CARBOHYDRATE STORAGE BY THE ORAL MICROFLORA IN VITRO

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CHAPTER I

INTRODUCTION

Many micro-organisms and cells, if not all, synthesize polysaccharides from extracellular sources of carbohydrate (Wilkinson, 1958). The formation and accumulation of polysaccharides, such as glycogen and starch, makes it possible for many types of cells to carry out their numerous metabolic processes in spite of wide variations in the availability of extracellular sources of carbohydrate (Lamanna and Mallette, 1959, p. 612).

Located on tooth surfaces in the mouth are films of bacteria usually referred to as dental plaque. These consist of numerous micro-organisms embedded in a matrix composed mostly of altered muco-substances (Winkler, 1958). Because the bacteria in these films have been implicated in both the caries and periodontal disease processes, numerous investigations have been carried out on their metabolism. Some of these studies have been on the plaque in situ; others have been on the plaque removed from the mouth; while others have been on saliva that has been collected by chewing paraffin and studied in vitro. Saliva collected in this way dislodges many organisms from the films covering the surfaces of the mouth, including those

covering the teeth (plaque), and also provides a medium similar to that found *in vivo*. When centrifuged, the organisms are concentrated into a pellet usually referred to as salivary sediment, which has been shown to behave in many ways like dental plaque. It is because of this similarity in behaviour that in this study salivary sediment was selected as the source of micro-organisms (see below).

Purpose of the Study

The purpose of the study was to (1) determine whether salivary sediment could accumulate cellular carbohydrate from glucose, and if so, how the level of the carbohydrate was related to the level of glucose in the medium; (2) determine if such a cellular storage carbohydrate could be utilized as a reserve nutrient by the micro-organisms in sediment; (3) determine if there is a relationship between changes in pH and changes in levels of carbohydrate in the medium and sediment of this system; (4) find out if low levels of fluoride have an effect on the accumulation of carbohydrate; and (5) determine whether sediment could store carbohydrate when lactic acid was used as a substrate.

Two additional purposes of a more general nature were involved, (1) to gain further information about the physiology of a natural population of micro-organisms

(comparatively few studies have been done on natural populations); and (2) to develop procedures, and obtain information, which would make it possible to study the same problems on dental plaque *in situ*.

Relation of the Study to Dental Caries

Most studies have indicated that caries occurs when sufficient acid is produced from carbohydrate by the plaque micro-organisms to drop plaque pH levels low enough to result in the dissolution of enamel. The availability of carbohydrate to the plaque bacteria is a major factor in determining both the degree and duration of the pH fall in plaque (Stephan, 1944).

The importance of the present study therefore lies primarily in the possible effect that carbohydrate storage might have in increasing carbohydrate availability to the oral micro-flora; thereby affecting the rate of progress of dental caries. The possible ways in which carbohydrate storage might increase dental caries are as follows:

Increasing the cariogenicity of carbohydrate foods rapidly cleared from the saliva. One of the theories regarding the cariogenicity of foods is that foods rapidly cleared from the mouth because of their physical properties and their ability to stimulate saliva flow are less likely

to cause caries (Lundquist, 1952). If short exposures of carbohydrates to micro-organisms enables them to store carbohydrate, these stores could enable the maintenance of low pHs for some time after the clearance of the foodstuff from the saliva. Thus rapidly-cleared foods may be more cariogenic than they are presently considered to be.

Selection of species of plaque micro-flora. Species of bacteria which are best able to store reserve carbohydrates would be more likely to have adequate energy sources during the periods between meals. They might also be more capable of surviving periods of low pH. Having these abilities would make them well fitted for survival in the dental plaque (Gibbons and Socransky, 1962). Therefore carbohydrate-storing micro-organisms would increase in number, resulting in a plaque capable of dropping to much lower pH levels for longer times than previously. This could then lead to the initiation and/or extension of dental caries lesions.

Lactic acid consumption. In the present investigation the possibility that lactic acid could be used as substrate was studied. This was done because it was felt that if the micro-flora of plaque can utilize lactic acid, this might provide a mechanism whereby the pH of the plaque could be

raised, with some protection against dental caries resulting (Stephan and Hemmens, 1947). Part of the utilized lactic acid could be converted to storage carbohydrate, as occurs with Escherichia coli (Palmstierna, 1956), and thus provide an additional method of removal of acid.

Effect of fluoride. The effect of fluoride was also studied because it was thought that if fluoride could affect this process then its well-recognized effect in reducing caries incidence might be at least partially explained.

Definitions of Terms Used

Several of the terms used in a special sense in the present study are defined as follows:

Salivary sediment. Salivary sediment was considered to be the sediment obtained from the centrifugation of paraffin-stimulated saliva for 15 minutes at 1740 x g. This would include nearly all of the micro-organisms in the original saliva since the remaining supernatant shows virtually no cellular activity.

Incubation mix (Culture). The terms "incubation mixture" and "culture" are used interchangeably. This is slightly different from the general usage of the term "culture" in that a culture usually implies growth from a

small inoculum. However, because total sediment nitrogen remained constant in the experiments reported in this study, it is unlikely that growth took place. This was probably because of the high cell concentrations used (16 2/3%, V/V).

Sediment carbohydrate. Sediment carbohydrate was interpreted as the total carbohydrate in sediment as measured by the anthrone method. Since glucose was used as a standard, the sediment carbohydrate concentration was expressed as micrograms of glucose. Because the carbohydrate estimated was not easily removed by washing with cold water, it would presumably include the intracellular, cell wall, capsular, and micro-capsular carbohydrates (Wilkinson, 1958), and would presumably include the less-soluble mucosubstances in saliva.

Reasons for Studying Salivary Sediment in a Glucose Medium

Dental plaque *in situ* is the ideal system for the study of the biochemical aspects of the possible role of the oral micro-flora in dental caries. Use of this system has been possible in some studies, but is limited because the small quantities of plaque available are not sufficient to enable the removal of a large number of samples for chemical analysis.

An alternative means of study is the isolation of

bacteria for growth in pure cultures. However, dental plaque is made up of a natural population of micro-organisms whose inter-relationships and inter-dependencies are lost when the organisms are isolated in a pure culture.

Also, study of the micro-organisms of dental plaque has often been done in a medium other than saliva, which is the medium present in the mouth. Separation of these organisms from saliva may induce changes in their metabolism which are not encountered *in vivo*. In addition, these changes in metabolism will probably cause the selection of those organisms best able to survive in the medium used, at the expense of those organisms not able to adapt to the new medium.

These problems are minimized by the *in vitro* use of salivary sediment with salivary supernatant. This system, however, is not without some disadvantages. Firstly, although the organisms comprise a natural population, the species may differ in some respects from those found in dental plaque. Secondly, because the system is a closed one, acids resulting from bacterial metabolism will be eliminated by further metabolism rather than by diffusion out of the system as is probably the case *in vivo*. However, using glucose as substrate, Kleinberg (1963a and 1963b) demonstrated that the changes in pH occurring *in vitro* in a culture

containing a high concentration of sediment, similar to the one used in the present study, closely resembled the changes occurring in plaque *in vivo*. It was therefore felt that the use of salivary sediment with salivary supernatant was the most promising method of predicting the behaviour of carbohydrate storage in plaque *in vivo*.

Organization of the Remainder of the Thesis

Chapter II will consider the contributions of previous investigators to (1) the knowledge of storage of carbohydrates by oral micro-organisms, and (2) a discussion of the factors that affect carbohydrate storage in micro-organisms in general which are pertinent to the present study. Chapter III will describe the methods used in the study; while some of the problems presented and experiments performed as the basis of the methods adopted will be covered in Chapter IV.

In Chapter V, the experimental work related to the effect of different concentrations of glucose on the carbohydrate storage by, and the pH changes in, cultures of salivary sediment will be reported and discussed. Chapter VI will report a similar study which determined the effect of low concentrations of fluoride on carbohydrate storage and pH changes at several glucose concentrations. Exper-

iments using lactic acid as substrate will be covered in Chapter VII. In Chapter VIII a summary of the findings of the study and the conclusions will be given.

CHAPTER II

LITERATURE RELATED TO CARBOHYDRATE STORAGE BY THE ORAL MICRO-ORGANISMS

Very few investigations have been carried out on the storage of carbohydrate by the oral micro-organisms. On the other hand, there have been a considerable number of investigations dealing with carbohydrate storage in micro-organisms not associated with the mouth and which are probably relevant to the present study. In the following resume, both oral and relevant non-oral studies have been reviewed, and their possible relationship to dental caries has been considered.

Relationship Between Acid Production From Exogenous Carbo-Hydrate and Dental Caries

Miller in 1890 presented his Chemico-Parasitic Theory which stated that carbohydrate food particles, when impacted in various sites in the mouth, could be decomposed by the oral bacteria to produce the acid which decalcified the teeth and started the caries process. It was later shown by Williams (1897) and Black (1898) that caries developed on those tooth surfaces which were covered with films or "plaques" of organisms. Stephan (1940) demonstrated that when carbohydrate was supplied to plaque the pH of this

plaque rapidly fell to a minimum and then slowly rose. The lowest pH (or pH minimum) to which the plaque fell was shown by Stephan (1944) to be inversely related to the caries incidence for a given individual. Stephan and Hemmens (1947) postulated that the rapid fall was the result of glycolysis being more rapid than the acid removal or "consumption" process whereas the pH rise was the result of the reverse. At the pH minimum the two were equal. In 1950, Stralfors proposed his Acid Production Diffusion Theory to explain why plaque could maintain a pH lower than saliva. Its main feature was that the plaque bacteria produced acid faster than it could diffuse into the adjacent saliva. The concepts of both Stephan and Hemmens and Stralfors were incorporated into a more general concept (Kleinberg, 1961) which also showed the indirect relationship between the carbohydrate concentration on the plaque surface and the plaque pH minimum.

Kleinberg and Jenkins (1963) postulated that the role of the dental plaque appears to be essentially that of an "environmental membrane" between tooth and saliva, in that the metabolism of the plaque bacteria has a marked effect on the pH of the plaque. Changes in pH would in turn affect the solubility of hydroxyapatite (the main calcium phosphate salt in teeth), which in turn would affect the net movement of calcium and phosphate into or out of the tooth.

The above, and numerous other studies, have been concerned with the acid produced by plaque from exogenous carbohydrate substrates. The use of endogenous materials by the plaque micro-organisms for acid production has received almost no attention to date. Indeed, endogenous metabolism in bacteriology in general has commanded little attention until the last decade (Lamanna, 1963). In the next section, the few studies that have been carried out having a relationship to the oral micro-organisms will be reviewed.

Carbohydrate Storage by the Oral Micro-organisms and its Relationship to Dental Caries

Gibbons and Socransky (1962) were the first to demonstrate that certain of the oral bacteria isolated from plaque could accumulate carbohydrate, and demonstrated that five strains of Streptococcus could use stored carbohydrates to produce acid. The stored carbohydrate was of the glycogen-amylopectin type. Since caries-active individuals were shown to contain a higher number of organisms in their plaque capable of storing this carbohydrate than caries-free individuals, they concluded that the plaques of the former would produce acid for longer periods of time after meals than the plaques of the latter. They postulated two possible effects of this on caries-active individuals, (1) more tooth substance

would dissolve, and (2) there would be a selective effect on the survival of the various bacterial species, in favor of acidophilic organisms.

Manly (1961) observed that salivary sediment caused a more prolonged pH drop after a short period of exposure to sucrose than after glucose. To explain this, he suggested from his data that the oral micro-organisms produced an extracellular levan-like material from the sucrose (cf. Osborne et al, 1951; Snyder, 1955). He postulated that this might act as a reserve source of substrate. His experiments did not rule out the possibility that polysaccharides of the glycogen-amylopectin type may have also been formed, with the glucose (from sucrose hydrolysis) supplying the necessary residues. Possibly the micro-organisms in the sediment hydrolyzed sucrose to its monosaccharide components (Sols and Fuente, 1960) and then used the glucose and any glycogen formed from it as its prime energy source while synthesizing the fructose into levan. Since Manly did not study changes in carbohydrate levels with time of incubation, the presence of levan may actually be support for its <u>not</u> prolonging the pH fall. However, this does not mean that when the glucose and any glycogen formed from it is reduced or gone, that the levan could not then become a source of energy.

Whether or not this natural population of micro-organisms