mixtures

For each experiment dental plaque was collected from the labial and buccal surfaces of the teeth of 4 subjects (about 50 µl from each) and dispersed in ice-cold distilled water as described in Chapter IV. After centrifugation at 1740g and 4°C for 15 minutes, the supernatant was discarded and the plaque pellet was washed twice before being made up in distilled water at a 25 per cent suspension (V/V). The plaque mixtures that were prepared consisted of 100 µl of 25 per cent (V/V) plaque suspension, 100 µl of salivary supernatant and 100 µl of dextran solution. The final concentration of plaque in these mixtures was 8.3 per cent (V/V) rather than 16.7 per cent as in sediment, since earlier studies had shown that the pH fall with glucose is comparable in plaque and sediment mixtures when the cell concentrations are 8.3 and 16.7 per cent, respectively (Singer and Kleinberg, 1970).

Preparation of C^{14} Supernatant and KOH-Sediment Extracellular Polysaccharides

The polymers formed from sucrose were prepared in radioactive form by adding 22.7 μCi of U-C^{14}-sucrose (obtained from Amersham/Searle, Toronto, Canada) to a sediment mixture consisting of 10 ml salivary supernatant, 10 ml sediment (50 per cent, V/V) and 10 ml sucrose (416.7 mM), and incubating at 37°C for 1 hour. The mixture was then centrifuged at 12,400g and 4°C for 20 minutes. The supernatant (A) was separated from the sediment; the sediment was then extracted with 10 ml 1 N KOH for 4 hours at 4°C. The resulting suspension was then centrifuged at 12,800g and 4°C for 30 minutes; the supernatant (B) was removed and the remaining sediment was discarded.

Both fractions A and B were dialyzed against distilled water for 48 hours at 4°C and then precipitated twice at an ethanol concentration of 70 per cent. Each precipitate was washed twice with 90 per cent ethanol, redissolved in distilled water and lyophylized.

Incubation Procedures

a. Utilization of fractions A and B by salivary sediment

In the first series of experiments, the glucose and fructose polymers synthesized from sucrose in the SSS system and released into the medium (fraction A) and those remaining with the sediment and extractable with cold KOH (fraction B), were examined for their ability to serve as substrates for acid formation by the bacteria in the system. At the

same time, the effect of sucrose (13.9 mM) on the degradation of fractions A and B was examined.

Utilization of fractions A and B by salivary sediment was determined by measuring formation of acid and $C^{14}O_2$ (see Sandham and Kleinberg, 1970b). Acid formation was measured by decrease in pH when the pH was permitted to decrease during the incubation and by recording the NaOH required to keep the pH constant when the pH was held at 7.0 during the incubation.

Where the pH was permitted to decrease, two series of incubation mixtures (36 in each), were prepared in 10 x 75 mm test tubes fitted with serum stoppers from each of which a teflon cup was suspended by a stainless steel wire to hold 100 µl of 0.1 N NaOH for trapping evolved CO_2 (Chapter III and Sandham and Kleinberg, 1970b). The mixtures in one series consisted of 50 µl salivary supernatant, 50 µl of 50 per cent sediment suspension and 50 µl of either fraction A or B. The composition of the mixtures in the second series was identical to the first, except for sucrose being added at a final concentration of 13.9 mM. Before and at each of several intervals during a 4 hour incubation at 37°C, 6 tubes were removed from each series (3 containing fraction A and 3 containing fraction B) and the $C^{14}O_2$ formed was estimated by liquid scintillation counting as reported previously by Sandham and Kleinberg (1970b).

Eight additional sediment mixtures (300 µl) were prepared, 4 with and 4 without sucrose. Two of each set contained either fraction A or fraction B. The mixtures were incubated alongside the mixtures for $C^{14}O_2$

determination and the pH measured at the same intervals with a glass electrode.

In these experiments, the pH of the mixtures containing sucrose decreased more rapidly and reached a lower pH than the mixtures containing either fraction A or B. To rule out any effects that pH might have on the rates of utilization of the polysaccharides in these fractions, the following type of experiment was carried out.

Four mixtures each having a total volume of 1.5 ml were prepared in 20 x 25 mm test tubes fitted with a rubber stopper penetrated by; (i) a pH glass electrode; (ii) a KCL salt bridge; (iii) a stainless steel hypodermic needle through which NaOH (0.5 N) was added from a microburette, and; (iv) a stainless steel needle through which HCl was added at the end of the incubation to stop the reaction and release any CO_2 still in solution in the incubation mixture (Fig. 5.1). In addition, a teflon cup containing 100 µl of 0.1 N NaOH was suspended from the rubber stopper by a stainless steel wire to trap the evolved $C^{14}O_2$.

The mixtures were incubated at 37°C for 2 hours and the pH kept constant at 7.0 using a pH-stat (Chapter IV). At the end of the incubation the metabolism of each mixture was stopped by injecting 1 ml of 0.1 N HCl into the medium. As before, the radioactivity of the contents of each teflon cup was determined by liquid scintillation counting.

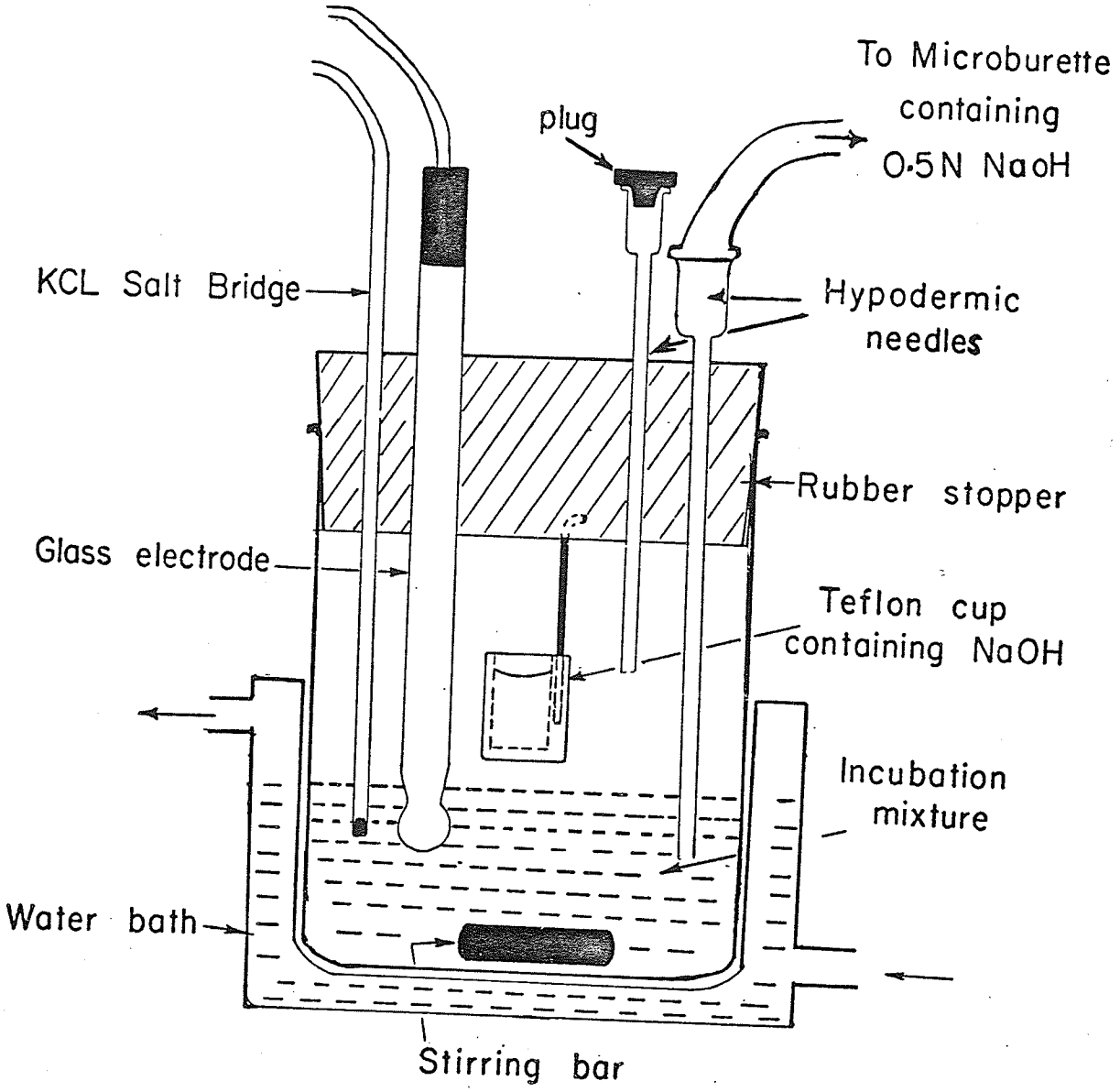

Figure 5.1. Schematic diagram showing the incubation chamber used to determine $C^{14}O_2$ formation at constant pH. At the end of the incubation the plug in the hypodermic needle was removed and a 2 c.c. syringe inserted to permit injection of 1 ml 0.1 N HCl to stop the metabolism of the cells in the incubation mixture.

b. Utilization of various dextrans and levans by salivary sediment and dental plaque

A variety of experiments were carried out to examine the utilization of the dextrans and levans shown in Table V.1 in salivary sediment and plaque-saliva mixtures (300 µl) incubated at 37°C for 18 hours; and concentrations tested were 0.1, 0.5 or 2.0 per cent (W/V); the parameters examined were the pH and in some experiments, the amounts of titratable acid formed.

Some experiments in this series examined the effect of salivary supernatant on the utilization of 5 dextrans and 4 levans at a substrate concentration of 0.1 per cent (Table V.1). Forty salivary sediment mixtures were prepared, half with and half without salivary supernatant. Two mixtures in each series contained one of the above dextrans or levans or distilled water. Only the pH was measured in these experiments.

Other experiments examined the utilization of a few of the above polysaccharides at a substrate concentration of 0.5 per cent. The polysaccharides selected were levans 1662 and 512 and dextrans 1191 and 512. In each experiment, five salivary sediment mixtures (300 µl) were prepared in duplicate; each set of duplicate mixtures contained one the 4 polysaccharides or distilled water. The pH was measured at intervals during the 18 hour incubation at the end of which, the total amounts of acid formed was determined by back titration of each mixture with 0.25 N NaOH (see Sandham and Kleinberg, 1970a).

Other experiments in this series compared the utilization of 4 levans

TABLE V.1

MICROBIAL SOURCE OF LEVANS AND DEXTRANS EXAMINED FOR THEIR UTILIZATION BY SALIVARY SEDIMENT AND DENTAL PLAQUE (*)

Dextran Producing Strains	Levan Producing Strains
B-1120	B-512
B-1254	B-523
B-512	B-1662
B-742	B-133
B-1191	
B-523	

* The above dextrans and levans were obtained from Northern Utilization Research Branch, Agricultural Research Service, United States Department of Agriculture, Peoria, Illinois, U.S.A.

and 4 dextrans, to that of sucrose. The polysaccharides selected were, levan 512, 1662, 523 and 133 and dextrans 742, 1191, 512 and 523. For each experiment, six sediment mixtures were prepared in duplicate. For the levan experiments, each pair of duplicates contained one of the 4 levans, sucrose or distilled water; similar mixtures were prepared when the 4 dextrans were the substrates. The final concentration of the polysaccharides and sucrose was 2 per cent.

The plaque experiments were carried out with dextran 512, dextran 742 and levan 1662. One type of experiment was as follows. Seven plaque mixtures were prepared; two with dextran 512, two with dextran 742 and two with levan 1662 and because of the small amounts of plaque usually available, one control containing distilled water was used. The polysaccharides concentrations were 2 per cent. As in the sediment experiments, mixture volumes were 300 µl and the pH was measured at several time intervals during incubation at 37°C for 18 hours. In another type, utilization of dextran (512) by plaque was compared in the same experiments with salivary sediment. For these, the plaque and sediment were obtained from the same subjects. The dextran concentration in the salivary sediment and plaque-saliva mixtures was 2 per cent; four sediment and four plaque mixtures were prepared; two of each set were with and two were without dextran.

c. Effect of pH on dextran and levan degradation

A third series of experiments was carried out to determine the effect of pH on dextran and levan degradation. For these experiments,

dextran 512 and levan 523 were selected. For each experiment, 4 sediment mixtures (3 ml) containing either dextran 512 or levan 523 at 0.5 per cent were each incubated for 5 hours at either pH 5.0, 6.0, 7.0 or 8.0. The NaOH added from a pH-stat to keep the pH constant was recorded and gave a measure of the acid formed (Chapter IV, Fig. 4.1).

At the end of an incubation, aliquots (100 µl) were removed from the mixtures, immediately centrifuged at 1740g and 4°C for 5 minutes, and the extent of degradation of the dextran or levan initially added determined by paper chromatography (Chapter III). The aliquots applied to the chromatograms were each 25 µl.

RESULTS

The polysaccharides synthesized by salivary sediment during incubation with sucrose (fractions A and B) were both utilized by the sediment bacteria. This was evident from the fall in the pH (Figs. 5.2a and 5.3a) and the $C^{14}O_2$ generated (Figs. 5.2b and 5.3b) with both fractions. The pH fall was comparable with fraction A (which contains mostly polyfructose) and fraction B (which contains mostly polyglucose). Comparable rates of formation of acid and $C^{14}O_2$ observed in the experiments where the pH was held constant at 7.0 (Figs. 5.4 and 5.5). Sucrose caused a more rapid pH fall and reached a lower pH than either fraction A or B, indicative of the slower rate of utilization of polyglucose and polyfructose compared to sucrose. Most important, in the presence of sucrose, the breakdown of

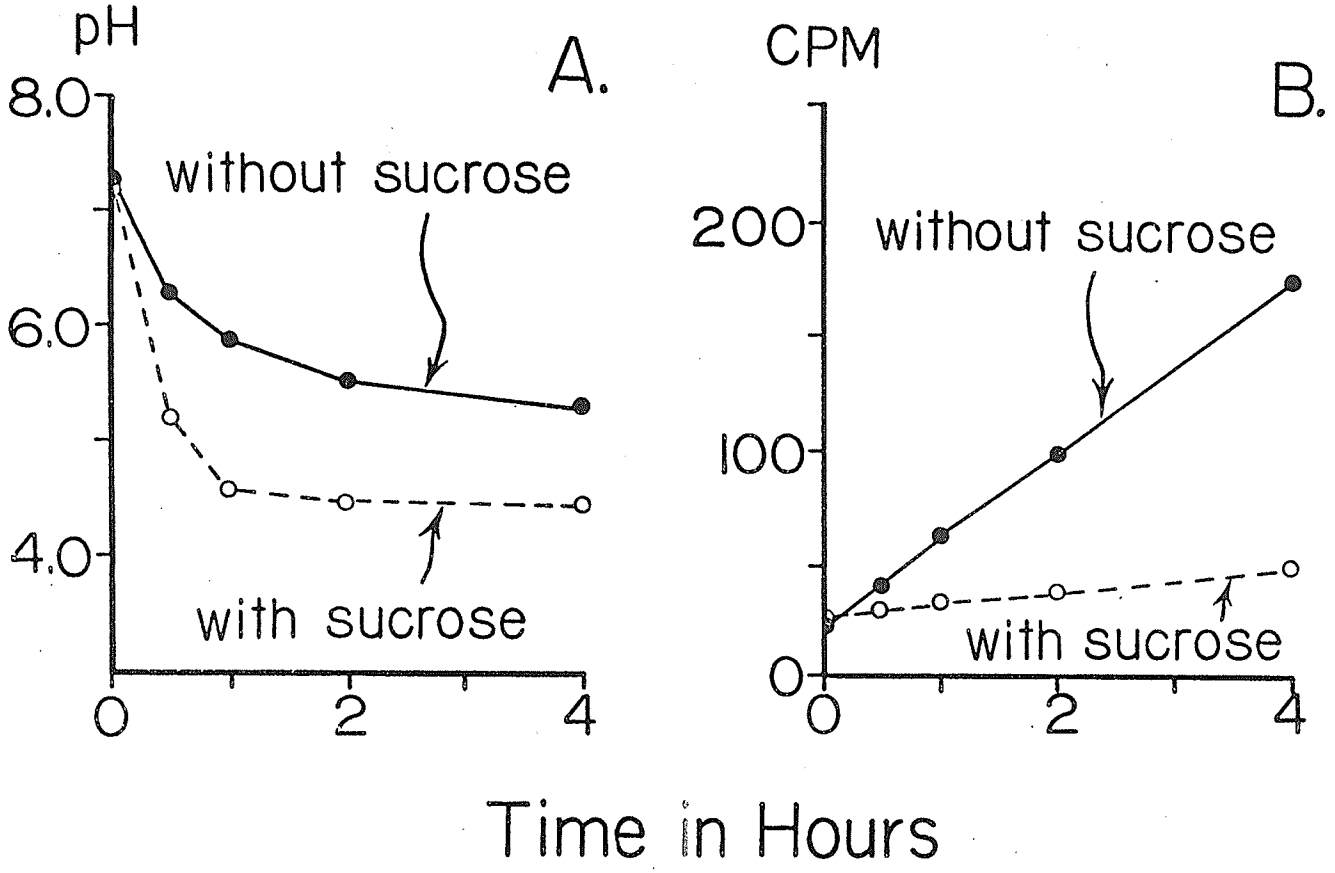

Figure 5.2. Decrease in pH (A) and formation of $C^{14}O_2$ (B) in salivary sediment mixtures incubated with C^{14}-labelled fraction A both in the presence and absence of sucrose. The sucrose concentration was 13.9 mM. Fraction A added initially to each incubation mixture (150 µl) contained 1525 CPM. Note that approximately 10 per cent of the C^{14}-label added at the start of the incubation appeared as $C^{14}O_2$.

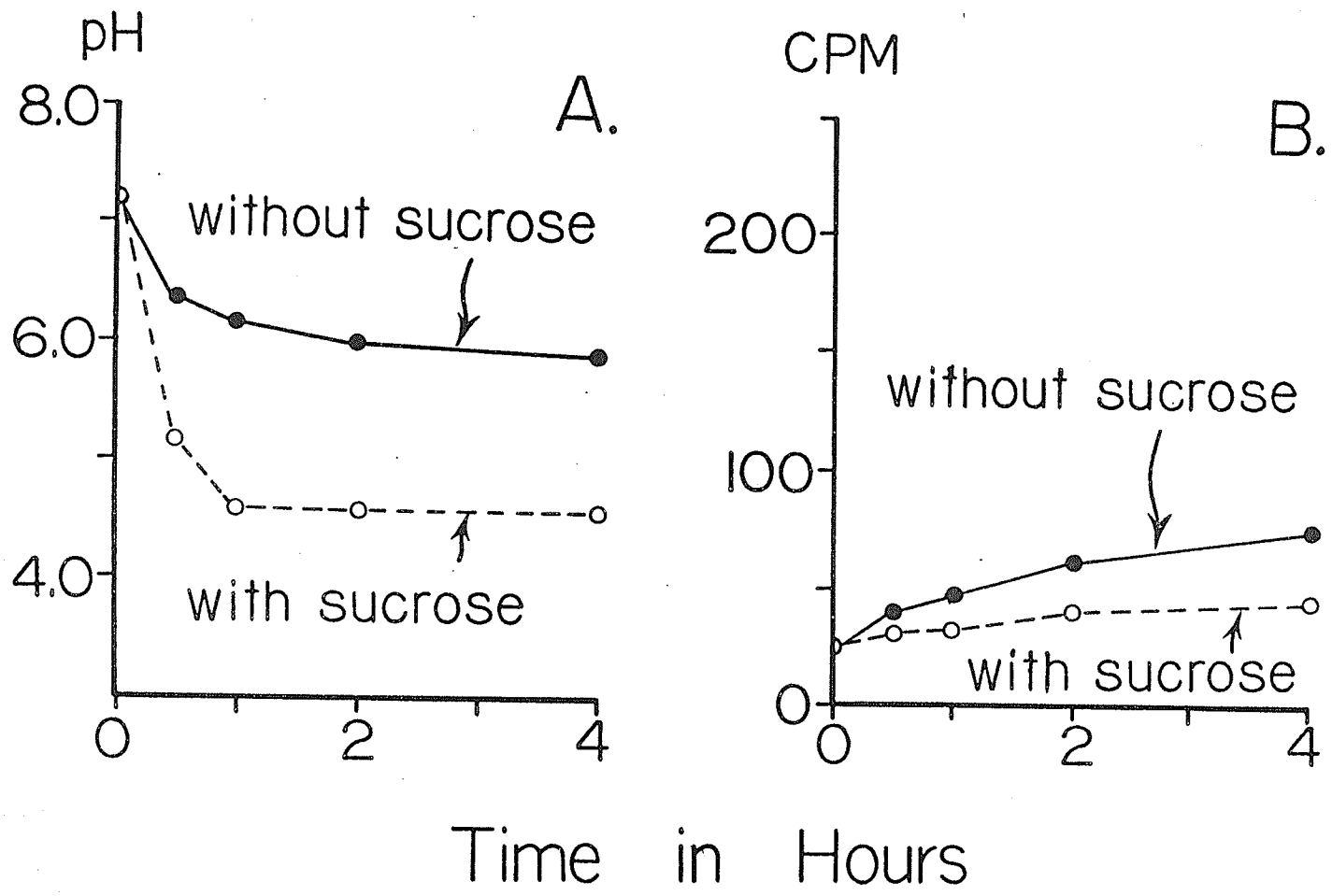

Figure 5.3. Decrease in pH (A) and formation of $C^{14}O_2$ (B) in salivary sediment mixtures incubated with C^{14}-labelled fraction B both in the presence and absence of sucrose. The sucrose concentration was 13.9 mM. 616 CPM was added initially to each incubation mixture (150 µl). Note that approximately 9.5 per cent of the C^{14}-label added at the start of the incubation as $C^{14}O_2$.

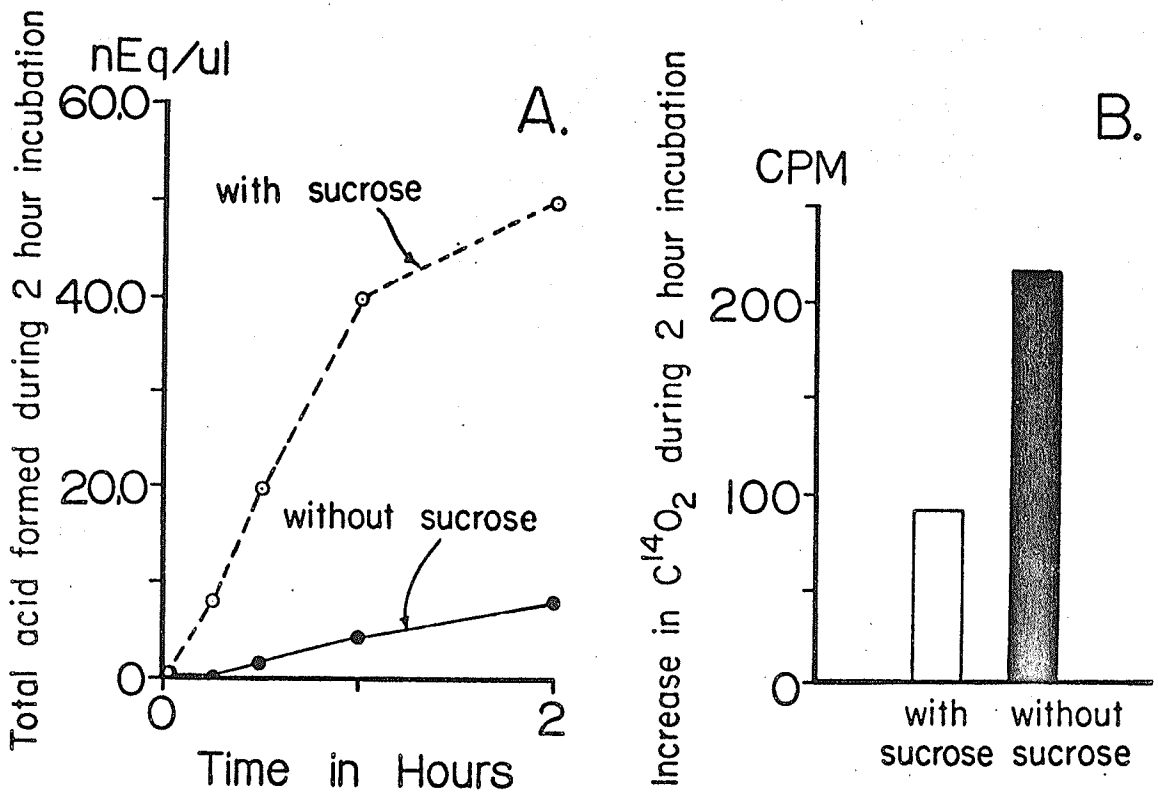

Figure 5.4. Formation of acid (A) and $C^{14}O_2$ (B) in salivary sediment mixtures (1.5 ml) incubated with C^{14}-labelled fraction A in the presence and absence of sucrose with the pH held constant at 7.0. The sucrose concentration was 13.9 mM. 15245 CPM was added initially to each incubation mixture (1.5 ml). Note that approximately 14 per cent of the C^{14}-label added at the start of the incubation appeared as $C^{14}O_2$.

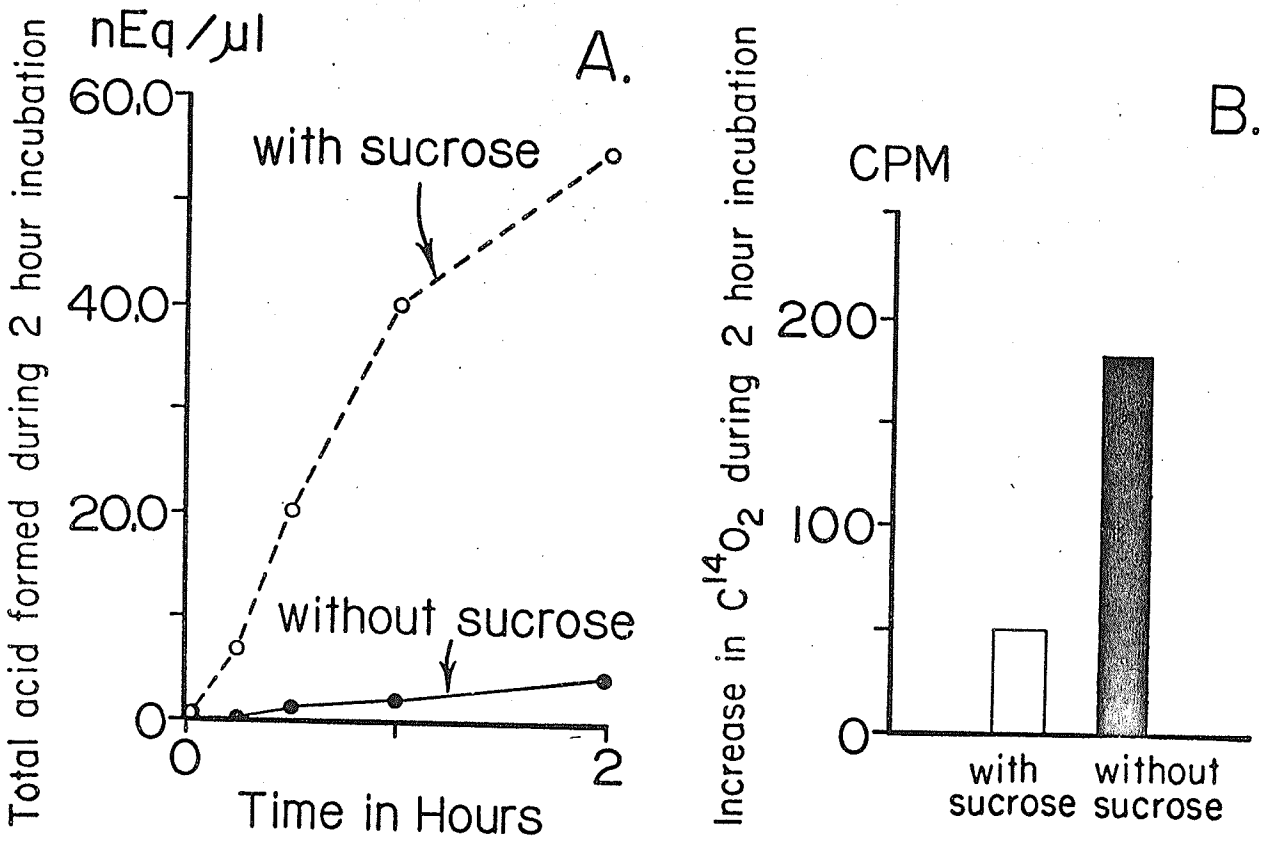

Figure 5.5. Formation of acid (A) and $C^{14}O_2$ (B) in salivary sediment mixtures incubated with C^{14}-labelled fraction B in the presence and absence of sucrose with the pH held constant at 7.0. The sucrose concentration was 13.9 mM. 6160 CPM was added initially to each incubation mixture (1.5 ml). Note that approximately 17 per cent of the C^{14}-label added at the start of the incubation appeared as $C^{14}O_2$.

both polymers (as shown by the amount of $C^{14}O_2$ formed) was suppressed (Figs. 5.2b and 5.3b). This was true whether the pH was allowed to fall or was held constant at pH 7.0 (Figs. 5.4b and 5.5b).

The results of the experiments in which utilization of the dextrans and levans listed in Table V.1 by the salivary sediment are shown in Figs. 5.6 to 5.9. At all concentrations tested, the dextrans and levans were utilized by the sediment bacteria.

In the absence of substrate, the pH fell during the first half hour and then slowly rose until the end of the incubation (Kleinberg, 1967). In the presence of 0.1 per cent levan or dextran the pH fell much further and also showed a subsequent rising phase (Figs. 5.6a and 5.7a). The fall in pH was slower with the various dextrans and levans than that observed with sucrose at the same concentration (Chapter III). The slower responses with levans and dextrans are indicative of slower rates of utilization. The fall and rise in the pH seen at 0.1 per cent in the presence of supernatant (Figs. 5.6a and 5.7a) did not occur in its absence (Figs. 5.6b and 5.7b).

At the higher substrate concentrations (Figs. 5.8 and 5.9) and with salivary supernatant present, no rise in the pH curve was seen and eventually the pH with dextran and levan reached as low a pH as that observed with sucrose (Chapter III). Again, compared to sucrose, the utilization of these polymers was quite slow.

In general, the rates of fall in pH with the various dextrans and levans were comparable although there were variations amongst the dextrans

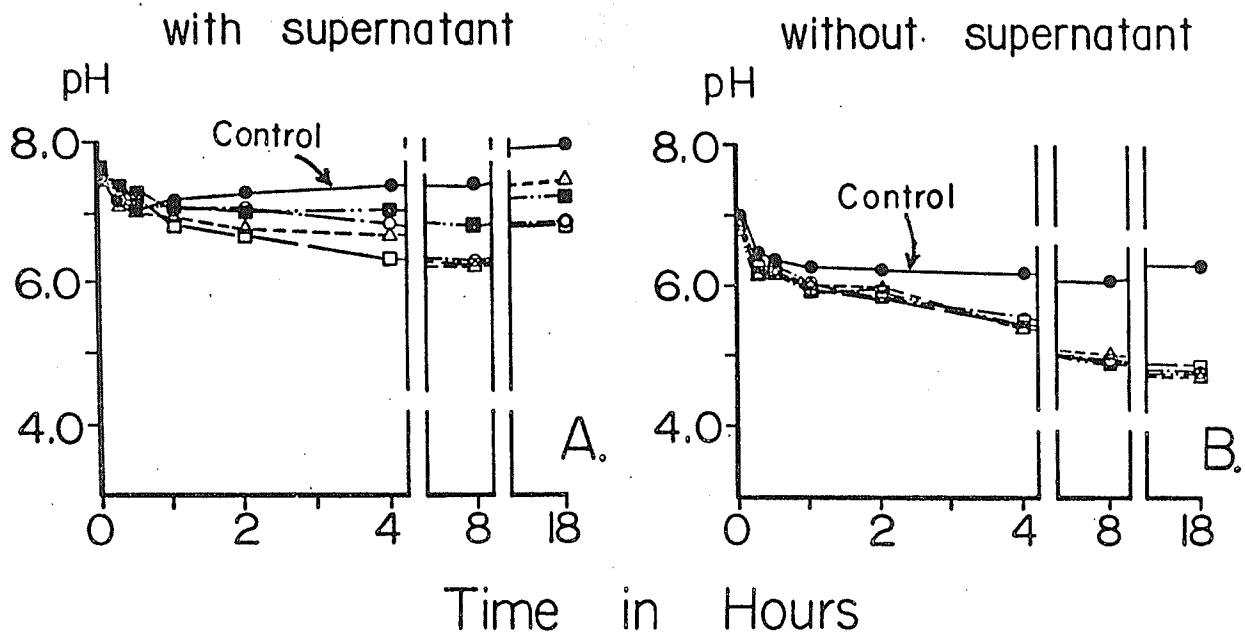

Figure 5.6. The pH of salivary sediment mixtures incubated with several levans each at a concentration of 0.1 per cent. (A) in the presence, and; (B) in the absence of salivary supernatant. □ - levan 1662; Δ - levan 133; o - levan 523; ■ - levan 512; ● - distilled water.

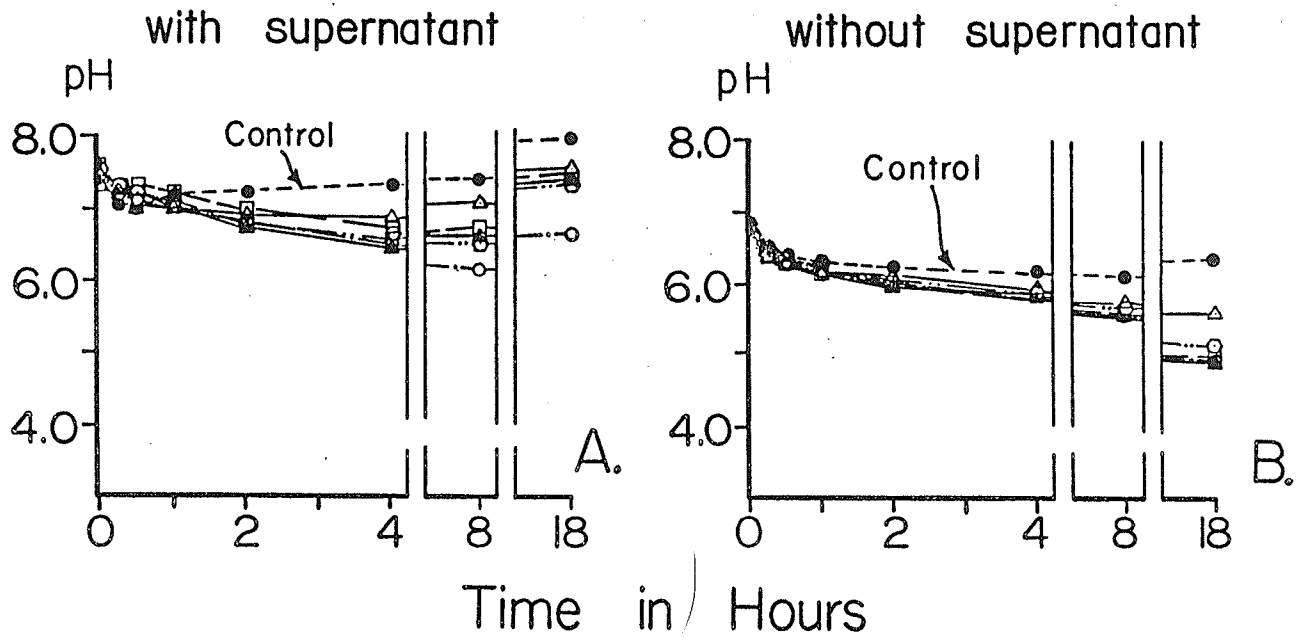

Figure 5.7. The pH of salivary sediment mixtures incubated with several dextrans each at a concentration of 0.1 per cent. (A) in the presence, and; (B) in the absence of salivary supernatant. o - dextran 512; ○ - dextran 1120; □ - dextran 1191; △ - dextran 742; ▲ - dextran 1254; ● - distilled water.

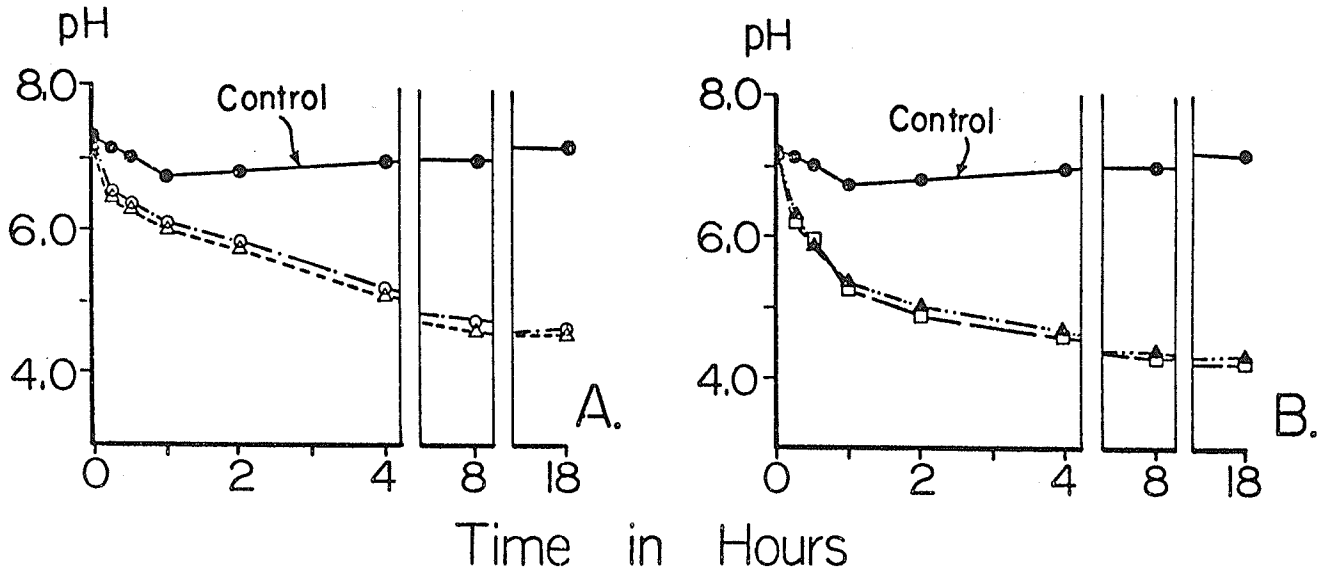

Figure 5.8. The pH of salivary sediment mixtures incubated with; (A) dextran 1191 and 512, and; (B) levan 512 and 1662, each at a concentration of 0.5 per cent. Titratable acid formed from the dextrans and levans at the end of the incubation was 23, 26, 33 and 32 nequivalent/µl of mixture, respectively. The value for the control was 1.7 nequivalent. o - dextran 1191; Δ - dextran 512; ▲ - levan 512; □ - levan 1662; ● - distilled water.

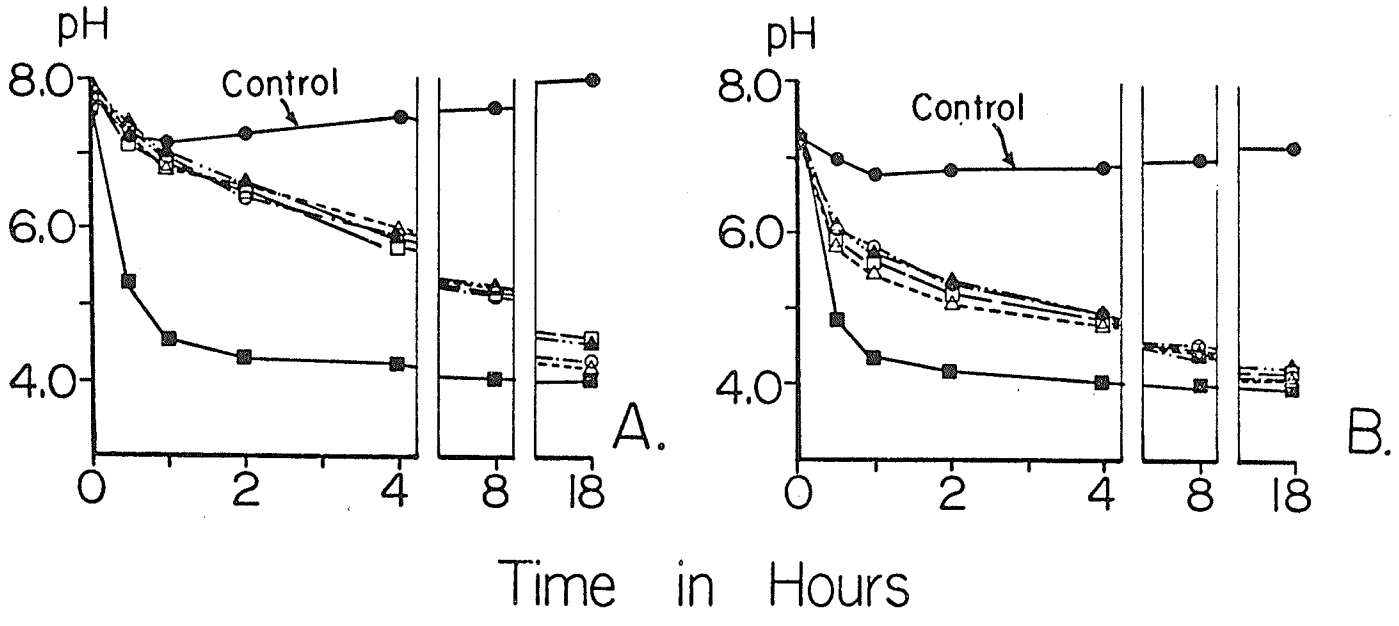

Figure 5.9. The pH of salivary sediment mixtures incubated with; (A) dextrans 512, 742, 1120 and 1254, and; (B) levan 512, 1662, 523 and 133, each at a concentration of 2 per cent. (A) Δ - dextran 512; o - dextran 523; ▲ - dextran 1191; □ - dextran 742. (B) Δ - levan 1662; o - levan 133; ▲ - levan 512; □ - levan 523; ■ - sucrose; ● - distilled water.

and levans.

In the experiments where the dextran and levan concentrations were 0.5 per cent the rate of decrease in the pH was faster with the levans than the dextrans tested. Measurement of the amounts of acid formed by the end of the incubation showed the same results.

In the experiments in which degradation of dextran 512 by dental plaque and salivary sediment was compared (Fig. 5.10a), the fall in pH was almost the same except perhaps for a slightly faster rate with plaque than with sediment. Both reached approximately the same pH at the end of the experiment, viz., pH 4.45 and 4.38.

Comparison of the rates of utilization of dextran 512, dextran 742 and levan 1662 by plaque suspensions showed that the plaque bacteria are capable of utilizing all three polysaccharides (Fig. 5.10b).

In the experiments in which the pH was kept constant at several levels, acid production with both the dextran and levan tested increased with increase in the pH (Fig. 5.11). At pH 5.0, little or no acid was formed from either substrates; with increase in the pH, acid production increased and of the 4 pH levels tested, was maximum at pH 8.0.

The results of the chromatography experiments showed that, only with levan, at pH 5.0 and 6.0 but not at 7.0 and 8.0 did traces of free fructose appear in the medium during the incubation. Levan and dextran were still present in the medium at the end of the 5 hour incubation.

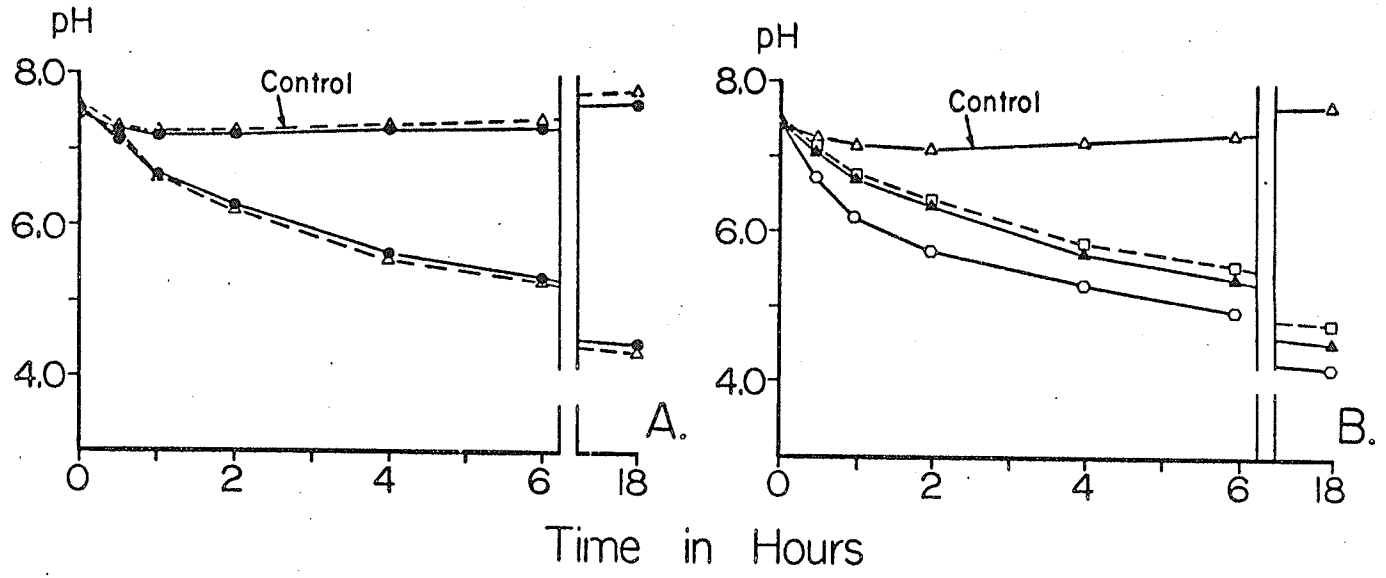

Figure 5.10. (A) pH of salivary sediment and plaque-saliva mixtures incubated with and without dextran 512; (B) pH of plaque-saliva mixtures incubated with either distilled water, dextran 512, dextran 742 or levan 1662. The dextrans and the levans were each at a concentration of 2 per cent. (A) ● - salivary sediment; Δ - plaque. (B) ○ - levan 1662; ▲ - dextran 512; □ - dextran 742; Δ - distilled water.

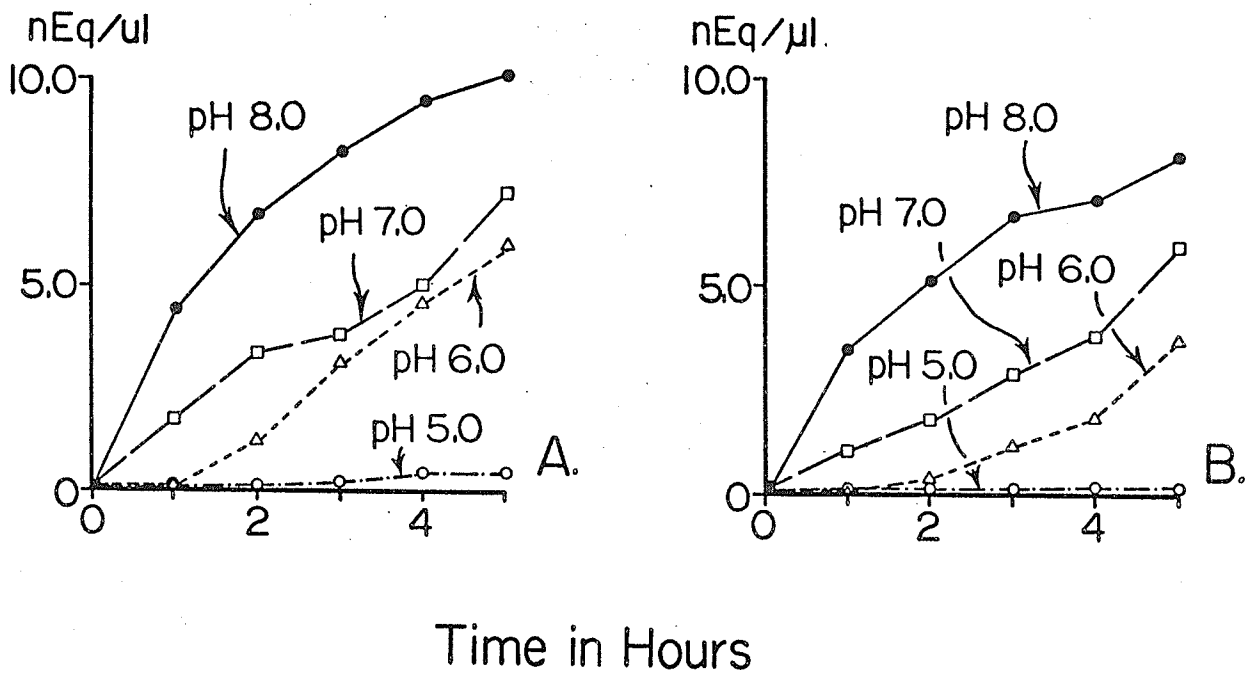

Figure 5.11. Acid formation in salivary sediment mixtures incubated with 0.5 per cent; (A) levan 523, and; (B) dextran 512, at constant pH.

DISCUSSION

The results show that the extracellular polysaccharides formed in salivary sediment mixtures incubated with sucrose can be utilized by the bacteria in the same system. This is evident from Figs. 5.2, 5.3, 5.4 and 5.5, where both acid and $C^{14}O_2$ are produced when the supernatant or the KOH-sediment fractions (A and B) are added as substrate. Acid and $C^{14}O_2$ formation were comparable from fraction A which contains mostly polyfructose, and from fraction B which contains mostly polyglucose. Comparable rates of utilization of fractions A and B suggest that dextrans may be utilized as rapidly as levans, assuming on quite good grounds that the polyfructose in the former is mainly levans and the polyglucose in the latter is mainly dextrans.

The experiments in which the fermentability of a variety of dextrans and levans was examined also showed that acid formation was comparable and slower than from sucrose (Fig. 5.9; see Chapter III for results with lower sucrose concentrations).

Although acid formation varied amongst the different dextrans and levans tested, they all showed a number of characteristics seen with glucose and sucrose under the same conditions. First, the rate of fall in pH increased with increase in substrate concentration. Second, the fall and rise in the pH seen at low but not at high glucose and sucrose concentrations (Chapter III), was also seen with levans and dextrans. Third, without salivary supernatant, the fall and rise in the pH observed at

low substrate concentrations was not seen; instead, the pH continued to fall and did not subsequently rise (Figs. 5.6b and 5.7b).

The simplest and most probable explanation of these results is that the hydrolysis of dextrans and levans by the sediment and plaque bacteria is a slow process yielding glucose and fructose at slow rates. This would lead in the scheme shown in Fig. 5.12, to steps I and II proceeding no faster than steps IV and V and acid formation occurring at the same comparatively slow rate. Accordingly, increasing the concentration of either dextran or levan would accelerate IV and V thereby cause a greater fall in the pH (Figs. 5.8 and 5.9). Since salivary supernatant stimulates the uptake of glucose (Kleinberg and Craw, 1969) and fructose (Chapter VI), at low concentrations of dextran and levan, more rapid uptake of glucose and fructose would lead to complete utilization of dextran and levan and the fall and rise type of pH curve seen in Figs. 5.6a and 5.7a.

In the experiments with C^{14} labelled fractions A and B, sucrose severely suppressed the utilization of the polysaccharides in both fractions. This inhibition was evidently due to sucrose and was not an effect due to pH (Figs. 5.4 and 5.5).

Sucrose may suppress dextran and levan degradation by inhibiting the activities of dextranase and levanase. Another and more probable explanation is that the glucose and fructose arising from sucrose hydrolysis are the vehicles, whereby sucrose inhibits the hydrolysis of levans and dextrans (Step III; Fig. 5.12). Hydrolysis of sucrose on the one hand

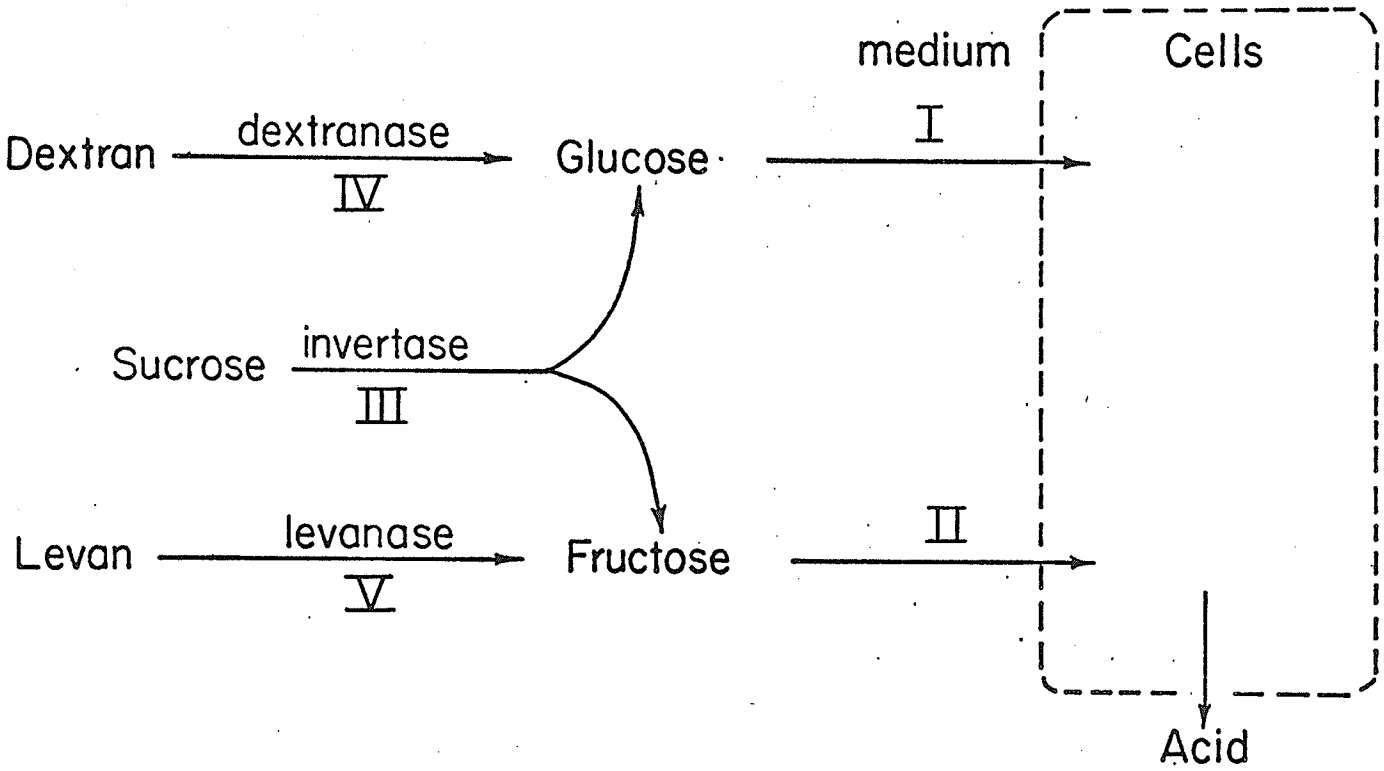

Figure 5.12. Interaction between sucrose and dextran and levan degradation.

and dextrans and levans on the other, both yield glucose and fructose. Since sucrose is hydrolyzed much more rapidly than levans and dextrans, (evident from the acid formation and chromatography experiments in Chapters III and IV), one can see how neatly the glucose and fructose formed from sucrose could suppress utilization of both types of extracellular polysaccharide. Moreover, suppression of both could occur simultaneously and continue until sucrose utilization is complete.

Regulation of dextran and levan utilization by sucrose is somewhat parallel to glucose regulation of intracellular polysaccharide utilization observed in the same and in other bacterial systems. Intracellular and extracellular polysaccharides are both synthesized while glucose on the one hand and sucrose on the other, is present in the medium. Both are also catabolized once the environment is depleted of their corresponding substrates (Gibbons and Socransky, 1962; Sandham and Kleinberg, 1969a). Because the intracellular polysaccharide formed from sucrose, glucose or fructose is mainly polyglucose, one would expect the free glucose in the medium to simultaneously affect the degradation of both intra- and extracellular polysaccharide. Once the glucose and fructose have been depleted the catabolism of extracellular polyfructose and polyglucose would proceed along with the catabolism of intracellular polysaccharide. As proposed earlier by several investigators (Gibbons and Socransky, 1962; Weiss et al, 1965; Sandham and Kleinberg, 1969b), this would prolong acid formation beyond the period that dietary carbohydrate is available to the microorganisms in the dental plaque as substrate.

Effect of pH on Dextran and Levan Utilization

Between pH 4.0 and 8.0, dextran and levan utilization both increased with increase in the pH. The effect of pH on the utilization of these polysaccharides parallels its effect on glucose (Korayem and Kleinberg, 1970) and sucrose utilization (Chapter IV) and strongly suggests, (and in keeping with the scheme shown in Fig. 5.12 for dextran and levan degradation) that the effect of pH on dextran and levan utilization is mainly an effect of pH on glucose and fructose uptake.

In contrast to that seen earlier with sucrose, no free glucose was observed chromatographically during the catabolism of dextran. This was also true for levans except for the appearance of trace amounts of free fructose at acidic pH. The dextran observations can be explained if one assumes that the two main dextranase systems described in other systems (Sery and Hehre, 1956; Tsuchiya et al, 1952) are present in sediment and plaque. Accordingly, one system degrades large molecular weight dextrans into smaller fragments between pH 5.0-5.5. Consequently at acidic pH, the larger fragments one would expect from dextran degradation would remain at the origin of the chromatogram along with unhydrolyzed dextran. The second dextranase system degrades dextran end-wise resulting in release of only glucose between pH 7.0-7.5 (Ingelman, 1948). At such a pH, the free glucose would be rapidly utilized by the microorganisms in the incubation mixture and result in no glucose appearing on the chromatograms.

Appearance of trace amounts of free fructose in the incubation medium when levan was utilized at pH 5.0 and 6.0 and not at higher pH may

be the result of fructose uptake at acidic pH being sufficiently inhibited so that the rate of uptake is slower than levan degradation.

Dextranases and Levanases in Sediment and Plaque

The present study clearly demonstrated that the mixed floras of both sediment and plaque contain the enzymes necessary to catabolize a variety of levans and dextrans.

Evidence for the presence of levanases in some oral microorganisms is not lacking. For example, Van Houte and Jansen (1968b) reported that 7 out of 17 strains of α-haemolytic streptococci and 6 out of 11 strains of Streptococcus salivarius isolated from human dental plaque were capable of hydrolyzing levans obtained from Streptococcus salivarius or Pseudomonas auerofeciens. Manly (1967 and 1968) demonstrated the presence of the enzyme levanase in both dental plaque and salivary sediment. Van Houte (1967) found that both under aerobic or anaerobic conditions several strains of Streptococcus mitis and Streptococcus salivarius (SS9) or Ps. auerofeciens. Da Costa and Gibbons (1968) showed that several streptococcal strains isolated from human dental plaque possess a fructan hydrolyzing enzyme.

On the other hand, a number of studies have suggested that plaque does not contain dextranases. For example, Gibbons and Banghart (1967) found that the microorganisms isolated from swabs of plaque, saliva and tongue of human subjects do not utilize dextran but do utilize levan. Also large amounts of dextran accumulate in the plaques of experimental

animals fed a high sucrose diet (Gibbons et al, 1966) and in human subjects whose diets were supplemented with an intake of one lump of sucrose every hour during the day time (50 grams per day) for one week (Carlsson and Egelberg, 1965). Also, consistent with levanases being present and dextranases being absent in dental plaque are the observations of Wood (1967b) and Critchley et al (1967) that dental plaque upon analysis contains much lower levels of levan than dextran.

The belief that there is little or no dextranase in plaque has been the main basis for recent attempts to use dextranases therapeutically either to prevent the formation of plaque and/or facilitate its removal. Recent findings indicate, however, that some of these interpretations and the basis for using dextranase to reduce plaque are no longer valid. First, as demonstrated in the present study and in those of others (Gold et al, 1969), the mixed bacteria in both salivary sediment and plaque are capable of degrading dextrans and consequently must possess the necessary dextranases. Second, the studies in animals and those in human subjects were carried out under conditions where sucrose was available at very high concentration and on a more or less continuous basis. These conditions would be favourable for the synthesis of extracellular dextrans and levans and at the same time, because of the continual presence of sucrose, would inhibit the breakdown of these polymers (Figs. 5.2-5.5). Both processes would favour accumulation of dextrans and levans. Third, the studies showing less levan than dextran in plaque did not take into account the greater lability and solubility of

levans than dextrans. Leach et al (1969) has pointed out that large amounts of levan may have been formed but that more rapid breakdown may have occurred and resulted in low levels of levan in plaque. Support for the occurrence of substantial synthesis of levan is indicated by the sediment and plaque studies in this thesis (Chapter IV) which also showed that most of the levan synthesized is soluble and is released into the incubation medium during synthesis. Critchley et al (1967), not aware of this point, had the subjects in their study, after rinsing their mouths with sucrose solution, rinse their mouths twice with distilled water prior to removing samples of plaque for dextran and levan analysis. Most probably, rinsing with distilled water would remove most of any levan synthesized during the period of rinsing with sucrose.

Relation Between Dextran and Levan Degradation and Plaque pH

It would appear from the experiments in the present study that both levan and dextran can act as substrates for acid formation and that their availability would depend upon the presence of sucrose. Once the sucrose is used up, the extracellular polymers can function as substrate reserves. Their degradation would cause slow release of glucose and fructose for acid formation, thereby retarding the return of the pH to neutrality. Interestingly, as the pH rises, the rate of degradation will also rise and slow the rate of pH rise even more.

CHAPTER VI

EFFECT OF SALIVARY SUPERNATANT AND FLUORIDE ON THE METABOLISM OF SUCROSE BY THE MICROFLORAS OF PLAQUE AND SALIVARY SEDIMENT

A number of studies have shown that both saliva and fluoride have strong inhibitory effects on the dental caries process. For example, surgical removal of the salivary glands of experimental animals results in a sharp increase in the severity of the disease (Klapper and Volker, 1953; Finn et al, 1955), the severity varying in accordance with the numbers and types of salivary glands removed (Schwartz and Shaw, 1955). In humans, conditions such as unilateral or bilateral xerostomia (Gurley, 1939; Suher et al, 1953), surgical removal or irradiation therapy of salivary glands result in little or no flow of saliva and a dramatic increase in the incidence of dental caries (Allington, 1950; Blackwell, 1955; Frank et al, 1965; Llory et al, 1971).

The inhibitory effect of fluoride on dental caries is well established. This effect appears to arise from the ability of fluoride to reduce the solubility of the hydroxyapatite of tooth enamel (Jenkins, 1963 and 1967) and the ability of the oral microorganisms to produce acid (Bibby and Van Kesteren, 1940).

Evidence has been presented indicating that the beneficial effect of saliva arises from several of its physical and chemical properties. First, flow of saliva facilitates clearance of fermentable substrate introduced into the oral cavity during eating as well as the dilution

and removal of the acidic end products that arise therefrom (Lanke, 1957). Second, saliva contains buffers such as bicarbonate that neutralize some of the acids formed (Wah Leung, 1951; Lilienthal, 1955). Third, saliva contains a factor that stimulates the formation of base at the same time that it stimulates glycolysis (Kleinberg, 1970b). As a result of the last property of saliva, one would expect utilization, and as a result the clearance of fermentable carbohydrate from the oral cavity to be accelerated.

The factor in saliva responsible for these effects has been isolated and identified as a peptide (Kleinberg and Craw, 1971). Because it favours an earlier rise in the pH after the pH minimum has been reached, it has been named salivary pH-rise factor.

Along with stimulation of the uptake of glucose and acid formation, salivary supernatant also stimulates the formation by the salivary sediment microflora of storage carbohydrate (Kleinberg and Craw, 1969). Fluoride has the opposite effect. Along with inhibition of glucose uptake, fluoride inhibits both acid formation and the formation of storage carbohydrate. These effects of fluoride have been demonstrated in bacterial systems containing pure cultures of microorganisms isolated from human mouths (Bibby and Van Kesteren, 1940; Clapper, 1947; Sims, 1966; Weiss et al, 1965; Hamilton, 1967 and 1969), in incubated whole saliva (Wright and Jenkins, 1954; Jenkins, 1959) and in incubation mixtures containing salivary sediment (Sandham and Kleinberg, 1969b). Interestingly, although salivary supernatant and fluoride have opposite effects on carbohydrate

metabolism, they have similar effects on the pH (Kleinberg and Craw, 1969). Both inhibit its decrease; salivary supernatant is more effective, however, above a pH of approximately 5.5, while fluoride is more effective below this pH (Kleinberg and Craw, 1969).

In the present chapter, examination of the effects of salivary supernatant and fluoride on the metabolism of glucose by the microorganisms in plaque and sediment has been extended to sucrose. The parameters of the carbohydrate metabolism of the microflora examined were sucrose uptake, decrease in pH and the formation of glucose and fructose polymers. Because of the similarity of the carbohydrate metabolisms of the microfloras in plaque and sediment demonstrated in a number of studies (Kleinberg, 1970c; Singer and Kleinberg, 1970; Korayem and Kleinberg, 1970 and 1971), and because of the much smaller supply of plaque as compared to sediment, a number of the experiments reported in the present chapter, were carried out with sediment.

Sucrose during its utilization by salivary sediment is hydrolyzed to glucose and fructose (Chapter III). This has also been demonstrated in this chapter in similar experiments carried out with dental plaque. Since any effects of salivary supernatant and fluoride on sucrose might simply be the result of their effects on glucose and fructose it was considered necessary for comparative purposes, in the present study to examine the effects of salivary supernatant and fluoride on the decrease in pH and the uptake and storage of carbohydrate with fructose. It was not necessary to examine the effects of salivary supernatant and fluoride

on these parameters with glucose since this has been done already (Sandham and Kleinberg, 1969b; Kleinberg and Craw, 1969).

METHODS

Preparation of Plaque and Salivary Sediment Mixtures

Plaque was collected from 8-10 human subjects as described in Chapter IV. The plaque was removed with a stainless steel spatula mainly from the labial and buccal surfaces of the anterior and posterior teeth. Each portion upon removal was transferred to and dispersed in distilled water in a 2 ml graduated centrifuge tube chilled in cracked ice. The plaque suspension was centrifuged at 1740g for 15 minutes, washed 3 times with cold distilled water and then suspended in distilled water as a 25 per cent (V/V) suspension.

Salivary sediment was prepared from wax-stimulated whole saliva as described previously (Chapter III) and suspended in distilled water as a 50 per cent (V/V) suspension.

Each plaque-saliva mixture prepared for the plaque experiments consisted of; 25 per cent plaque suspension, sucrose solution with or without added fluoride and either salivary supernatant or distilled water in 1:1:1 ratio; the volume of each was either 450 or 600 µl. The final plaque concentration was 8.3% (V/V); the final sucrose concentration was 13.9 or 138.9 mM and the fluoride level was 5 ppm. Fluoride was added as sodium fluoride (Baker analyzed).

For the sediment experiments, the volume of each salivary sediment mixture was either 1.5 or 3 ml and was similar in composition to the plaque saliva mixtures, except that the final cell concentration was 16.7 per cent (V/V) rather than 8.3 per cent (V/V). All of the experiments were carried out for 2 hours and incubations were at 37°C.

Effect of Salivary Supernatant and Fluoride on Sucrose Utilization, Decrease in pH and Formation of Polyglucose and Polyfructose in Plaque-Saliva Mixtures Incubated with 13.9 mM and 138.9 mM Sucrose

For each experiment, four plaque-saliva mixtures (450 µl) were prepared. Before and at regular intervals during an experiment, the pH was measured in each mixture and aliquots (15 µl) were removed in triplicate for determination of the carbohydrate in both the supernatant and the plaque pellet. The pH was measured with a glass electrode and the electrode arrangement described in Chapter III. Each aliquot removed from an incubation mixture was pipetted into a 10 x 75 mm test tube chilled in cracked ice and containing either 0.4 ml 1 per cent NaF (if samples were obtained from mixtures where the sucrose concentration was 13.9 mM) or 1 ml 1 per cent NaF (if samples were obtained from mixtures where the concentration was 138.9 mM).

The contents of each 10 x 75 mm test tube were mixed by vibrating on a vortex mixer. After centrifugation at 1740g for 5 minutes at 4°C, aliquots (50 µl) were removed from the supernatant and the pellet was washed with NaF. The carbohydrate contents of both were then analyzed

for glucose and fructose by the procedures described in Chapters II and III.

Chromatographic Examination of Plaque Mixtures Incubated with Sucrose

For these experiments, two plaque saliva mixtures (450 µl) were prepared containing plaque suspension, salivary supernatant and sucrose at final concentrations of 13.9 and 138.9 mM. At regular intervals during a 2 hour incubation, aliquots (50 µl) were transferred to 6 x 50 mm Kimax tubes held in cracked ice and centrifuged immediately at 1740g for 5 minutes at 4°C. Aliquots (15 µl) of supernatant were then examined by paper chromatography for carbohydrate as described in Chapter III.

The Effects of Supernatant and Fluoride on Formation of Acid and Sediment Carbohydrate in Plaque and Sediment Mixtures Incubated at Constant pH with Sucrose

The pH has a major effect on the rate of utilization of glucose by both sediment and plaque (Korayem and Kleinberg, 1970 and 1971). The stimulatory and inhibitory effects of salivary supernatant and fluoride on glucose catabolism are also pH dependent (Sandham and Kleinberg, 1969b; Kleinberg and Craw, 1969). Supernatant is more effective above whereas fluoride is more effective below approximately pH 5.5.

Consequently, the effect of pH on the supernatant and fluoride effects on the formation of acid in mixtures containing either plaque or sediment incubated with sucrose were compared in the following type of

experiment.

Plaque mixtures were prepared consisting of; (i) 200 µl of 25 per cent plaque suspension; (ii) 200 µl of 416.7 mM sucrose either with or without added fluoride, and; (iii) 200 µl of either 1740g salivary supernatant or distilled water. The final sucrose concentration was 138.9 mM. Similar sediment mixtures were prepared except that each incubation mixture was 3 ml and the final sediment concentration was 16.7 rather than 8.3 per cent (V/V).

The pH was held constant at 5.0 and 6.0 by frequent addition of 0.5 N NaOH from a microburette controlled by a pH-stat (see Chapter IV). At the same intervals as in the first experiments in this chapter where the pH was allowed to fall, the amounts of NaOH required to keep the pH constant, were recorded.

In the incubations with salivary sediment, the carbohydrate accumulated by the bacteria by the end of the 2 hour incubation was measured. Three aliquots (25 µl) were removed from each mixture at the beginning and end of the incubation and delivered into 10 x 75 mm test tubes containing 1.8 ml 1 per cent NaF. After centrifugation at 1740g and washing the sediment 3 times with 2 ml of 1 per cent NaF, the sediment pellet was analyzed for carbohydrate (polyglucose and polyfructose) as described in Chapter III.

Effect of Salivary Supernatant and Fluoride on the Formation of Supernatant and KOH-Sediment Polysaccharides

For each experiment, 4 sediment mixtures (3 ml) were prepared in which the final sucrose concentration was 138.9 mM. Two mixtures contained salivary supernatant; two contained distilled water. One of each pair contained fluoride. After incubation for 2 hours, each mixture was centrifuged at 12,400g and 4°C for 20 minutes. Three aliquots (70 µl) were removed from each supernatant and the supernatant polysaccharide precipitated at 4°C with ethanol (final concentration of 70 per cent). To obtain the KOH-sediment component of the extracellular polysaccharide formed during the incubation, the sediments of each mixture were extracted with 1 N KOH for 4 hours at 4°C and then centrifuged at 12,800g and 4°C for 20 minutes. Three aliquots (30 µl) were removed from the extract and precipitated at 4°C with ethanol (final concentration of 70 per cent). After standing overnight, the precipitates were analyzed for polyglucose and polyfructose as described in Chapter IV.

Effect of Salivary Supernatant and Fluoride on Fructose Utilization, Decrease in pH and Formation of Polysaccharides in Sediment-Saliva Mixtures Incubated with 27.8 mM Fructose

In the experiments carried out to determine the effect of salivary supernatant and fluoride on fructose utilization, 4 salivary sediment mixtures (1.5 ml) were prepared in which the fructose concentration was

27.8 mM. Each mixture was incubated for 2 hours. At regular intervals, the pH was measured and 3 aliquots (25 µl) were removed from each mixture. Each aliquot was pipetted into a 10 x 75 mm test tube containing 0.6 ml of 1 per cent NaF previously chilled in cracked ice. After mixing the contents of each tube by vibrating on a vortex mixer, sediment and supernatant were separated by centrifuging at 1740g for 5 minutes at 4°C. The fructose content of the supernatant and the carbohydrate content of the sediment were determined as described in Chapter III.

RESULTS

Effects of Salivary Supernatant and Fluoride on the pH, Uptake of Sucrose and the Formation of Plaque Carbohydrate in Incubated Plaque Mixtures

The effects of salivary supernatant and fluoride on the pH, utilization of sucrose and accumulation of polysaccharide in the plaque pellet of mixtures incubated with 13.9 mM sucrose are shown in Figs. 6.1, 6.2 and 6.3. Salivary supernatant stimulated only a slight decrease in the pH, but the utilization of sucrose and the formation of plaque carbohydrate were greatly enhanced. Both in the presence and absence of salivary supernatant, fluoride inhibited all three. Maximum inhibition of all three parameters of sucrose metabolism occurred when fluoride was present and salivary supernatant was absent.

Fluoride inhibited the utilization of both of the glucose and fructose residues of the sucrose molecule, whereas, in the absence of fluo-

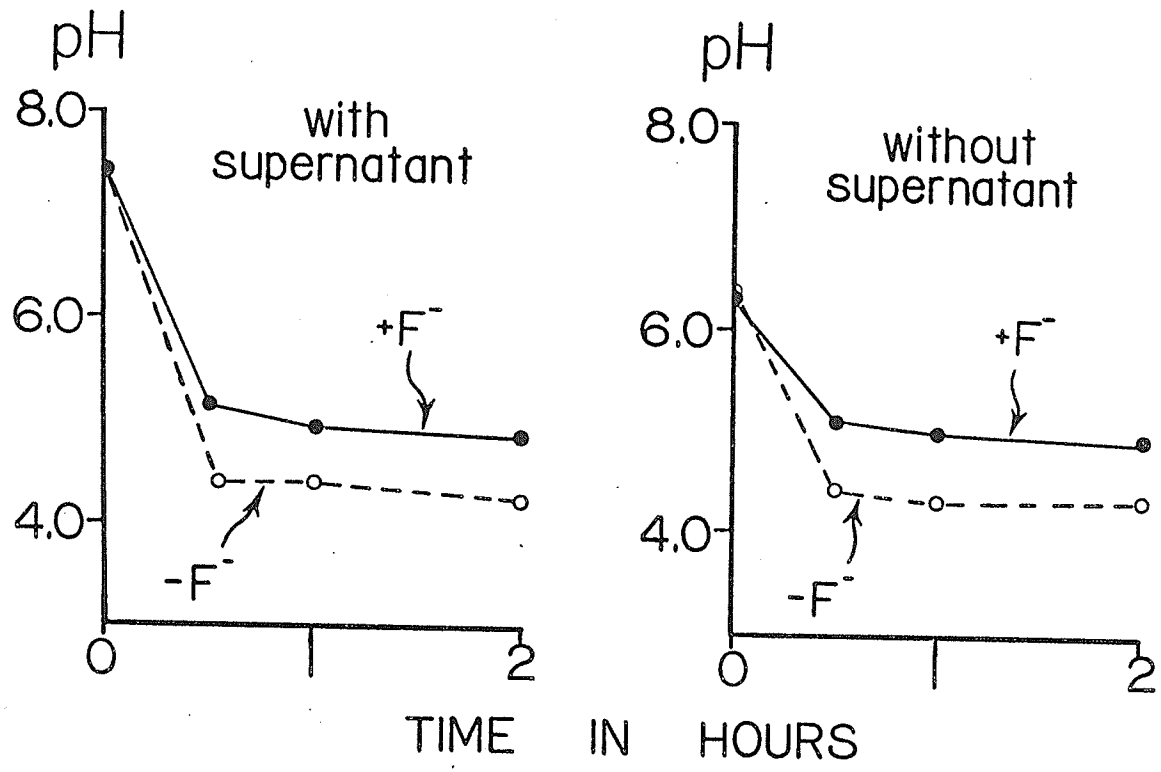

Figure 6.1. Effect of salivary supernatant and fluoride on the pH of plaque mixtures incubated with 13.9 mM sucrose.

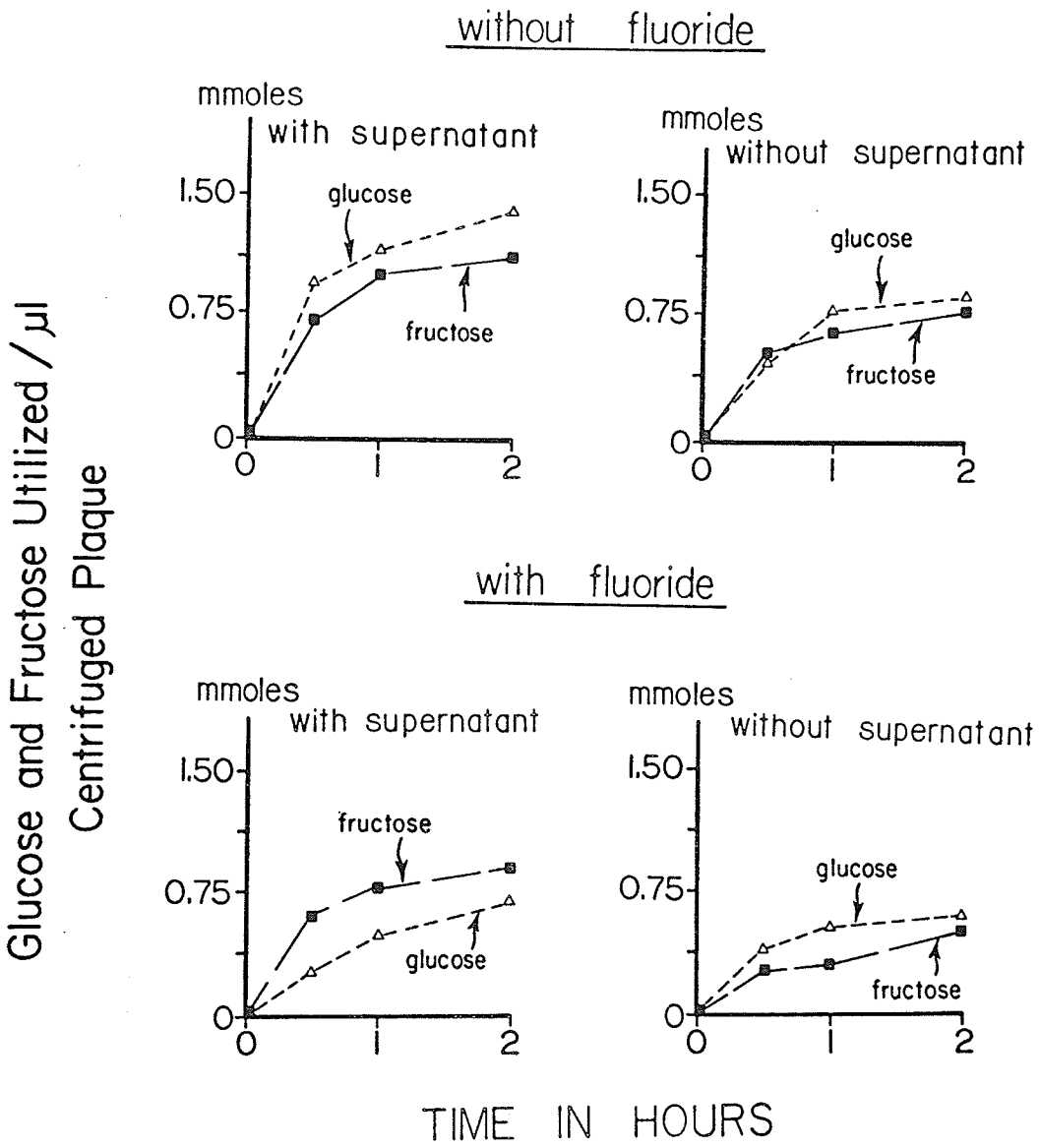

Figure 6.2. Effect of salivary supernatant and fluoride on the glucose and fructose residues utilized by centrifuged plaque in plaque mixtures incubated with 13.9 mM sucrose.

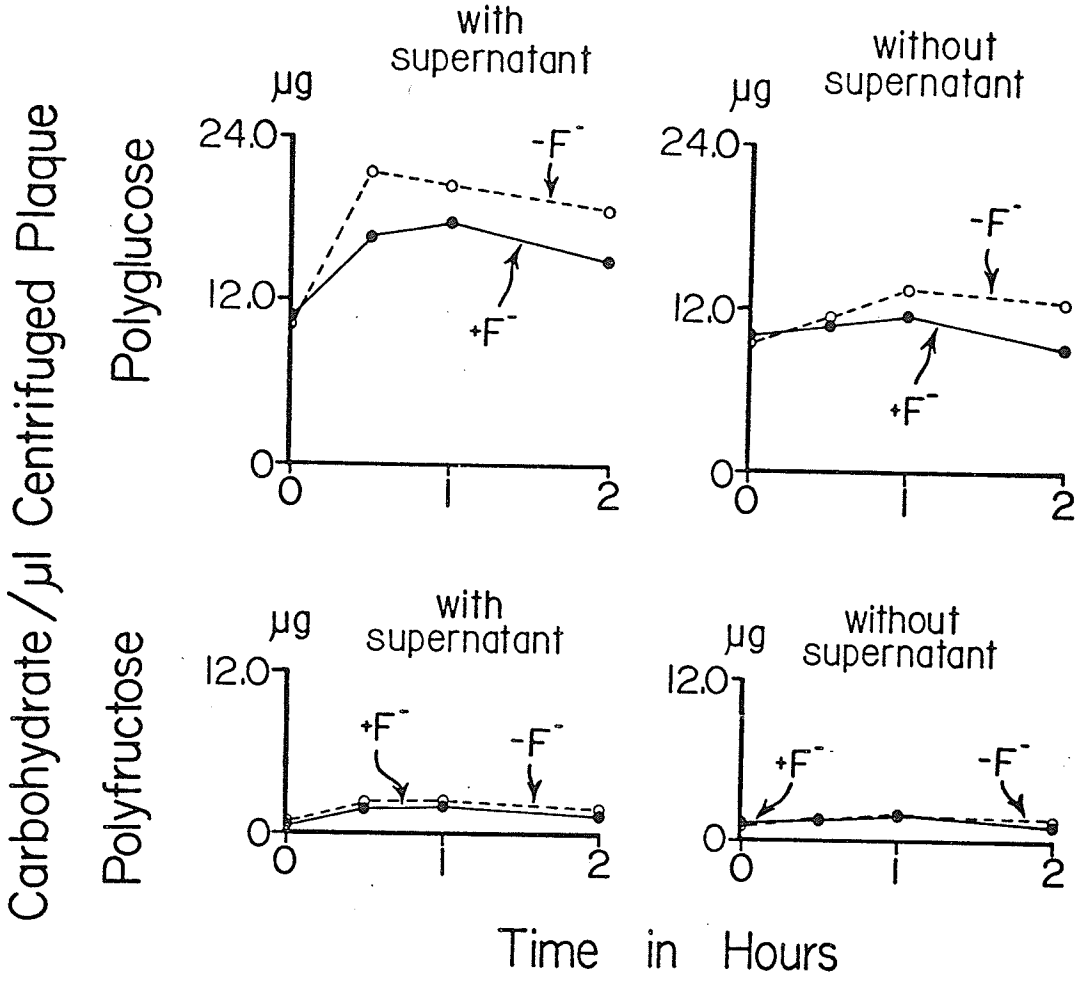

Figure 6.3. Effect of salivary supernatant and fluoride on the carbohydrate content of centrifuged plaque in plaque mixtures incubated with 13.9 mM sucrose.

ride, supernatant stimulated their utilization (Fig. 6.2).

The effects of salivary supernatant and fluoride on the formation of storage carbohydrate was mainly on plaque pellet polyglucose; plaque pellet polyfructose was hardly affected by either salivary supernatant or fluoride (Fig. 6.3).

When the sucrose concentration was 138.9 mM, the results were similar to those with 13.9 mM (Figs. 6.4 and 6.5), viz., salivary supernatant enhanced whereas fluoride inhibited the pH fall and the accumulation of carbohydrate. Also, the effects of fluoride and supernatant were mainly on the formation of plaque pellet polyglucose rather than on the formation of plaque pellet polyfructose (Fig. 6.5b).

As found earlier in salivary sediment mixtures (Chapter III), sucrose was hydrolyzed extracellularly, resulting in the appearance of its monomer constituents, glucose and fructose, in the medium (Fig. 6.6).

Effect of Salivary Supernatant and Fluoride on Acid Formation and Polysaccharide Formation in Plaque and Sediment Mixtures Incubated with Sucrose at pH 5.0 and 6.0

Both in plaque and sediment, less acid was formed at pH 5.0 than at pH 6.0 (Figs. 6.7 and 6.8) and much less in the absence than in the presence of salivary supernatant. The inhibition of acid formation by fluoride was evident more at pH 5.0 than at pH 6.0, particularly in the absence of supernatant.

The carbohydrate storage results for the sediment mixtures are shown

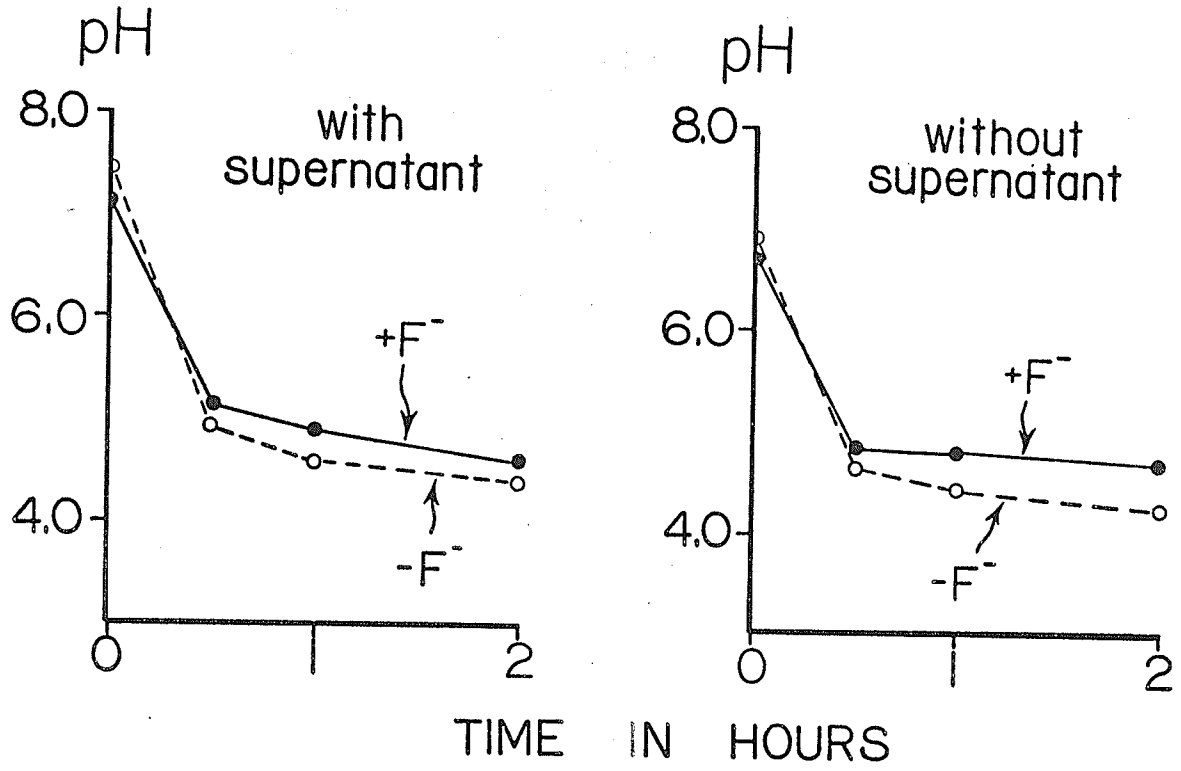

Figure 6.4. Effect of salivary supernatant and fluoride on the pH of plaque mixtures incubated with 138.9 mM sucrose.

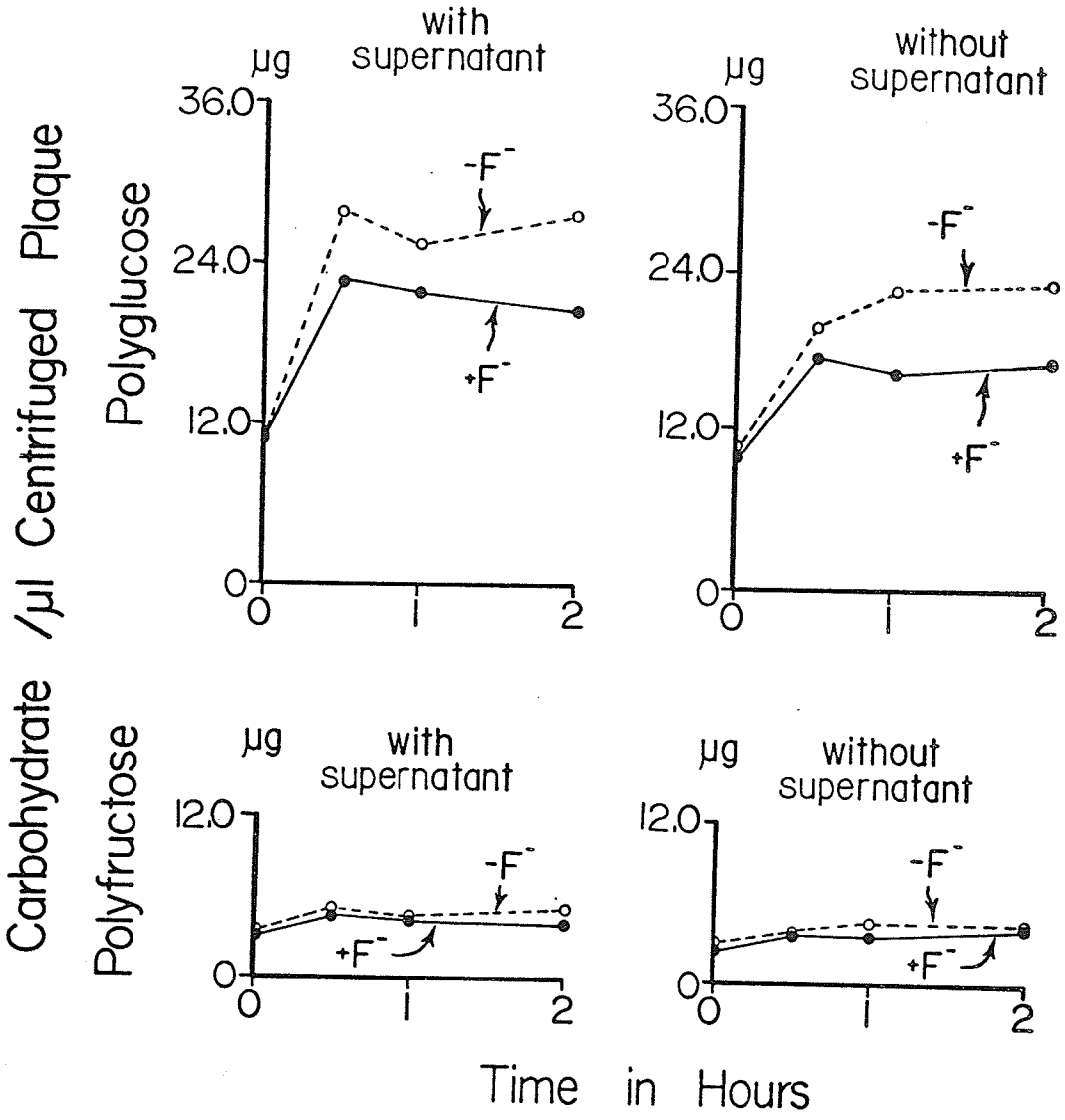

Figure 6.5. Effect of salivary supernatant and fluoride on the carbohydrate content of centrifuged plaque in plaque mixtures incubated with 138.9 mM sucrose.

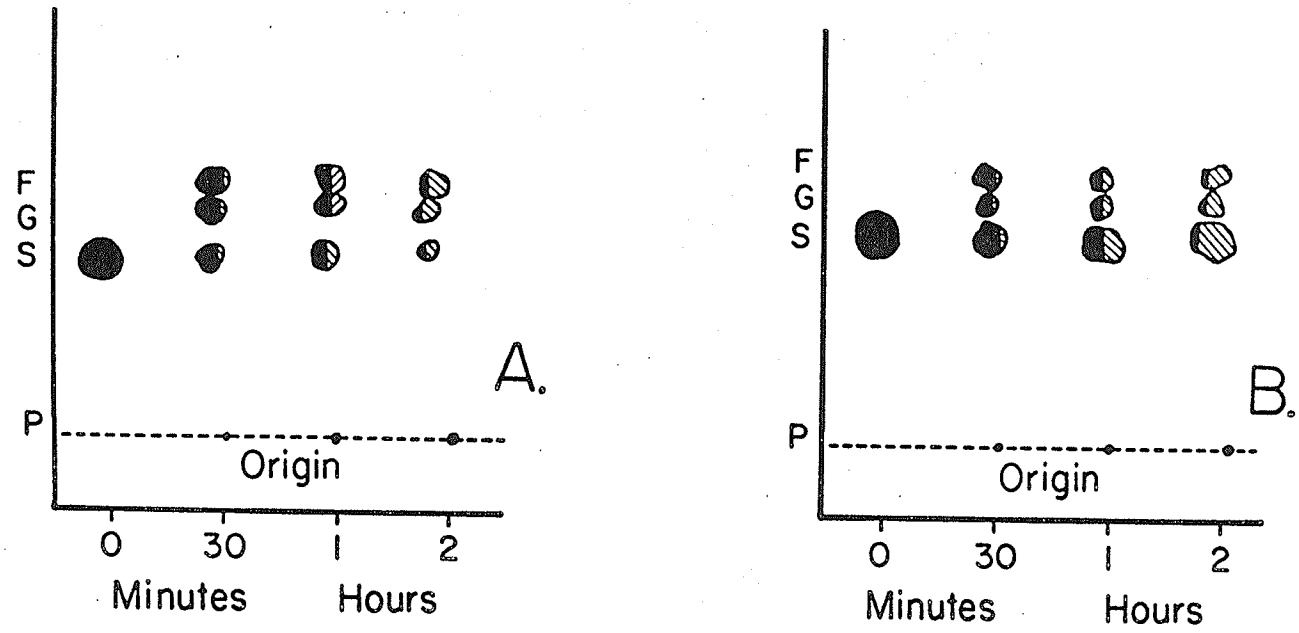

Figure 6.6. Copy of chromatograms obtained with aliquots removed from plaque saliva mixtures incubated with; (A) 13.9 mM, and; (B) 138.9 mM sucrose. F-fructose; G-glucose; S-sucrose; P-polysaccharide.

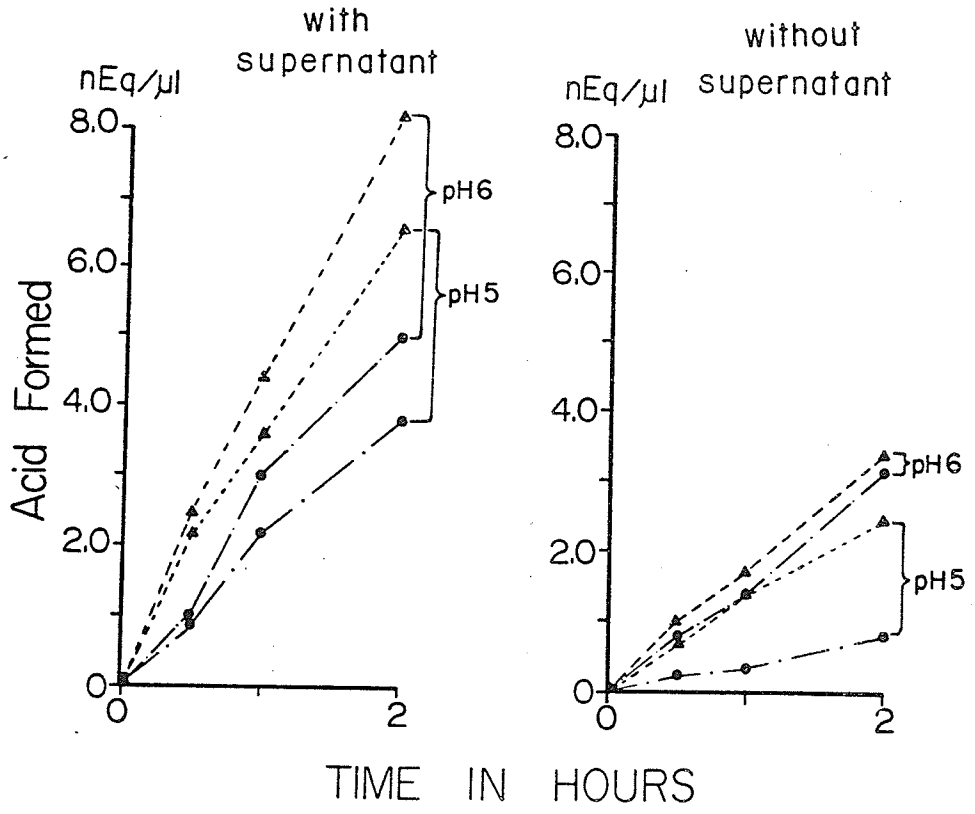

Figure 6.7. Effect of salivary supernatant and fluoride on acid formation in plaque mixtures incubated with 138.9 mM sucrose at pH 5.0 and pH 6.0. ● - +F; ▲ - -F. Concentrations are expressed in nequivalents per µl of plaque mixture.

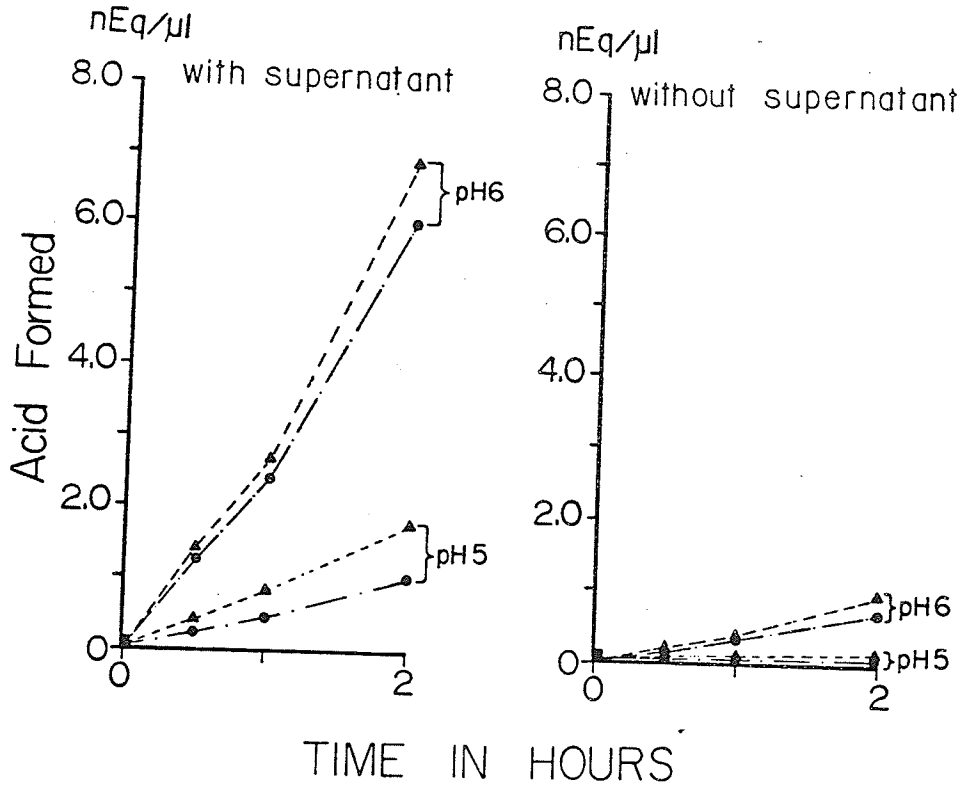

Figure 6.8. Effect of salivary supernatant and fluoride on acid formation in sediment mixtures incubated with 138.9 mM sucrose at pH 5.0 and pH 6.0. ● - +F; ▲ - -F. Concentrations are expressed in nequivalents per µl of sediment mixture.

in Fig. 6.9. The total amount of sediment polysaccharide formed (polyglucose plus polyfructose) was more at pH 6.0 than at pH 5.0. Formation was stimulated with supernatant and inhibited with fluoride. Fluoride inhibition was more at pH 6.0 than at pH 5.0.

Effect of Salivary Supernatant and Fluoride on the Formation of Extracellular Polysaccharide in Incubated Salivary Sediment Mixtures

The distribution of polysaccharide between the supernatant and KOH-sediment components of the extracellular polysaccharide formed in salivary sediment mixtures incubated with sucrose is shown in Fig. 6.10. The relative proportions of polyglucose and polyfructose in the supernatant and KOH-sediment fractions appeared to be about the same, with or without salivary supernatant or fluoride present. However, the amounts of glucose and fructose polymer in the two fractions was lower in the absence of salivary supernatant and higher in the presence of fluoride.

In each case and as found previously (Chapter IV) more polyfructose was observed in the supernatant than polyglucose, while more polyglucose than polyfructose was found in the sediment.

Effect of Salivary Supernatant on Fructose Uptake, pH and Polysaccharide Storage

The results for fructose are shown in Figs. 6.11, 6.12a and 6.12b. As was observed earlier for glucose (Sandham and Kleinberg, 1969b; Kleinberg and Craw, 1969), salivary supernatant enhanced the pH drop

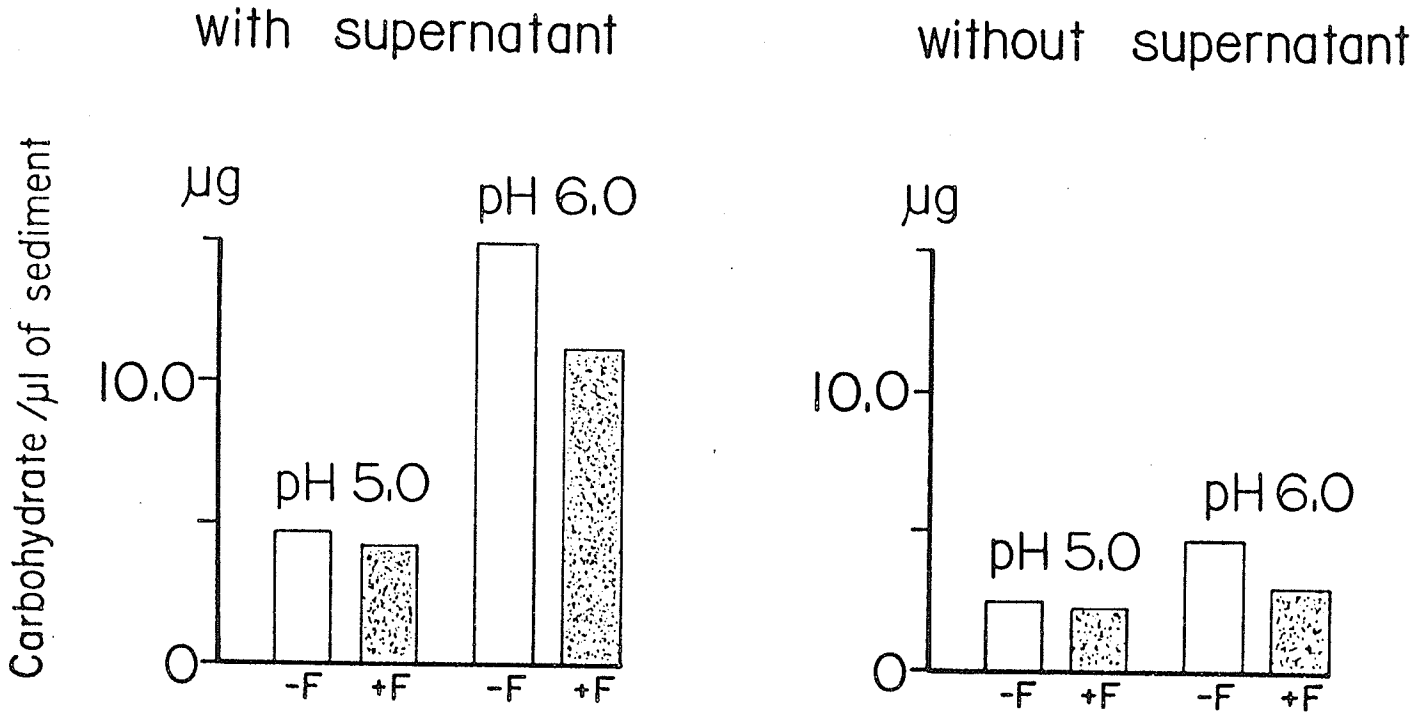

Figure 6.9. Effect of salivary supernatant and fluoride on the amount of carbohydrate synthesized by sediment in sediment mixtures incubated with 138.9 mM sucrose at pH 5.0 and pH 6.0.

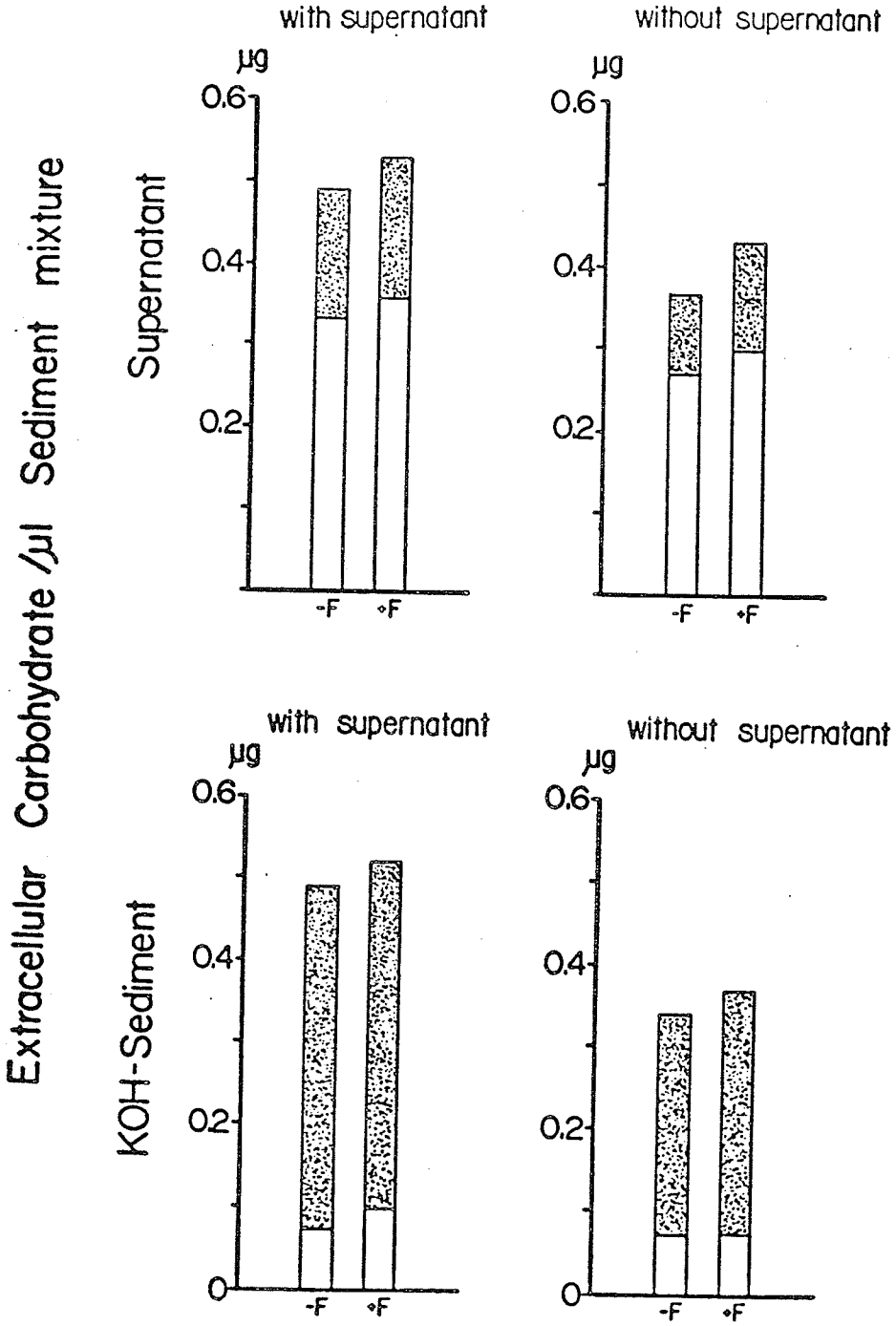

Figure 6.10. Effect of salivary supernatant and fluoride on extracellular polysaccharide formation in salivary sediment mixtures incubated with 138.9 mM sucrose. A-supernatant component; B-KOH-sediment component. ▨ - polyglucose; □- polyfructose.

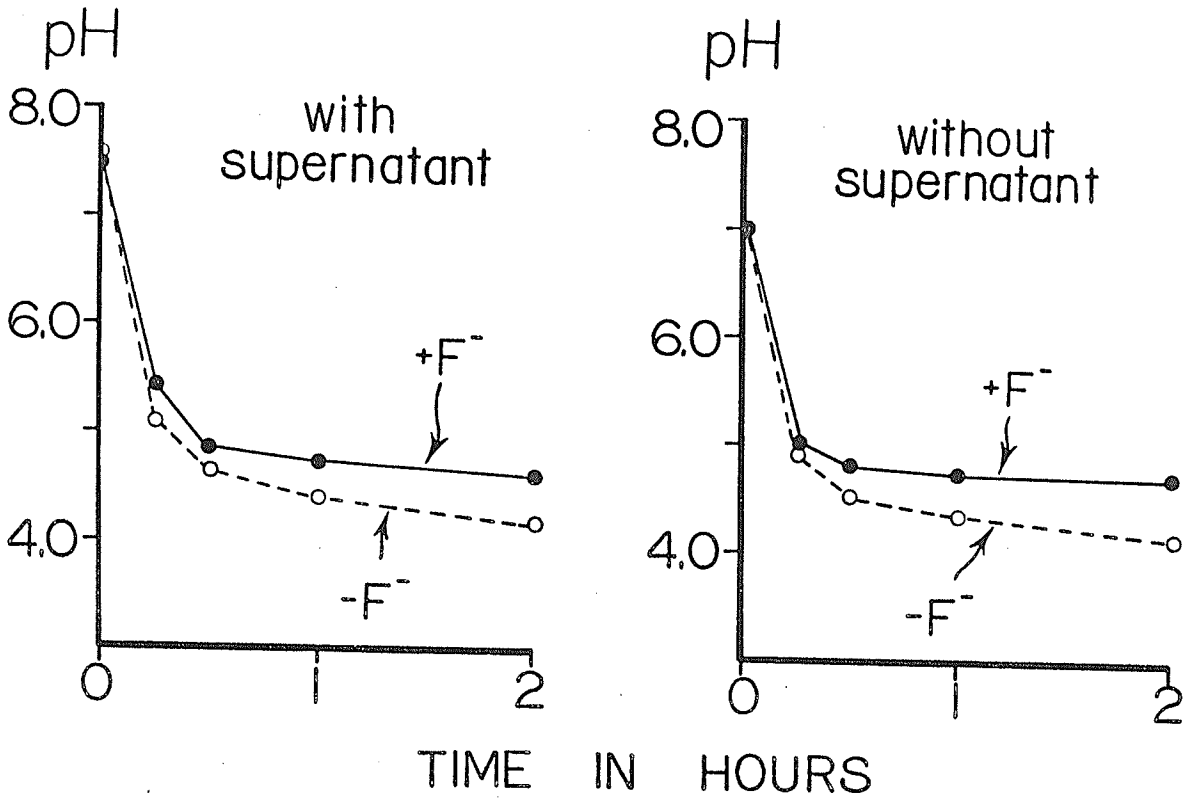

Figure 6.11. Effect of salivary supernatant and fluoride on the pH of salivary sediment mixtures incubated with 27.8 mM fructose.

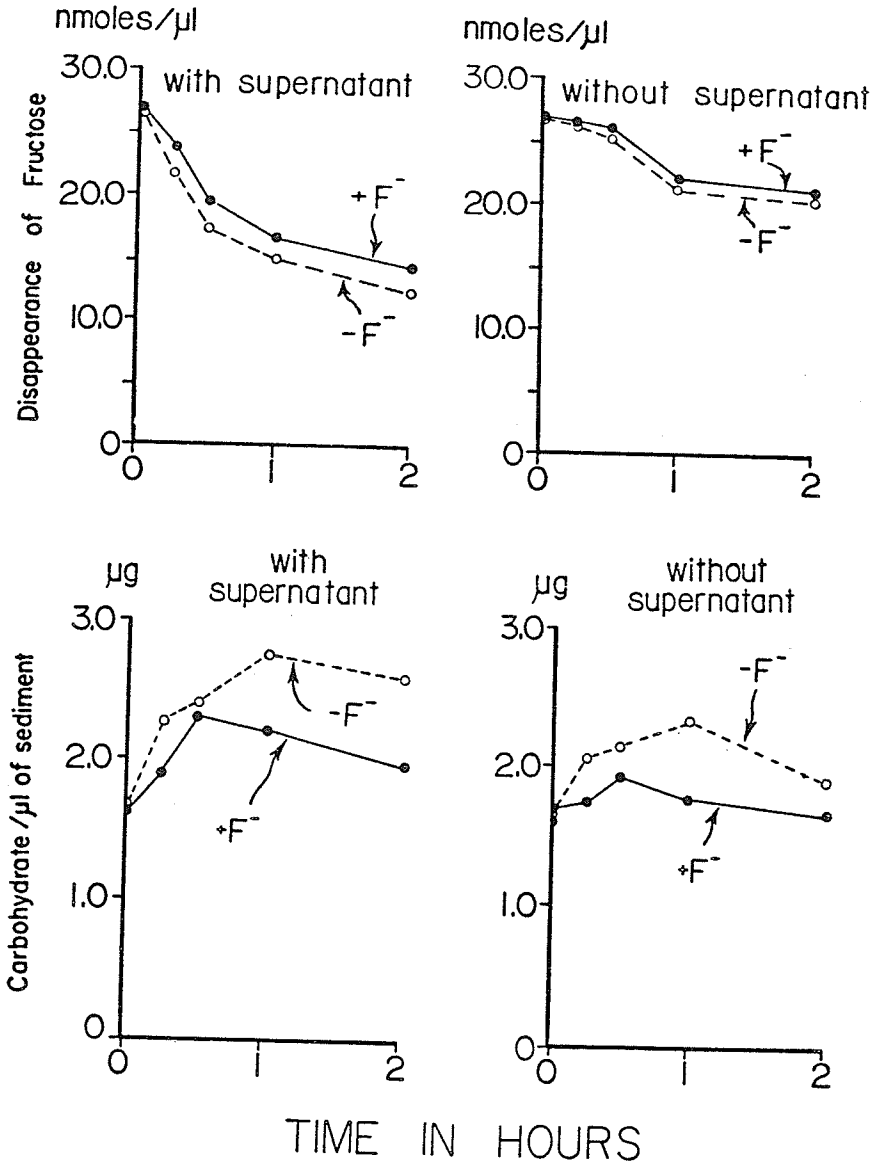

Figure 6.12. Effect of salivary supernatant and fluoride on (a) fructose disappearance and (b) carbohydrate formed in salivary sediment mixtures incubated with 27.8 mM fructose.

slightly, uptake of fructose and formation of sediment carbohydrate considerably. On the other hand, fluoride inhibited these processes.

DISCUSSION

As was shown earlier for glucose (Sandham and Kleinberg, 1969b; Kleinberg and Craw, 1969) and observed in the present study for fructose (Figs. 6.11 and 6.12), salivary supernatant stimulates whereas fluoride inhibits the catabolism of these sugars by the bacteria in salivary sediment. The present study also demonstrated that salivary supernatant and fluoride have the same effects on the catabolism of sucrose by the microbial flora found in dental plaque (Fig. 6.1-6.5). With all three sugars, supernatant stimulated whereas fluoride inhibited sugar utilization, acid formation and the formation of sediment or plaque carbohydrate. This similarity between sucrose on the one hand and glucose and fructose on the other can be attributed to salivary supernatant and fluoride primarily affecting the cellular uptake of glucose (Kleinberg and Craw, 1969) and fructose (Fig. 6.12a), the two monosaccharides produced upon hydrolysis of the sucrose molecule. The probability of this being so is enhanced by the observation made in the present study that like salivary sediment (Chapters III and IV), the bacteria in plaque rapidly hydrolyze sucrose to glucose and fructose (Fig. 6.6).

Salivary supernatant and fluoride also favoured extracellular polysaccharide formation from sucrose. Both enhanced formation of polyglucose

and polyfructose in both the supernatant and KOH-sediment fractions of the extracellular carbohydrate formed. Supernatant had a much greater stimulatory effect than fluoride.

The effect of salivary supernatant on extracellular polysaccharide formation was the same as that on total sediment or plaque polysaccharide but the effect of fluoride was opposite. A possible explanation for these observations is as follows.

From Fig. 6.13, synthesis of dextrans and levans from sucrose yields fructose and glucose as by-products; accumulation and removal of these monosaccharides should indirectly regulate the synthesis of dextrans and levans. Accordingly, supernatant by stimulating uptake of glucose and fructose by the sediment or plaque bacteria would increase dextran and levan synthesis as was observed in Fig. 6.10. Fluoride on the other hand by inhibiting the uptake of these sugars should have decreased extracellular polysaccharide synthesis rather than increasing it. One explanation for this discrepancy with fluoride is that fluoride inhibits the pH fall (Figs. 6.1, 6.4 and 6.11) and a higher pH is more favourable for the synthesis of extracellular polysaccharide (Chapter IV). If the magnitude of the latter effect be greater than the inhibitory effect of fluoride on uptake (and on synthesis; Fig. 6.10) then stimulation rather than inhibition would occur.

The inhibitory effect of glucose and fructose on extracellular polysaccharide formation has been demonstrated in several studies. For example, fructose depresses the activity of dextransucrase isolated from

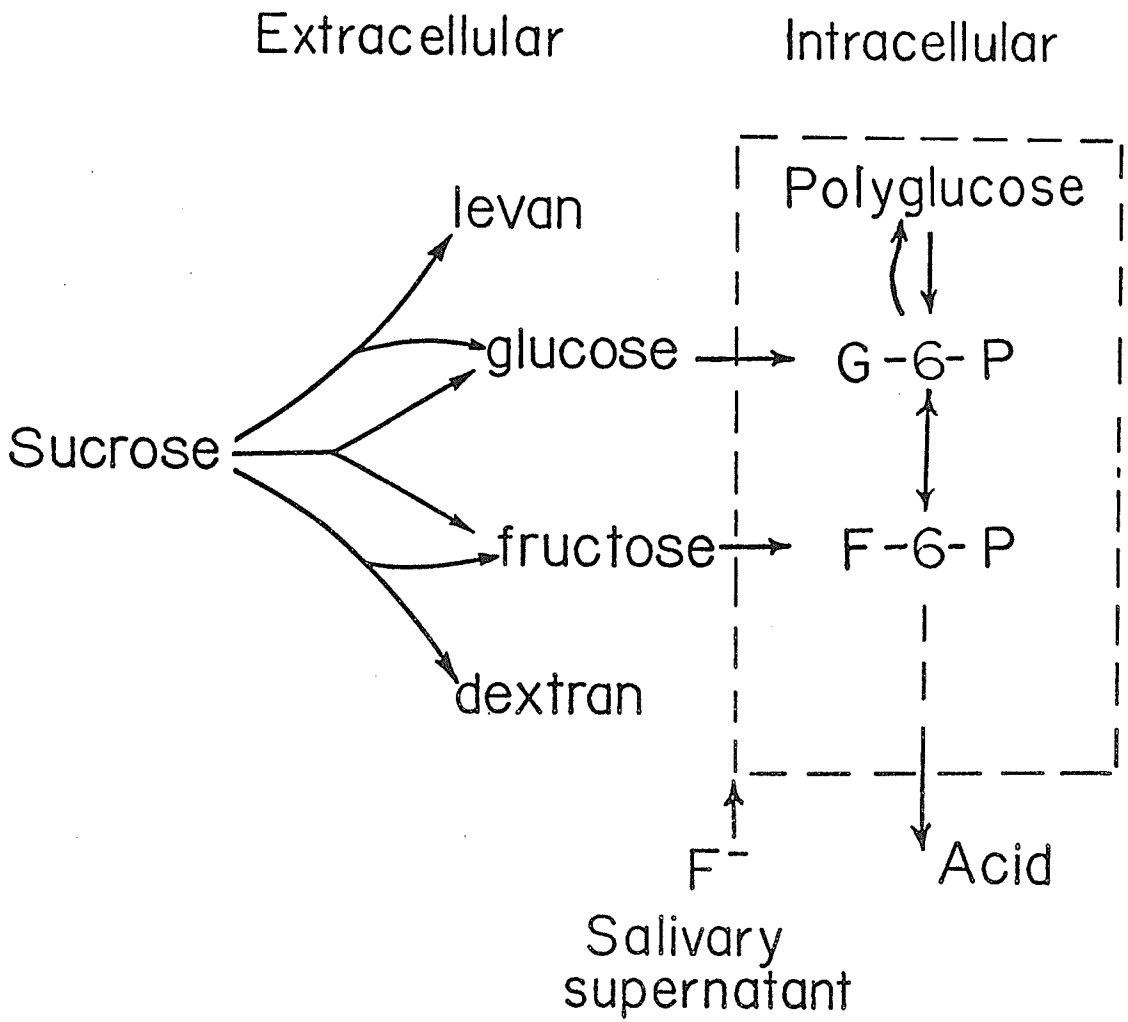

Figure 6.13. Schematic diagram to show the effect of salivary supernatant and fluoride on the relation between intra- and extracellular polysaccharide formation with sucrose.

Streptococcus bovis (Bailey, 1959) and Leuconostoc mesenteroides (Koepsell et al, 1953). A dextransucrase isolated from Streptococcus strain GS5 is inhibited by both fructose and glucose (Gibbons and Nygaard, 1968). A levansucrase from Aerobacter levanicum is inhibited by glucose (Hestrin and Shapiro, 1944). Both fructose and glucose added along with sucrose to a culture medium containing streptococcus strain GS5 inhibited the formation of extracellular polysaccharide (Gibbons and Banghart, 1967).

Effect of pH

The present study has shown that both in sediment and plaque, acid formation from sucrose was stimulated by salivary supernatant and inhibited by fluoride (Figs. 6.7 and 6.8). The stimulatory effect of salivary supernatant was less at pH 5.0 than at pH 6.0 (Figs. 6.7 and 6.8) confirming with sucrose and plaque what was observed earlier with glucose and sediment (Korayem and Kleinberg, 1970). However, greater inhibition of acid formation by fluoride at pH 5.0 than at pH 6.0 (Figs. 6.7 and 6.8) confirms the observations of Jenkins (1959) and others (Sandham and Kleinberg, 1969b; Weiss et al, 1965) that fluoride inhibition is more effective at acidic than at a higher pH. Any difference between sediment and plaque in the degree of inhibition of acid formation may be due to a difference in the buffering capacity of the two systems at acidic pH (Reddy and Kleinberg, 1971). Noteworthy is the observation that fluoride inhibited the formation of carbohydrate more at pH 6.0 than at pH 5.0, which is opposite to the effect of fluoride on acid formation at these

two pH levels. The reason for this at the present time is not readily apparent.

Site of Fluoride Action

A number of studies have shown that fluoride inhibits the uptake of glucose by the bacteria in plaque, sediment, saliva and pure cultures of Streptococcus salivarius and mitis (Sandham and Kleinberg, 1969b; Hamilton, 1969; Weiss et al, 1965). Fluoride also inhibits the uptake of lactic acid (Sandham and Kleinberg, 1971) and urea (Reddy and Kleinberg, 1971). In the present study, fluoride inhibited uptake of fructose by the bacteria in salivary sediment and the uptake of sucrose by the bacteria in both plaque and sediment. Táken as a whole, these effects of fluoride are consistent with the hypothesis that the site of fluoride inhibition is associated with membrane transport (Sandham and Kleinberg, 1969b).

In support of the effect of fluoride being on the uptake of glucose and fructose and not on the enzymes involved in the formation of these sugars (viz., dextransucrase, levansucrase and β-fructofuranosidase) are the observations that fluoride has no effect on the activity of; (i) the levansucrase isolated from Streptococcus mutans, a microorganism found in large numbers in dental plaque (Carlsson, 1970); (ii) the dextransucrases isolated from Streptococcus sanguis (Carlsson et al, 1969) and from Streptococcus strains, GS5 and LM7 (Gibbons and Nygaard, 1968), and; (iii) the β-fructofuranosidase isolated from Baker's Yeast (Hestrin et al,

1955). The observation that fluoride did not alter the distribution of extracellular polysaccharide between supernatant and KOH-sediment nor the proportion of polyglucose and polyfructose in these fractions can be viewed as support for fluoride primarily affecting glucose and fructose uptake.

The Relation of the Present Findings to Dental Caries

Because of the stimulatory effect of salivary supernatant on the glucose metabolism of salivary sediment, it was proposed earlier that the plaque found in regions of the dentition having better access to saliva would undergo a more vigorous carbohydrate metabolism than plaque in the more protected regions of the dentitión (Kleinberg, 1970a). The finding in the present study that salivary supernatant also stimulates the sucrose metabolism of plaque provides additional support for this hypothesis.

The results in the present study suggest that in areas of the dentition accessible to saliva, presence of saliva should stimulate the formation of dextrans and levans from ingested sucrose. However, dilution of the sucrose by the saliva should counteract this tendency since a high sucrose concentration is necessary to ensure that unhydrolyzed sucrose molecules are present for dextran and levan synthesis to occur (Chapters III and IV).

On the other hand, in the regions of the dentition poorly accessible to saliva, the stimulatory effect of saliva on extracellular polysaccharide

formation would be small or absent. As a consequence, synthesis of dextran and levan would proceed at a slow rate and would require that sucrose be available continuously as for example, if it were part of a sticky carbohydrate.

CHAPTER VII

SUMMARY AND CONCLUSIONS

Sucrose is the sugar found in greatest abundance in human diets and has been implicated both in human and animal studies as a causative agent in the initiation and progression of dental caries.

A review of the literature has disclosed that although numerous studies have been carried out to determine the properties of sucrose responsible for its cariogenicity, information on its metabolism particularly that by the mixed bacterial populations found in dental plaque and saliva, is fragmentary.

The studies reported in this thesis have examined those aspects of the metabolism of sucrose by the bacteria in salivary sediment and dental plaque likely to be applicable to the ability of the latter to form acid on the tooth surface and the development of the caries lesion.

Sucrose during its metabolism by the oral bacteria can be hydrolyzed into its constituent monosaccharides, glucose and fructose, or it can be synthesized into polymers of these sugars. In addition, the bacteria found in the human mouth can degrade sucrose or polymers formed from sucrose into acidic end products. To be able to follow these different processes with a single and relatively simple analytical procedure, the anthrone method for the estimation of carbohydrate was adapted to the estimation of glucose and fructose, either free as monomers or as residues of sucrose or polymers of glucose and fructose. This was possible not only for mixtures containing glucose and fructose, but also for mixtures

of their polymers without prior treatment.

A basic study was then carried out to compare the metabolism of sucrose by the microorganisms in salivary sediment to that of its constituent monosaccharides, glucose and fructose. The parameters examined were decrease in pH, sugar utilization and polysaccharide, carbon dioxide, lactic acid and hetero-acid formation.

Comparison was made at several sugar concentrations in mixtures incubated for four hours at 37°C and in which the concentration of salivary sediment was 16.7 per cent (V/V).

With all the sugars tested, a fall and rise in the pH occurred at low sugar concentration and a fall without the subsequent rise at high concentration. The pH curves were almost idéntical except for a slightly larger pH fall with low and a slightly smaller pH fall with higher levels of fructose.

The rates of utilization at each of the sugar concentrations examined were almost identical, except again, for fructose; fructose was utilized at a slightly slower rate. Paper chromatography demonstrated in this and in a later study with plaque that sucrose was rapidly hydrolyzed extracellularly to glucose and fructose and that complete hydrolysis occurs within the first few minutes of an experiment when the sucrose concentration is low.

The amount of carbohydrate stored by the bacteria in the sediment was much higher with sucrose than with any of the other sugars. Amongst the others, least storage occurred with fructose. The much greater

storage with sucrose was largely the result of sucrose being the main sugar from which extracellular polyglucose (dextran) and polyfructose (levan) could be synthesized. Most of the polyfructose formed was released into the medium, whereas most of the polyglucose formed remained with the sediment and could be extracted with cold KOH.

In another series of experiments in the same study, acid and CO_2 formation from the same sugars were determined. With all the sugars, formation of lactic acid, hetero-acid (acid other than lactic) and CO_2 were essentially the same. In all cases, when no sugar was supplied to the sediment system, no lactic acid and only a small amount of hetero-acid was formed. As the sugar concentration was increased, both lactic and hetero-acid correspondingly increased. In sediment mixtures containing low initial concentrations of sugar, the lactic acid concentration rose and fell. These lactic acid changes corresponded to the changes in sediment carbohydrate in that both reached their maximum values at the same time that the sugars disappeared from the medium, indicating that the formation of both lactic acid and sediment carbohydrate depended upon the presence of sugar in the medium. With higher sugar concentrations, the lactic acid curves did not show the rise and fall observed at lower sugar concentrations, instead their shapes were asymptomatic.

The major findings that came out of these experiments were; (i) that sucrose is rapidly hydrolyzed extracellularly and has to be present in high concentration for sucrose molecules to be present for any length

of time; (ii) acid formation was the same with all of the sugars; sucrose only differed in that it caused the formation of larger amounts of stored carbohydrate, and; (iii) sucrose was the only sugar that supported the formation of extracellular polyglucose and polyfructose.

The next study was restricted to sucrose and attention was focused on the effect of sucrose concentration and pH on the formation of extracellular polysaccharide. At low sucrose concentration, small amounts of polyglucose were formed and remained with the sediment fraction; on the other hand, only trace amounts of polyfructose were formed and this was released into the medium. As the sucrose concentration was increased, the polyglucose associated with the sediment and the polyfructose released into the medium, both increased. At very high concentration, some polyglucose appeared in the medium along with polyfructose, while some polyfructose appeared along with polyglucose in the sediment.

Alcohol fractionation of these polymers disclosed that the fructose polymers formed from sucrose were more soluble and homogenous than the glucose polymers, since nearly all of the former was precipitated at 70 per cent and not at lower alcohol concentrations. On the other hand, polyglucose was precipitated at several but mainly at lower alcohol concentrations, indicating that it was more heterogeneous and less soluble than the polyfructose.

In the experiments in which the effect of pH was examined, maximum formation of supernatant and sediment polysaccharide occurred around pH

6. A higher pH favoured more supernatant carbohydrate, while a lower pH favoured more sediment carbohydrate. More polyfructose than polyglucose was present in the supernatant at all levels of pH, whereas their relative amounts were reversed in the sediment. Regardless of the pH, polyfructose precipitated mainly at higher ethanol concentration (70 per cent), whereas polyglucose precipitated mainly at lower ethanol concentration (30 per cent).

It was evident from this study that; (i) synthesis increases with increase in sucrose concentration; (ii) that free and attached polysaccharide and the polyglucose and polyfructose they contain are dependent upon the sucrose concentration, and; (iii) synthesis and the types of polymers formed are also dependent upon the pH.

Previous studies with pure cultures suggested that bacteria cannot utilize their own extracellular polysaccharide. Studies particularly with cariogenic and non-cariogenic streptococci, isolated from dental plaque, have indicated that dextrans are metabolically inert while levans are not. Whether this was true for mixed populations was examined in the next study. Also examined was whether the presence of sucrose or such factors as salivary supernatant and pH might affect the degradation of extracellular polysaccharide.

Bacteria in salivary sediment utilized all of the several dextrans and levans tested; generally levans were more labile than dextrans. Acid formation from the various dextrans and levans were dependent upon their concentration and molecular structure. Interestingly, the pH curves

observed when these extracellular polysaccharides were added to sediment mixtures showed certain similarities to those seen with glucose and sucrose. First, the rate of fall in pH increased with increase in the dextran and levan concentration. Second, the fall and rise in the pH seen at low and not at high substrate concentration was also seen with levans and dextrans. Third, without salivary supernatant, the fall and rise did not occur; rather, the pH fell further without supernatant and did not show a rising phase.

It was also shown in this study that the ability of the bacteria in plaque to utilize dextrans was comparable to that of the bacteria in salivary sediment.

The extracellular polysaccharides synthesized in salivary sediment mixtures incubated with sucrose when isolated and supplied as substrate, were utilized by the bacteria in the same system. A most important observation was that sucrose inhibited utilization of both extracellular polyfructose and polyglucose. This inhibition was evidently an effect due to substrate, rather than an effect due to pH.

Clearly, the mixed flora of both sediment and plaque contain the enzymes necessary to catabolize a wide variety of levans and dextrans.

Previous studies had shown that metabolism of glucose by salivary sediment and dental plaque is regulated by salivary supernatant, fluoride and the pH. In the next study, the effect of these factors on the metabolism of sucrose was examined. As with glucose, salivary supernatant stimulated utilization of sucrose, formation of plaque carbohydrate and

at the sugar concentration tested, a slight decrease in the pH. Both in the presence and absence of salivary supernatant, fluoride inhibited the same three parameters of sucrose metabolism. Both in plaque and sediment mixtures incubated with sucrose, salivary supernatant stimulated while fluoride inhibited acid formation. Acid formation was less in both cases at pH 5.0 than at pH 6.0. Moreover, the stimulatory effect of salivary supernatant was greater at 6.0 than 5.0, whereas the inhibitory effect of fluoride was more at pH 5.0 than pH 6.0.

More sediment carbohydrate was formed in the presence than in the absence of supernatant and more formed at pH 6.0 than at pH 5.0. On the other hand, fluoride inhibited formation of sediment carbohydrate. Both the supernatant and sediment components of the extracellular carbohydrate decreased in the absence of supernatant and increased with fluoride whether supernatant was present or not.

Metabolism of fructose was affected in the same way by salivary supernatant and fluoride as the metabolisms of sucrose and glucose. This plus the fact that sucrose is hydrolyzed extracellularly, indicates that the effect of salivary supernatant and fluoride on sucrose metabolism is essentially through their effects on its glucose and fructose monomers.

In general, the studies in this thesis have clarified several aspects of the metabolism of sucrose by the oral microflora associated with the formation of glucose and fructose polymers and acid formation. Major factors regulating the metabolism of sucrose have been identified and related to the caries process. Additional similarities between the carbo-

hydrate metabolisms of the sediment and dental plaque microfloras have been demonstrated and provide additional support for salivary sediment being a suitable model for studying the metabolism of dental plaque.

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