THE EFFECT OF OSMOTIC SHOCK ON THE RELEASE OF ACID PHOSPHATASE ACTIVITY FROM *STREPTOCOCCUS MUTANS*

by

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ABSTRACT

The "osmotic shock" treatment of bacterial cells has proven to be an effective procedure for releasing hydrolytic enzymes located in the periplasmic compartment of the cell. It was the object of the present study to examine the mechanism of the "osmotic shock" procedure on the *Streptococcus mutans* strain PR-89 and the measurement of the acid phosphatase activity of the shocked cells. This enzyme could be a primary etiological agent in dental caries formation, and a simple method of releasing the enzyme would greatly facilitate the characterization of its properties.

During the course of the study several important characteristics of the enzyme were observed. First, the enzyme activity increases linearly with the growth of the bacteria. Secondly, in the presence of inorganic phosphate the enzyme is observedly repressed. Finally, during the period of bacterial growth in minimal media supplemented with various concentrations of phosphate, the nature of the enzyme is constituitive.

The "osmotic shock" procedure allowed a limited examination of the properties of the acid phosphatase enzyme produced by the *Streptococcus mutans*. However, the enzyme activity was not successfully separated from the bacterial cell to prove that it had been released.

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Finally, I would simply like to offer my sincere thanks to all those who have participated in the Oral Science program this year, as they have all contributed to making this a most enjoyable educational experience for me.

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I. INTRODUCTION

The oral bacteria found in the dental plaque are essential for the formation of carious lesions. This fact influenced early researchers to investigate bacterial products, attempting to isolate the specific factors responsible for dental caries. Recognizing the ability of acids to dissolve minerals, the researcher's emphasis has focused on the bacterial acids, particularly lactic acid, as the potentially dominant etiological agent in dental caries. Even though the potential of acids has been under extensive study for many years, nevertheless the evidence is far from conclusive that they are primarily responsible for the cariogenicity of certain oral bacteria. This simplistic hypothesis is contradicted by the non-cariogenic status of many acid-producing bacteria located in the plaque.

An alternative mechanism to explain carious lesions proposes the existence of a bacterially produced, enzymatically active protein capable of accelerating the release of mineral from the tooth enamel. The attractive nature of this concept becomes clear after analysis of the enamel structure reveals it contains phosphorylated proteins which enable calcium ions to bind to specific proteins at specific sites. This is a structure that should be susceptible to the action of a "phosphoprotein phosphatase".

Stephen Kreitzman, in his doctoral dissertation (1969), demonstrated the similarity in subsurface destruction of tooth enamel found in enzymatically induced caries to those of natural caries.

The investigation of the properties of the enzyme would be greatly facilitated if a relatively simple method of isolation could be developed. In the present study the "osmotic shock" method was explored as a potentially effective means to liberate the enzyme under investigation for future isolation and characterization.

II. LITERATURE SURVEY

A. Etiology of Dental Caries

Dental caries has been defined as "a localized, posteruptive, pathological process of external origin involving softening of the hard tissue and proceeding to the formation of a cavity" (Report of the WHO Scientific Group, 1970). The ability of teeth to resist the onset and progress of caries may reside in the intrinsic structure and composition of the tooth, in the oral environment, and in systemic factors. Clearly, the causative factors in dental caries are analogous to the basic factors proposed in the modern theory of infectious diseases, which include the interaction of host, environment, and agent.

The generally accepted theory of dental caries etiology today, the acidogenic theory, dates from the work of W.D. Miller in the nineteenth century. Miller (1890) observed that carious lesions were primarily the result of the action of bacterial acids which were produced at the tooth surface after the ingestion of carbohydrate diets.

Since the work of Miller, the acid theory has been under exhaustive study; during this research, many problems have been uncovered that prevent the complete acceptance of the acid theory as an explanation of the

etiology of the disease. Manly and Bibby (1949) studied the effects of substances that would inhibit the glycolytic pathway in bacteria. They found that there is no correlation between the production of acid and the ability of the bacterial strain to produce carious lesions in the germ-free animal. In addition, the ability of a strain of cariogenic bacteria to produce acid is not attenuated by being subjected to repeated subculture, although the cariogenicity is lost by repeated subculture in artificial media (Gibbons, 1968). Finally, many investigators have found the strongest acid formation in caries resistant plaques (Eggars-Lura, 1958; Manly, 1962; Rosen and Weisenstein, 1963; Gibbons, 1964).

Both the proteolytic theory and the chelation theory of the etiology of dental caries have also received a great deal of attention from researchers. Fleischmann (1914) first proposed the proteolytic theory when he stated that microorganisms attacked the organic constituents on the surface of the enamel destroying the organic matrix. The microorganisms produce acid which can destroy the mineral phase. Schatz (1951) proposed that proteolytic bacteria attacked the protein matrix of the enamel, releasing from it various breakdown products which then formed chelation compounds with the mineral matter of the enamel. Thus, both organic and mineral phases of the enamel would be destroyed simultaneously. The

controversy that exists in the literature suggests that the etiology of dental caries still calls for further investigation.

The studies of Orland (1955) firmly established that plaque bacteria are required for dental caries to occur. This evidence allowed laboratories with gnotobiotic facilities to infect germ-free animals and identify specific strains of microorganisms that can induce caries in sterile animals. Strains of streptococci and lactobacilli have been shown to be cariogenic in mono-infected animals (Zinner and Jablon, 1968; Krasse and Carlsson, 1970; Littleton *et al.*, 1970). In particular, *Streptococcus mutans* was one of the causative agents in smooth surface caries; it was associated with the earliest detectable lesions, and its absence was noticeable on intact tooth surfaces in man (Shklar *et al.*, 1972; Hoerman *et al.*, 1972).

Kreitzman (1969) tested four strains of streptococci that had been previously characterized with respect to their caries promoting potential. The results showed a correlation between the relative caries potential of the strain and the production of phosphatase by the strain. Because matrices of enamel, dentin, and cementum are phosphoproteins, a phosphatase that removes phosphate residues from the matrix phosphoprotein could cause a large loss of mineral from the tooth structure (Yamauchi *et al.*, 1967). Kreitzman has demonstrated in his doctoral thesis

that phosphoprotein phosphatase could well be an important factor in the process of dental caries.

B. Enamel Composition

X-ray crystallography reveals enamel mineral to be crystalline with a lattice structure characteristic of hydroxyapatite. However, the living tooth is more than hydroxyapatite minerals. In the formation of dental caries, the protein matrix must be removed as well as the mineral. It is possible that, for the etiology of dental caries, the matrix is more important than the mineral. Chemical analysis reveals that enamel is about 96% mineral and 4% organic matter and water (Orban's Oral Histology and Embryology). Brudevold (1967) states that the chemistry of the mineral phase is governed by the physical-chemical properties of the apatite crystals and by the water that is located in the intercrystalline spaces which serves as a vehicle for diffusion.

There is evidence that the surface enamel differs from the subsurface enamel. Newbrun (1959) showed that the surface enamel is harder than the subsurface enamel. A greater concentration of mineral at the surface of enamel than in subsurface enamel has been reported (Soni, 1959; Angmar, 1963). Furthermore, the organic material is unevenly distributed in the enamel (Brudevold, 1967).

The protein matrix of enamel consists of an acid insoluble phase and an acid soluble component. Glimcher (1964) identified peptides in the enamel containing serine phosphate. These proteins containing serine phosphate belong to the class of proteins known as phosphoproteins. The ability to bind calcium to specific proteins at specific sites is a property of phosphoproteins.

It is established that enzymes exist that are capable of adding or removing phosphate from fully formed proteins (Judah *et al.*, 1962; Jackson *et al.*, 1965; Kreitzman, 1969).

Therefore, the presence of phosphoproteins in the matrix of mineralized tissues indicates that there ought to be experimentation with these enzymes on the mineralized tissues to determine whether phosphoprotein phosphatases are able to remove the phosphate from the proteins after they have been fully mineralized.

C. Phosphatases

The class of enzymes known as phosphatases was first reported by Suzuki *et al.* in 1907, when he discovered an enzyme in rice and wheat bran capable of decomposing phytin. Grosser and Husler (1912) found that an enzyme that would hydrolyze glycerophosphate was widely distributed in animal organs with the kidney and intestine having the highest activity. Plimmer (1913) tested extracts of intestine, kidney and lung tissue, and found they could

successfully hydrolyze a variety of phosphoric esters.

Enzymes with phosphatase-like activity are reportedly present in the carious lesions (Makinen *et al.*, 1969). Since serine and threonine phosphates are subject to enzyme hydrolysis, the polymer may also be dephosphorylated resulting in the destruction of the protein matrix of the enamel. As the protein matrix of the enamel contains these phosphate esters, it is reasonable to assume that the activity observed in the carious lesions at an acid pH is that of an acid phosphatase.

The phosphatase enzyme has been extracted from numerous microorganisms and characterized, and it was shown to exhibit a broad range of substrate specificity (Simpson and Vallee, 1969; Takeda and Tsugita, 1969; Knuuttila *et al.*, 1972). Knuuttila and coworkers have successfully isolated a magnesium-dependent phosphatase from a cariogenic strain of *Streptococcus mutans* using ion exchange chromatography and isoelectric focusing as the primary techniques. In this case, solubilization experiments supported the hypothesis of a membrane associated enzyme.

The accessibility to substrates in the intact cell of the enzyme indicates that the phosphatase may be located in the periplasmic space (from the present study). The location of acid phosphatase and other hydrolytic enzymes have been associated with this space in bacteria by histochemical procedures as well (Arnold, 1972).

Furthermore, a hypothesis that degradative enzymes are separated from the part of the cell where synthetic processes occur, has been proposed by Heppel (1967).

In summary, the presence of the enzyme in cariogenic streptococci and its association with the caries potential of bacterial strains strongly suggests that it is related to the etiology of dental caries.

D. Osmotic Shock

The mechanical strength of the bacterial cell wall and the ability to withstand the physico-chemical effects of various natural and synthetic agents are important factors in the survival of bacteria. Heppel (1967) utilized an "osmotic shock" procedure to release proteins from the periplasmic space of a gram-negative bacteria without impairing their viability. These proteins are not extracellular enzymes as they are not excreted into the medium during any phase of the growth cycle of the bacteria.

The mechanism of "osmotic shock" has not been adequately defined, but the key to the process appears to be in the structure of the cell wall. Schwencke *et al.* (1971) employed electron microscopic techniques and was unable to detect a visual difference between shocked and unshocked cells. It is known that the cell wall acts as a sieve to allow molecules to diffuse through the aqueous channels that are an integral part of the structure of the

cell wall (Scherrer and Gerhardt, 1971). The openings exist not as uniformly sized perforations, but as tortuous, heterogeneously sized pathways through a 3-dimensional fenestrated matrix (Gerhardt and Judge, 1964). The relative geometries are not fixed properties (Trevithick *et al.*, 1966) and the sieving properties are complex relationships governed mainly by the relative sizes and shapes of both the openings and the diffusable molecules. It has been demonstrated that bacterial walls flex and change volume in response to a changed ionic environment, a process possibly accompanied by dilation and contraction of the openings in the matrix (Marquis, 1968).

The method of "osmotic shock" appears to make the enzymes of the periplasmic space available to the substrate by a complex mechanism. The enzymes become available to the external environment by the combined action of expansion of the protoplasmic membrane and dilation of the pores in the cell wall. If the "osmotic shock" treatment can be shown to release acid phosphatase from the streptococcal cell, it would prove to be a very simple procedure for extracting this potentially important factor in dental caries.

E. Summary

There is general agreement among oral scientists that dental caries result from an external attack on the tooth surface by microorganisms. A known cariogenic microorganism *Streptococcus mutans*, has been shown to possess acid phosphatase activity that may be responsible for the dephosphorylation of phosphoproteins in the organic matrix of enamel. Therefore, this enzyme deserves special attention as a potential causative factor in the etiology of dental caries.

The streptococcal cell wall is quite difficult to rupture by standard physical and chemical techniques, and the release of the enzyme in an active state is enhanced by the delicacy of the releasing procedure. The method of "osmotic shock" has proven to be a subtle means of exposing proteins commonly located in the periplasmic space of bacteria and was used on the cariogenic strain PR-89.

The association of phosphatase activity to the caries potential of a cariogenic bacterial strain strongly suggests the importance of studying the enzyme in its relation to the problem of dental caries. The isolation of this enzyme is crucial for a meaningful evaluation of the properties and "osmotic shock" may play the role of the first primary step in accomplishing this goal.

III. MATERIALS AND METHODS

A. Bacteriological Methods

1. Cariogenic Organism

Streptococcus mutans strain PR-89, a bacterial strain of known caries potency, was obtained from Dr. George E. White. The original source of this strain of bacteria was Dr. Robert Fitzgerald (V.A. Hospital, Miami, Florida).

2. Culture Media

The bacteria were cultured in either 3% Trypticase Soy Broth (Baltimore Biological Laboratories, Division of Bioquest, Cockeysville, Md.) or in Carlsson's Minimal Medium (Carlsson, 1970; Table I). Modifications of the media will be specifically noted where they occur. The flasks were placed in a Gas Pak Anaerobic System with a Disposable Gas Generator Envelope (Baltimore Biological Laboratories, Division of Bioquest, Cockeysville, Md.) and incubated, unshaken at 37°C.

B. Enzyme Assay

Acid phosphatase hydrolyzes a colorless compound, p-nitrophenyl phosphate (Sigma Chemical Co., St. Louis, Mo.), to a yellow salt of p-nitrophenol at pH 4.8. The colorimetric reaction is read at 410 mμ, and the amount of acid phosphatase activity is determined from the calibration curve of p-nitrophenol standards.

Table I

Carlsson's Minimal Medium

Components	mg/liter
Glucose	10,000.00
L-cysteine	50.00
$(NH_4)_2SO_4$	1,320.00
$MgSO_4 \cdot 7H_2O$	200.00
KH_2PO_4	13,600.00
Pyridoxine HCl	36.00
Nicotinic Acid	0.69
Biotin	0.0018
p-aminobenzoic acid	0.003
Thiamine HCl	0.015
Riboflavin	0.06
Ca pantothenate	0.36

C. Cell Preparation for the Enzyme Assay

The preparation of *Streptococcus mutans* for measurement of acid phosphatase activity has been accomplished by using osmotic shock (Heppel, 1967).

The preparation of the cells was done as follows: the cells were harvested from the broth, washed in 1.0 M Tris buffer (pH 7.2 at 25°C), harvested again and resuspended in triple distilled water for a specified time (usually 30 min.). The cell suspension, called the "shock fluid", is then ready for enzyme analysis.

D. Cell Dry Weight

A Millipore membrane (Millipore Filter Corp., Bedford, Mass.) is stored in a desiccator, to remain thoroughly dry and ready for use while retaining its full filtering capacity. The membrane is then weighed, and wetted with distilled water which is followed by the application of the cell pellet suspended in 5 ml of distilled water. The cell pellet is obtained from 43 ml of the bacterial culture, centrifuged at 10,000 rpm for 10 minutes, harvested and washed in 0.1 M Tris buffer (pH 7.2). Following this procedure the membrane is dried in an oven at 37°C for 24 hours. Control membranes were treated in a similar fashion, with duplicate determinations made on both control and test membranes. The dried membranes were weighed on a Mettler analytical balance to the nearest 10^{-4} gram.

E. Growth of *Streptococcus mutans*

The method of Carlsson (1970) was used in determining the absorbance of the cell broth at 420 mμ (Bausch and Lomb Spectronic 20). The samples were diluted when necessary to read in the absorbance range of 0.00 to 0.05.

F. Scanning Electron Microscopy

The cells were fixed, for 3 hours, in a solution of 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2). Then, .2 ml of the fixed cells were added to 2 ml of fixative and centrifuged for 5 minutes at 1,600 rpm. The cell pellet was then washed in 1 ml of sodium cacodylate buffer, followed by three consecutive washings in distilled water. Finally, the cells were resuspended in 2 ml of triple distilled water. Eight 20 λ drops were placed on a polished aluminum disc in a dust-free environment. The disc was then placed in an evaporator and a thin coat of gold applied (approx. 100 Å thick). The cells were then viewed using the scanning electron microscope apparatus (Jelco).

IV. EXPERIMENTAL RESULTS

A. Release of Acid Phosphatase from Cells as a Result of "Osmotic Shock"

1. Procedure

Streptococcus mutans were grown in trypticase soy broth and harvested from the broth when the cells had reached the maximum stationary phase of their growth. They were washed in 0.1 M Tris buffer (pH 7.2), harvested again, and suspended in 3 ml of triple distilled water or 0.1 M Tris buffer (pH 7.2) for 30 minutes. Samples from each suspension were then assayed for acid phosphatase activity. The remainder of each cell suspension was centrifuged to separate the cells from the supernatant. Both cell-free supernatants were immediately tested for enzyme activity. The cell pellets were resuspended in either Tris buffer or triple distilled water for 30 minutes, and then tested to determine the acid phosphatase activity still present.

2. Results

From Table II, it can be seen that the enzyme activity is significantly greater in the cells suspended in triple distilled water, "shock fluid", then the activity shown by the cells suspended in Tris buffer. It is also apparent that, when the cells are separated from the "shock fluid", the cell-free supernatant loses all phosphatase activity. The cell fraction retains the full enzyme

Table II

Test for Release of Enzyme

Sample	µMoles p-nitrophenol/hr/g
"Shock fluid", cells in triple distilled water	150.4
Cell-free supernatant obtained from centrifugation of "shock fluid"	0.0
Resuspended cell pellet from "shock fluid"	161.8
Cells suspended in 0.1 M Tris buffer (pH 7.2)	60.5
Cell-free supernatant obtained from cells originally suspended in Tris buffer	1.6
Resuspended cell pellet from Tris buffer suspension	76.6

activity, ruling out the possibility that the techniques which were used destroyed the active enzyme.

3. Comments

The apparent relationship between acid phosphatase activity and the necessary presence of the bacterial cells, leads to the conclusion that the "osmotic shock" treatment does not liberate the enzyme from the cell. Rather, an increase in the cell wall permeability to the p-nitrophenyl phosphate substrate is,in all likelihood, the effect of placing the cells in a hypotonic solution. Kreitzman (1969) successfully attempted to measure phosphatase activity of a cariogenic strain of streptococci, using toluene. Considering Kreitzman's work and my own, has led me to believe that the "osmotic shock" would produce the observed subtle changes in cell wall permeability that would permit the measurement of enzyme activity.

B. Optimum Conditions for the "Osmotic Shock" Preparation of the Cells

1. Procedure

Cells were grown in trypticase soy broth for 24 hours, harvested, and washed in 0.1 M Tris buffer. The cell culture is then divided into four subgroups one of which is suspended for 30 minutes in 3 ml of deionized distilled water; the second is suspended in triple distilled water, the third in triple distilled water and 0.01 M KH_2PO_4, and the fourth in triple distilled water and 0.1 M KH_2PO_4.

Samples of each subgroup were then assayed for enzyme activity. Another culture of cells were grown and washed similarly, then suspended in triple distilled water for 30 minutes. Before samples were taken to be assayed, 0.01 M KH_2PO_4 was added to one sample and 0.1 M KH_2PO_4 to another.

To examine the effect of the duration of "osmotic shock" on the cell, the cells were grown in trypticase soy broth for 24 hours (maximum stationary phase) and for 48 hours (death phase). The cells were harvested, washed in Tris buffer and samples were taken at specific time intervals of fifteen minutes to be tested for enzyme activity.

2. Results

Tables III and IV show that the presence of phosphate reduces the acid phosphatase activity of the cell. Table V indicates the optimum period of "osmotic shock" treatment would be 30 minutes for both the 24 and 48 hour cultures, the 24 hour culture showing the maximum activity.

3. Comments

In the experience of the writer, the optimum procedure developed for the cell preparation for the acid phosphatase assay was as follows: the cells are harvested from the broth, washed in 0.1 M Tris buffer, harvested again and suspended in triple distilled water for 30 minutes.

Table III

Effect of Phosphate in Cell Suspension During "Osmotic Shock" Treatment

Sample	μMoles p-nitrophenol/hr/g
Deionized water	135.4
Triple distilled water	142.2
Triple distilled water + 0.01 M KH_2PO_4	101.7
Triple distilled water + 0.1 M KH_2PO_4	34.5

Table IV

Effect of Phosphate on Cell Enzyme Activity
After "Osmotic Shock" Treatment

Sample	μMoles p-nitrophenol/hr/g
No additional phosphate	115.9
+ 0.01 M KH_2PO_4	58.9
+ 0.1 M KH_2PO_4	19.6

Table V

Effect of the Duration of "Osmotic Shock" on the Cell Enzyme Activity

Time of Osmotic Shock (min.)	μMoles p-nitrophenol/hr/g	
	24 hr. culture	48 hr. culture
0	96.2	94.5
15	129.2	110.2
30	192.3	141.7
45	124.6	141.7
60	115.4	114.2

C. Acid Phosphatase Activity of PR-89

1. Production of Enzyme

With many enzymes, the optimum activity is reached after the exponential phase of bacterial growth is completed. This may be due to some essential growth factor that approaches exhaustion, or to the accumulation of toxic metabolic products in the medium. Other enzymes are produced during the growth phase of the culture and the enzyme activity reflects the number of viable cells at any given time. This is often an indication of the role of the enzyme in the physiology of the microorganism.

The production of acid phosphatase was tested in a culture of cariogenic strain PR-89 grown in trypticase soy broth.

2. Procedure

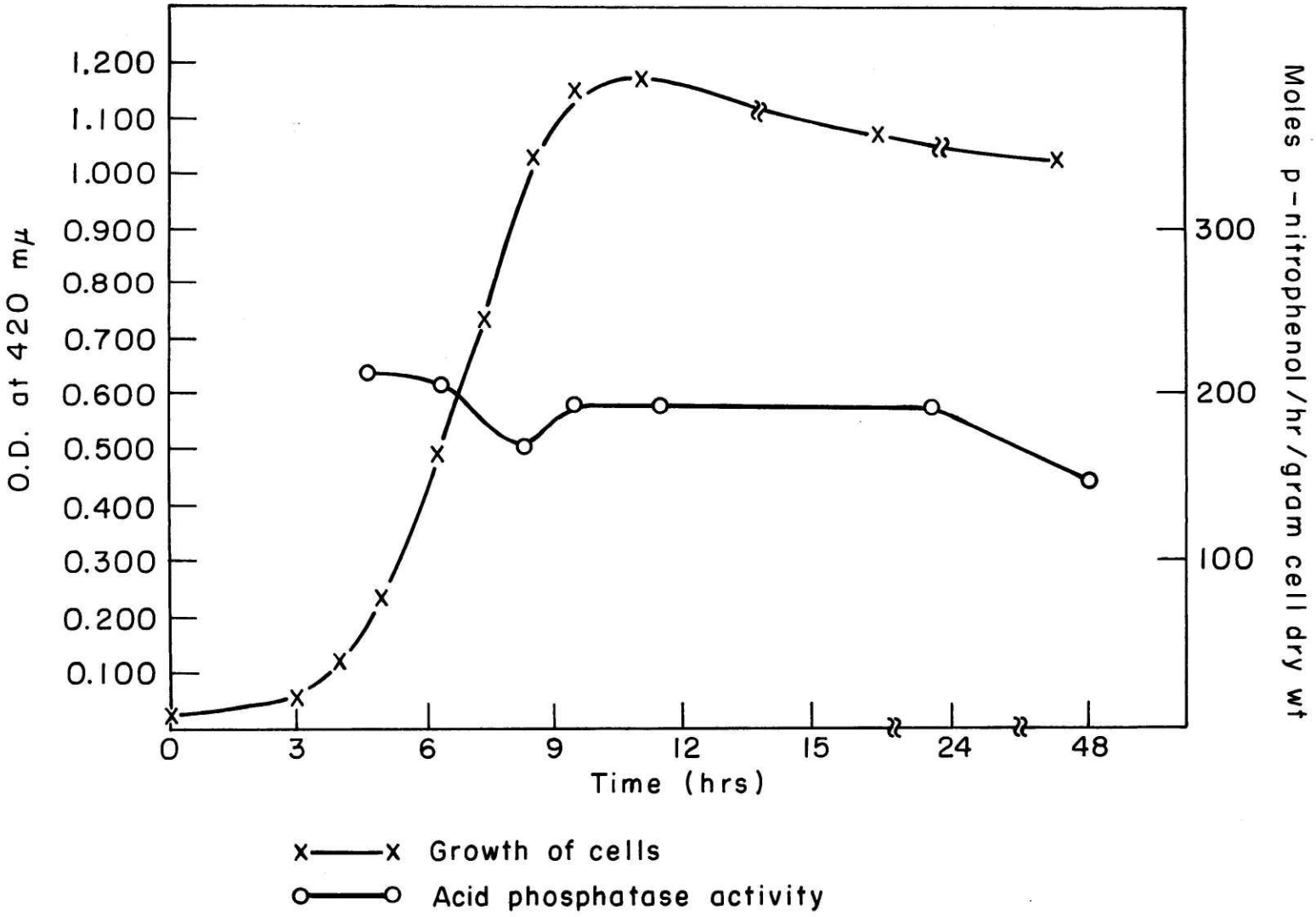
Ten ml of an actively growing log phase culture of streptococci were inoculated into 500 ml of sterile trypticase soy broth medium. The culture was incubated at 37°C under anaerobic conditions and aliquots were removed at varying time intervals and assayed for acid phosphatase activity.

3. Results

The measured enzyme activity appears to remain approximately constant throughout the growth phase of the culture and does not drop off appreciably until the culture has entered its death phase (Figure 1).

FIGURE 1. Growth of PR-89 Streptococci and the Appearance of Acid Phosphatase.

Figure 1 Growth of Streptococcus mutans (PR-8g) and the appearance of acid phosphatase activity.



4. Comments

The constant enzyme activity per cell during the growth period indicates that the enzyme is constitutive. The total enzyme activity reflects the number of cells from the growth phase through the stationary phase.

D. Effect of Phosphate Concentration on the Bacterial Acid Phosphatase Activity

1. Procedure

50 ml of log phase culture, grown in trypticase soy broth medium, is harvested and washed three times in Carlsson's Minimal Medium, modified to contain the appropriate concentration of phosphate and Tris buffer. The cells are then used to inoculate Carlsson's Minimal Media containing KH_2PO_4 concentrations of 0.1 M, 0.01 M, 0.001 M and 0.0001 M, and additional Tris buffer to make the total buffer molarity 0.1 M. Each culture is allowed to reach its maximum stationary phase and is then assayed.

2. Results

While the molarity of the media ranges from 10^{-1} M phosphate to 10^{-4} M phosphate, the enzyme activity remains almost constant.

3. Comments

The nature of the enzyme appears to be constitutive with respect to the phosphate content of the medium (see Table VI). This study would appear to be physiologically significant as the inorganic phosphate concentration of

Table VI

Effect of Phosphate Concentration on Phosphatase Activity

Sample	μMoles p-nitrophenol/hr/g
C.M.M. + 0.1 M KH_2PO_4	152.5
C.M.M. + 0.01 M KH_2PO_4	167.6
C.M.M. + 0.001 M KH_2PO_4	132.1
C.M.M. + 0.0001 M KH_2PO_4	175.0

saliva is within the range studied, which McCann (1968) determined as between 4 to 20 x 10^{-3} M.

E. Scanning Electron Microscopy of *Streptococcus mutans*

1. Procedure

The bacteria were grown for 24 hours. The control group was harvested and immediately prepared for the scanning electron microscope. Another group of cells was harvested and subjected to thirty minutes of "osmotic shock", then prepared for the microscopic study.

2. Results

Clearly, the integrity of both the control cells and the osmotically shocked cells was maintained by their intact cell walls (Figures 2 and 3). Because of the limitations in the magnification of the apparatus, it was impossible to distinguish the fine structure of the cell wall.

3. Comments

The micrographs show that the "osmotic shock" treatment does not make the enzyme available to the substrate by physically destroying the cell wall and exposing the periplasmic space. Smirnova *et al.* (1971) applied the scanning electron microscope to bacterial cells and found that pores in the wall surface influenced the passage of both endogenous and exogenous materials. The magnification achieved in Figures 2 and 3 suggest the existence of pores, but greater magnification is necessary to study the effect of "osmotic shock" on these pores.

FIGURE 2. Scanning Micrograph of Normal *Streptococcus mutans* Cells.

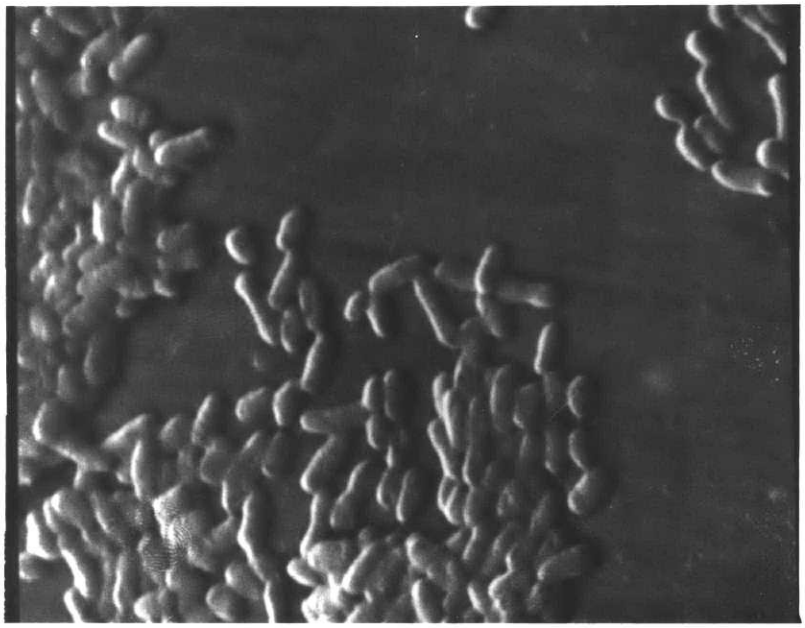
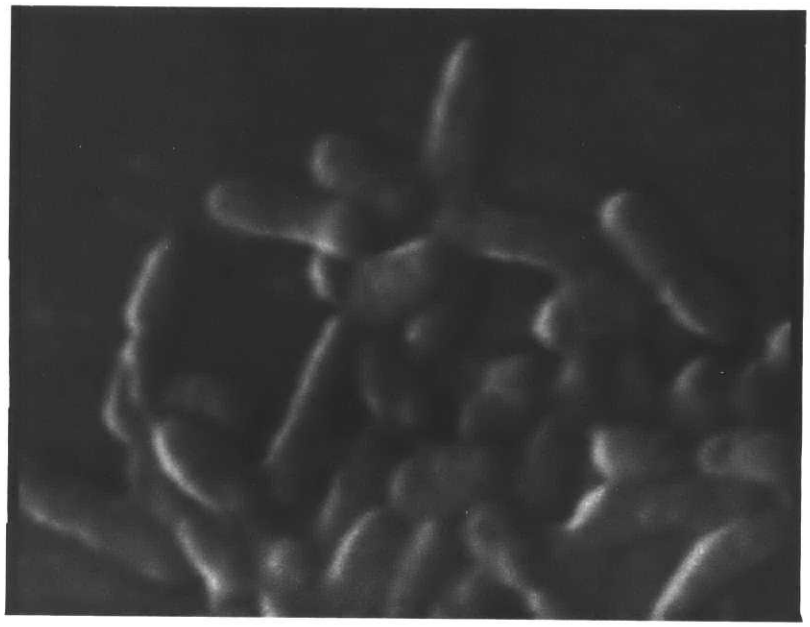


FIGURE 3. Scanning Micrograph of *Streptococcus mutans* Cells After Subjection to "Osmotic Shock".



V. DISCUSSION

Dental caries remains a major health problem in the world today and factors other than acid remain to be examined to discover their role in the etiology of the disease. One such factor to be considered is the phosphatase activity of cariogenic microorganisms that are capable of demineralizing tooth enamel. The acid phosphatase activity produced by Streptococcus mutans is of interest as it might be associated with the cariogenicity of the bacterial strain.

As "osmotic shock" treatment has been reported to release acid phosphatase activity from Streptococcus mutans, I chose to analyze the mechanism of action to facilitate the further study of the enzyme. The results of my work do not confirm the release of an active phosphatase enzyme from within the cell. Rather the enzyme activity remains closely associated with the bacteria. This is unfortunate as it had been hoped that the reduced osmotic pressure would easily release the enzyme and allow for the isolation and purification of the acid phosphatase. However, the phosphatase activity measured in the intact cell is evidence that the enzyme is located near the cell surface, as a substrate possessing a phosphate ester is presumed to be unable to penetrate the cytoplasmic membrane of the bacterial cell. The mechanism of action appears to occur through an increase in the cell wall permeability to the p-nitrophenyl phosphate substrate, permitting the measurement of phosphatase activity.

Analysis of the results strengthens the correlation between phosphatase activity and dental caries formation. The total phosphatase activity has been shown to be an increasing function of cell growth. Therefore, the caries problems associated with an increasing accumulation of cariogenic bacteria may be related to the equivalent increase in total phosphatase activity. Furthermore, the data shows inorganic phosphate to be a potent inhibitor of the enzyme, which indicates that the enzyme activity may be responsible for the effect of a high phosphate diet decreasing caries formation (Harris and Nizel, 1955). Certainly, the implications of the study appear to suggest a role for phosphatase in promoting dental caries.

In summary, while "osmotic shock" has proven to be a disappointment as a simple preparatory step in the release of the acid phosphatase for further purification procedures, it does allow the identification of enzyme activity in an otherwise inactive cell. This should be a valuable method to quickly survey the acid phosphatase activity of a wide range of bacterial strains. The properties of the streptococcal enzyme that were studied are consistent with its postulated role in dental caries.

VI. SUMMARY AND CONCLUSIONS

A. Summary

1. Subjecting a strain of cariogenic Streptococcus mutans to "osmotic shock" allowed the measurement of acid phosphatase activity without disrupting the integrity of the cell wall.

2. The optimum conditions for the "osmotic shock preparation of Streptococcus mutans for the determination of acid phosphatase activity were defined as: the cells are harvested from the broth, washed in 0.1 M Tris buffer, harvested again and suspended in triple distilled water for 30 minutes.

3. The presence of the bacterial cells was found to be essential for the measurement of enzyme activity after the cells had been exposed to the shock treatment.

4. The enzyme activity is constant throughout the growth phase and stationary phase of the bacteria.

5. The enzyme was found to be constituitive when the bacterial cells were cultured in phosphorus-limited media.

6. The presence of inorganic phosphate in the "shock fluid" has an inhibitory effect on the acid phosphatase activity of the cells.

B. Conclusions

1. The identification and partial characterization of acid phosphatase activity produced by a cariogenic strain of Streptococcus mutans has been made in this study.

2. The results of this thesis support the hypothesis that "osmotic shock" has an effect on the streptococcal

cell wall permeability, all owing the acid phosphatase activity to be measured in the intact cells.

VII. SUGGESTIONS FOR FUTURE RESEARCH

Following the work developed in this thesis, future consi-eration ought to be extended into several different aspects of the work.

1. The presence of phosphoproteins in the matrix of enamel makes the effect of acid phosphatase on phosphoproteins an interesting area of experimentation. It would be beneficial to study the ability of the phosphatase to remove phosphate from such phosphoprotein substrates as phosvitin (derived from egg yolk), casein and pepsin.

2. Disruption of the streptococcal cell wall should be attempted by more conventional mechanical and chemical means such as sonication, rotary cell homogenization, pressure cell disruption and lysozyme treatment. A comparison of the total acid phosphatase activity released from the cells after their complete disruption by conventional methods with that produced by the "osmotic shock" method will provide a basis for measuring the relative effectiveness of the "osmotic shock" procedure on the acid phosphatase activity of the bacteria.

3. The correlation between the relative caries potency of the cariogenic streptococci and the production of acid phosphatase by streptococci should be examined. Further, other strains of cariogenic bacteria should be examined to see if the correlation can be generalized.

4. The comparison between shocked and normal bacterial cells should be viewed under greater magnification by the scanning electron microscope to observe the fine structure of the cell wall surface. Transmission electron microscopy would disclose changes in the channels leading through the cell wall and into the periplasmic space.

5. The determination of inhibitors of the phosphatase activity is necessary and the inhibitors should be tested clinically for a controlling influence on dental caries. If this enzyme is responsible for the promotion of caries then inhibitors should help control the disease.

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BIOGRAPHICAL NOTE

I was born in Brooklyn on November 22, 1950, to George and Fay Fleisher. Since my father was and still is practicing dentistry in Brooklyn, I can trace my interest in oral science back to a very early time in my life. I attended elementary and junior high school in Brooklyn, where I had the usual interests for a boy of my age, i.e. sports, particularly basketball. I went on to James Madison High School in Brooklyn where I developed interests in mathematics and political science in which I took some college level courses. I was accepted by the Massachusetts Institute of Technology, and began my studies there somewhat undecided as to which field of concentration I would like to pursue. My original intention was to major in mathematics, but I soon discovered that my interest was not strong enough and switched to political science. I received the Bachelor of Science degree in Political Science from M.I.T. in 1972. However, my interest in science had not abated, and I continued to take courses in biology, nutrition and chemistry, as an undergraduate.

During my senior year in college, I worked in the Oral Science Laboratories at M.I.T. learning research techniques and basic approaches to the bacteriology of dental caries. Upon graduation from college, I again found myself in a position of indecision. My interests

at this point led me to consider careers in both oral science and medicine. I decided to work for the Master's degree in Nutritional Biochemistry which was greatly facilitated by receiving a grant to work in the Oral Science Training Program at M.I.T.

Although, upon the completion of and acceptance of this thesis, I plan to pursue a career in medicine, I consider my time and studies in the Oral Science Training Program to have been an extremely worthwhile experience for me, and one which I know will help me in my future work.