LACTIC ACID UTILIZATION BY STREPTOCOCCAL STRAIN AHT

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ABSTRACT

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Under conditions of a high cell concentration and a low glucose substrate level, lactic acid was formed and then degraded by Streptococcus AHT. The lactic acid was converted to acetic acid, ethanol and carbon dioxide.

Lactic acid utilization was greatly suppressed by anaerobic incubation indicating that oxygen was required for the process. The end products of glucose fermentation under anaerobic incubation were lactic, acetic, butyric, isobutyric, propionic acids, ethanol and carbon dioxide.

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CHAPTER I

INTRODUCTION

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INTRODUCTION

Present on most human tooth surfaces are concentrated masses of bacteria which, together with an inter-bacterial matrix, make up dental plaque. The bacteria in the plaque can readily convert many carbohydrates to acid, causing a rapid fall in the pH of the plaque. Subsequently the pH slowly rises again to fasting levels (Stephan, 1944; Kleinberg, 1961; Kleinberg and Jenkins, 1964). Dental caries is generally accepted to be initiated by dissolution of the inorganic components of the enamel of the tooth surface when the plaque pH drops below some critical level known as the "critical pH" (Englander et al, 1956). Below this pH the concentrations of calcium and phosphate in the plaque and in the saliva are thought to be insufficient to prevent the solubilization of the enamel.

A prominent portion of the plaque microflora consists of the Streptococci. Estimates of their number vary from 27 to 98 per cent of the total flora (Stralfors, 1950; Gibbons, 1964; McCarthy, 1965). It is generally accepted that only streptococci produce acid at a sufficient rate and occur in sufficient numbers in dental plaque to have any major importance in producing the pH fall which occurs in dental plaque in vivo (Bibby, Volker, and Van Kesteren, 1942; Sims, 1965). This suggests that studies of the carbohydrate metabolism of streptococci are important to provide a greater understanding of the dental caries process.

Investigations of carbohydrate metabolism in streptococci are usually carried out using low concentrations of cells and levels of

carbohydrate substrates that are too high to be completely utilized during the experimental period. Under such conditions of incubation, streptococci carry out a typical homolactic fermentation of glucose with only small amounts of products formed other than lactic acid (e.g., Hewitt, 1932; Tatum and Peterson, 1935).

In 1947, Stephan and Hemmens pointed out that these cultural conditions have only limited relevance to the situation occurring in the human oral cavity, where streptococci and other microorganisms exist as dense masses of cells, which are only intermittently supplied with carbohydrate. When these workers investigated the pH change produced *in vitro* when a high concentration of a pure strain of streptococcus isolated from a human mouth utilized a low level of glucose, they observed that the pH initially fell rapidly, but then reached a minimum and, surprisingly, subsequently rose by nearly one pH unit. When they incubated the cell suspension with an equivalent amount of lactic acid instead of glucose, the pH again rose. The extent of the pH rise was approximately equal to that occurring with glucose.

On the basis of these findings, Stephan and Hemmens suggested that the pH rise resulted from the consumption of lactic acid. However, their experiments did not include measurements of lactic acid, nor did they rule out the possibility that the pH rise might have resulted from other factors, such as from the formation of base by the breakdown of nitrogenous material, such as amino acids (Gale, 1946).

To the present, research into dental caries has been almost entirely directed at the falling portion of the "Stephan Curve" in dental plaque. Almost no attention has been paid to the rising portion of the

curve and to the fact that dental caries might be averted if the organisms on the teeth should cause a pH rise by a mechanism such as the consumption of lactic acid. If many streptococci are able to raise the plaque pH by utilizing lactic acid, they might contribute significantly to this process.

The object of the present study was to determine the correctness of Stephan and Hemmens' hypothesis that some streptococci, which are generally accepted to be homolactic, can utilize lactic acid and thereby cause a pH rise. Additional studies were undertaken to clarify the fate of the utilized glucose and lactic acid and to determine some of the conditions that might affect that process.

The main streptococcal strain studied in this thesis, AHT, was originally isolated from human dental caries lesions by Zinner and his co-workers (1964). It is of some interest that of numerous strains of oral streptococci they tested in hamsters, AHT had the greatest activity in producing caries. The last letters of the name of the organism, HT, signify the source of the organism, human teeth, while A is the order of isolation.

Most of the experiments reported in the thesis were carried out using a high cell concentration and a low initial glucose level, to duplicate as nearly as possible the situation occurring in the dental plaque *in vivo*.

Outline of the Remainder of the Thesis

Chapter II is a review of the literature about lactic acid production and subsequent degradation in both mixed cultures and pure cultures of microorganisms. Chapter III will describe the materials and

methods used in this study. In Chapter IV, the experimental work will be reported and the results discussed. A summary of the findings of the study and conclusions will be given in Chapter V.

CHAPTER II

LITERATURE REVIEW

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LITERATURE REVIEW

Oral microorganisms were first observed by Antony Van Leeuwenhoeck in 1683 (cited by Fitzgerald, 1963), and were postulated to have an important role in the initiation of dental caries by Miller in 1890, in his "Chemo-Parasitic Theory". The theory stated that the decalcification of teeth results from the action of acids produced from the breakdown of dietary carbohydrates by plaque bacteria.

Acid formation by the oral microflora was subsequently studied by Neuwirth and Klosterman (1940), who observed the rapid formation of lactic acid from carbohydrate by microorganisms in saliva, both *in vivo* and *in vitro*. They established that bacteria were the responsible agents in the saliva when they showed that saliva which had been passed through a Seitz filter failed to produce acid from glucose. Later, Neuwirth and Summerson (1942) found that lactic acid accounted for no more than 50 per cent of the total acid formed, and that lactic acid was rapidly metabolized by the bacteria in saliva with the production of other unidentified acids. Similar observations were made with plaque *in vitro* by Muntz (1943), who observed that lactic acid was formed and then degraded, while the concentration of other acids progressively rose. He demonstrated that the destruction of lactic acid occurred most rapidly at neutrality and under aerobic conditions. When plaque was incubated under anaerobic conditions, the lactic acid concentration did not fall.

Later, Neuwirth and Summerson (1951) observed that when paraffin-stimulated saliva was incubated with a low concentration of glucose,

lactic acid accumulated only as long as glucose was present in the medium. When the glucose was gone, the lactic acid began to disappear. On the basis of their experiments with saliva, they predicted that the rapid production of lactic acid in dental plaque would account for the rapid fall in pH previously reported by Stephan (1940).

The oxidation of lactic acid has been reported in many diverse types of microorganisms such as Escherichia coli (Haugarrd, 1959), Mycobacteria, (Cousins, 1956), Pseudomonas (Walker and Eagon, 1964) and yeast (Appleby and Morton, 1954). Lactobacilli have also been shown to utilize lactic acid; Peterson et al.(1920) found that Lactobacillus pentaceticus, a heterolactic fermenter, could stoichiometrically convert lactic acid to acetic acid.

More recently, even two homolactic fermentors, Lactobacillus delbrueckii and Lactobacillus arabinosus, were found to contain lactic oxidases (Hager, Geller and Lipman, 1954; Snoswell, 1959). These lactic oxidases were NAD-independent. Snoswell (1963) found that L. arabinosus contains two pairs of lactic dehydrogenases, $NAD^{\pm}$-dependent and $NAD^{\pm}$-independent pairs. The former pair of enzymes were involved with the production of D(-)- and L(+)- lactate from pyruvate while the latter pair were involved with the oxidation of D(-)- and L(+)- lactate back to pyruvate.

As reported in the previous chapter, the utilization of lactic acid in oral streptococci was first suggested by Stephan and Hemmens (1947) when they incubated a high concentration of a pure strain of an unidentified oral streptococcus with low initial concentration of glucose. They found that the pH in the medium fell rapidly and then rose slowly, and

postulated that the pH rise occurred because of the disappearance of lactic acid. Lamanna (1965, p. 698) had reported that Streptococcus lactis had been found to excrete lactate into the medium while glucose was being metabolized, and then utilized it after the glucose had disappeared from the medium. Evidence of lactic acid degradation by streptococci was also provided by London and Appleman (1961), who reported that Streptococcus faecium strain NCTC 7171 possessed a specific flavin-linked L(+)-lactic dehydrogenase which converted L(+)-lactic acid to acetic acid and carbon dioxide. This enzyme system was capable of oxidizing lactic acid when either oxygen or an appropriate dye was provided as an electron acceptor.

More recently, London (1968) investigated the regulation of the synthesis and function of the L(+)-lactic dehydrogenase. He found that the majority of the enzyme activity appeared during the last hour of exponential growth, when the glucose in the medium was nearly exhausted. This enzyme system enabled the organism to grow at the expense of L(+)-lactate and to survive in a medium devoid of carbohydrate substrate. Presumably, as postulated by Snoswell (1959) for L. arabinosus, the oxidation of lactate was coupled to ATP production, providing a source of energy when the primary energy source in the medium, glucose, was used up. In addition, the presence of an NAD^+-independent lactic dehydrogenase could conceivably enable the bacteria to regenerate pyruvate which could be used for synthetic purposes through its conversion to acetyl-CoA.

Since little is known about lactic acid degradation by bacteria in the oral cavity, the importance of such degradation to dental caries

is not well understood. It was because of this lack of information that the present investigation was carried out.

CHAPTER III

MATERIALS AND METHODS

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Cultures and Growth Media

The AHT streptococcal strain (Zinner, 1965) used in this study were obtained from Dr. J. Donohue of Colgate-Palmolive Company, New Jersey and from Dr. M. Goldner, Faculty of Dentistry, University of Toronto. Additional streptococcal strains, BHT, CHT, HHT (Zinner, 1965); E-49, FA-1, 2F2, GF-71, HS-10, 2M2 (Fitzgerald isolates); and PK-1 and GS-5 (Gibbons isolates) were also obtained from Dr. Donohue. Strains of streptococci of Lancefield groups A, C, D, E, F, G, H, and K were obtained from the Laboratory of Hygiene of the Department of National Health and Welfare in Ottawa.

All cultures were maintained at 0-4°C in trypticase agar (Jordan et al, 1960) having the following composition per litre: trypticase (Baltimore Biological Laboratory), 10 gm; yeast extract, 10 gm; K_2HPO_4, 10 gm; salt solution, 1 ml; sucrose, 10 gm; and agar, 10 gm. The salt solution consisted of $MgSO_4.7H_2O$, 0.8 gm; $FeCl_3.6H_2O$, 0.04 gm; and $MnCl_2$ 0.012 gm; in 100 ml of distilled water. Cultures were transferred every six weeks. All cultures except the Lancefield strains were also freeze-dried as soon as received.

Preparation of Incubation Mixtures

For all experiments in the investigation, cells were grown in trypticase soy broth (Baltimore Biological Laboratory, Baltimore, MD.) in an atmosphere of 95 per cent nitrogen and 5 per cent

carbon dioxide at 37°C for 18 hours, at which time they were in the exponential growth phase. They were harvested by centrifugation at 7710 x g for 20 minutes. The supernatant was carefully poured off and the pellet washed twice with approximately 10 ml of sterile cold trypticase medium, by centrifuging at 1470 x g for 20 minutes, and decanting the supernatant. Finally the cells were resuspended in sufficient sterile cold trypticase medium to give the desired final cell concentration, in most cases, 16.7% (V/V).

Incubation mixtures were prepared by combining the cell suspension and the substrate to make a final volume of 3 ml. The glucose used as substrate was introduced at zero time of each experiment. All preparation and handling of the incubation mixtures prior to incubation were carried out at 0-4°C in an ice-packed beaker to limit cellular metabolism.

Incubation Procedure and pH Measurement

All incubations were carried out at 37°C in 25 x 55 mm test tubes fitted with rubber stoppers with a hole through which a glass electrode was inserted. The mixtures were incubated in a Dubnoff metabolic shaking incubator (Precision Scientific Co., Chicago, Ill.) for four hours.

The pH of the incubation mixtures was measured at regular intervals using a model PHM 4c Radiometer pH meter. Samples for other chemical analyses were placed in 10 x 75 mm test tubes in ice to stop metabolism.

Chemical Analyses

Lactic acid analysis. For lactic acid analysis, 25 µl of incubation mixture was removed and delivered into 10 x 75 mm test tubes which contained

the volume of cold distilled water required to give the correct dilution for analysis. The mixtures were vibrated on a mixer (Vortex Jr.) and centrifuged at 1470 x g for 10 minutes at 4°C. Aliquots of 200 µl containing 0-1.5 mM lactic acid were removed from the supernatant and transferred to 10 x 75 mm test tubes. Each tube was capped with parafilm and the contents frozen until assayed for lactic acid by the method of Horn and Brun (1956), as modified by Cohen and Noel (1960). In this method, the enzyme lactic dehydrogenase is added, together with nicotinamide adenine dinucleotide (NAD) to the sample containing lactic acid. The enzyme catalyzes the oxidation of lactic acid to pyruvic acid by NAD. The $NADH_2$ formed was measured spectrophotometrically at 366 mµ (Fig. 1).

Total acid. To determine total acid, 25 µl of sample were removed and delivered into 200 µl of distilled water. The mixture was then titrated with 0.01 N NaOH using phenol blue (pK 8.9) as indicator. Titration values obtained for the mixtures at zero hour were subtracted from the values for each time point to give the value of the total acid produced.

Glucose analysis. For glucose analysis, 100 µl of culture was removed and delivered into 10 x 75 mm test tubes which contained 900 µl of distilled water. The mixtures were vibrated on a mixer (Vortex Jr.) and incubated for 30 minutes at 37°C. Aliquots of 200 µl containing 0-300 µg/ml glucose were transferred to 10 x 75 mm test tubes and the contents assayed for glucose using the "Glucostat" reagent (Worthington Biochemical Corp., Freehold, N.J.). The assay was measured spectrophotometrically at 500 mµ (Fig. 2).