Some Environmental Factors Affecting the Morphology of Streptococcus mutans 10449

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SOME ENVIRONMENTAL FACTORS
AFFECTING THE MORPHOLOGY OF STREPTOCOCCUS MUTANS 10449

by
Ruth G. Emyanitoff

A thesis submitted to the Faculty of the Graduate School of Loyola University in partial fulfillment of the Requirements for the Degree of Master of Science

June 1975
Dedication

To all the good people in my life.
Acknowledgements

I would like to thank Dr. Dale Birdsell for his guidance, his faith in my abilities and his friendship. To Dr. Tadayo Hashimoto goes my deepest gratitude and thanks for his help and guidance during the completion of this thesis.

My thanks and appreciation to Dr. H. J. Blumenthal and Dr. R. J. Doyle for the time and effort they spend in critical evaluation of my work. Finally, my thanks to Mrs. T. Hashimoto for her generosity and skill in preparing the figures used in this thesis and to Christine Kallal for typing the manuscript.
Life

Ruth Gail Emyanitoff was born to Zolman and Julia Emyanitoff in Chicago, Illinois on January 10, 1951. She graduated from the Theodore Roosevelt High School, Chicago, Illinois, in June 1968, and received a Bachelor of Arts degree from the Division of Biological Sciences, University of Chicago, Chicago, Illinois in June 1972. She began her studies for a Master's degree in the Department of Microbiology, The Loyola University of Chicago, Stritch School of Medicine in July, 1973. Ms. Emyanitoff was the recipient of an American Society for Microbiology President's Fellowship in October, 1974.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>CHAPTER</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>II. MATERIALS AND METHODS</td>
<td>10</td>
</tr>
<tr>
<td>III. RESULTS</td>
<td>16</td>
</tr>
<tr>
<td>IV. DISCUSSION</td>
<td>42</td>
</tr>
<tr>
<td>V. SUMMARY</td>
<td>53</td>
</tr>
<tr>
<td>VI. REFERENCES</td>
<td>55</td>
</tr>
</tbody>
</table>
ABBREVIATIONS

BHIG ...................... brain heart infusion with 0.5% glucose
CaCO₃ ..................... calcium carbonate
CAMG ..................... caries active medium with glucose
CAMM ..................... caries active medium with mannitol
CAMS ..................... caries active medium with sorbitol
C ......................... centigrade
cm ........................ centimeter
CFU ........................ colony forming units
CKU ........................ corrected Klett units
\( \text{H}_2\text{O}_2 \) ................ hydrogen peroxide
hr .......................... hour
kV .......................... kilovolt
l ............................ liter
\( \mu \text{m} \) .................. micrometer
\( \mu \text{M} \) .................. micromolar
ml .......................... milliliter
mm .......................... millimeter
min ........................ minute
M ............................ molar
\( \text{N}_2:\text{CO}_2 \) .............. 95% nitrogen 5% carbon dioxide
iv
ABBREVIATIONS (continued)

N ....................... normal

$O_2:CO_2$ ................... 95% oxygen 5% carbon dioxide

% ....................... percent
List of Tables and Figures

Table

1. Growth characteristics of *S. mutans* species 20
2. Volume distributions mean cell volumes of selected samples 36

Figure

1. Schematic diagram of apparatus used for culture growth 12
2. Relative amounts of oxygen in solution in CAMG 17
3. Standard chart illustrating correlation between either viable or direct chain counts and corrected Klett units (CKU) 18
4. Growth curves of representative strains of the four subspecies of *S. mutans* in CAMG 21
5. Growth curves of *N$_2$*:C0$_2$ CAMG cultures of *S. mutans* 10449 23
6. Growth curves of *O$_2$*:C0$_2$ CAMG cultures of *S. mutans* 10449 25
7. Phase contrast and electron micrographs of *S. mutans* 10449 in *N$_2$*:C0$_2$ CAMG culture 26
8. Phase contrast and electron micrographs of *S. mutans* 10449 in *O$_2$*:C0$_2$ CAMG culture 27
9. Growth curves and phase contrast micrographs of *S. mutans* 10449 in CAMM cultures 29
10. Growth curves of *N$_2$*:C0$_2$ CAMM cultures of *S. mutans* 10449 31
11. Growth curves of *O$_2$*:C0$_2$ CAMM cultures of *S. mutans* 10449 32
12. Electron micrographs of *S. mutans* 10449 in CAMM cultures 33
13. Growth curves of *S. mutans* 10449 in *N$_2$*:C0$_2$ CAMG with varying concentrations of H$_2$:O$_2$ 37
### List of Tables and Figures (continued)

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>14.</td>
<td>Growth curves of <em>S. mutans</em> 10449 in N$_2$:C$_2$O CAMG with varying concentrations of ethanol</td>
<td>39</td>
</tr>
<tr>
<td>15.</td>
<td>Growth curve and phase contrast micrographs of <em>S. mutans</em> O$_2$:C$_2$O to N$_2$:C$_2$O shift</td>
<td>41</td>
</tr>
</tbody>
</table>
I. Introduction

Organisms which can be shown to undergo morphological changes under specific defined conditions have been very useful in the study of bacterial growth and division and the cellular control of these processes. Several recent reviews presenting current work and thought in this area have been published (Adler et al., 1969; Rogers, 1970; Donachie and Beggs, 1970; Higgins and Schockman, 1971; Donachie, 1973; Slater and Schaeacter, 1974). Mutants of a number of different species, both rods and cocci, have been used in these studies.

Rods. A number of workers have studied mutants of Escherichia coli. In 1967, Adler et al. described a mutant of E. coli which produced miniature, anucleated cells in addition to normal cells. These spherical cells were about 1/10 the volume of normal cells. They appeared to be formed by normal but misplaced septation during exponential growth of the organisms.

The morphology of a class of conditional mutants which exhibited inhibition of septum formation was characterized by Walker and Pardee (1967). After ultraviolet light irradiation, these mutants, known as lon⁻, grew as long filaments due to the inhibition of septum formation. Adler et al. (1968) studied the effect of introducing the lon⁻ mutation into a radiation resistant mutant which exhibited an amorphous, spherical morphology. They found
that, upon irradiation, the progeny of this cross developed into giant amorphous cells, 500-1000 X the volume of normal E. coli.

A mutation in a gene referred to as rod A led to a mutant of E. coli which exhibited a rounded morphology at 30, 37, or 42 C in a variety of complex or synthetic media (Matsuzawa, et al., 1973). A chain forming mutant of E. coli has also been described (Normark et al., 1971). This mutation, env A⁻, results in E. coli which form complete septa but fail to separate.

Mutations which are particularly useful to study are those which are temperature sensitive, exhibiting the mutant phenotype under a defined, limited range of temperature. Three classes of temperature sensitive mutants of E. coli have been described (Van de Putte, et al., 1964). They are:

I. Those mutants with complete inhibition of cell division and no colony forming ability at non-permissive temperatures.

II. Those mutants with incomplete inhibition of cell division which form colonies consisting of long filaments (or other aberrant forms) at non-permissive temperatures.

III. Those mutants with no colony forming ability at non-permissive temperatures accompanied
by, and probably caused by, inhibition of nucleic acid synthesis.

Several temperature sensitive mutants of *E. coli* which exhibited incomplete inhibition and morphologically aberrant cells were subsequently characterized. Reeve *et al.* (1970) isolated a mutant, BUG-6, which grows normally at 30°C, but stops dividing almost immediately to form long filaments when shifted to 42°C. These filaments divide abruptly into unit length *E. coli* when shifted back to 30°C. The BUG-6 mutant was subsequently crossed with a mutant which produced minicells (Khachatourian *et al.*, 1973). At 30°C, this hybrid mutant yielded normal progeny and minicells. At 42°C, cell division stopped, filaments with some constriction were produced, and no minicells were formed. This implied that production of minicells has one or more steps in common with normal septation.

Other temperature sensitive mutants of *E. coli* include rod to sphere mutants such as that described by Henning *et al.* (1972). This mutant was a rod at 30°C and would lyse at 42°C unless supplied with at least 2 mM Mg++. When the Mg++ was supplied, the cells were spherical. When shifted from 42°C to 30°C the cells reverted to rod form.

Another bacillary species in which many division mutants have been characterized is *Bacillus subtilis*. A number of these appear, morphologically, to be the same as those seen
in *E. coli*. Fourteen mutants, which mapped at four loci, were described by Van Alstyne and Simon (1971). Div A mutants synthesized septa with a high frequency error of placement which resulted in minicell formation. Div B mutants developed abnormal septa when grown at 45°C. A temperature sensitive mutant at Div C showed small balloon-like structures protruding from membrane thickenings, while Div D mutations resulted in septumless filaments at non-permissive temperatures.

Rogers *et al.* (1968, 1970) isolated and characterized rod− mutants of *B. subtilis* and *B. licheniformis*. These were spherical mutants which grew in groups or strings of coccal bodies. Ultrastructural examination revealed gross distortions in walls and membranes and septum formation was greatly disorganized. A temperature sensitive variant of these mutants was also isolated and characterized (Boylan and Mendelson, 1969; Cole *et al.*, 1970).

The effect of genetic background on the expression of a rod− mutation of *B. subtilis*, tag 1, has been studied (Reeve and Mendelson, 1972). Two patterns of expression were found:

1. Irregular, unstable, spherical cells unable to form colonies.

2. Regular, spherical cells which grow and divide and form colonies.
Tag 1 was also crossed with ts132, a temperature sensitive DNA\textsuperscript{-} mutation. This resulted in cells which were partially spherical and partially bacillary at 45 C.

Strains of other rod shaped species exhibiting abnormalities in division processes have also been studied. Two such mutants of *Salmonella typhimurium*, Div A and Div C were investigated (Ciesla et al., 1972). These were temperature sensitive mutants in which cell division stopped completely at non-permissive temperatures resulting in septumless filaments. DNA and protein synthesis were unaffected. Shifting to a permissive temperature allowed the cells to divide after a short lag period.

Grula and Grula (1962) described a species of *Erwinia* in which division could be inhibited either by 6 amino acids, penicillin, or ultraviolet light. These effects could be reversed by the addition of pantoyl lactone to the culture medium.

Conditional cell division mutants of *Agmenellum quadruplicatum*, a marine unicellular blue-green bacterium which has a wall structure similar to that of a gram negative rod, were isolated by Ingram and Fisher (1973a). One of these, SN29, formed filaments at non-permissive temperatures (35 C) but developed normally at permissive temperatures (44 C). Another strain, SN12, grew as multinucleoid filaments in dilute suspensions, but divided spontaneously into unit lengths as the culture density increased, implying
the possible accumulation of a division promoting substance (Ingram and Fisher, 1973b).

Cocci. Among the cocci, Cripps and Work (1967) described a strain of *Staphylococcus aureus* which showed morphological aberrations at the end of exponential growth when grown in the presence of 4% NaCl. They noted such abnormalities as irregular shapes of whole cells, multiple incipient septa in a single cell, and irregular wall thickening. Membranes were seen contracting away from the cell wall and small membrane-enclosed bodies were seen in the periplasmic space. In some cases there was no cytoplasm remaining, and only the small, membrane-enclosed bodies were seen.

Cultures grown under these conditions lysed rapidly. After lysis had started, cytoplasm was often seen extruded from points of rupture. Half septa (invaginations seen on only one side of the cell) and debris of a fibrous nature were also seen in these cultures.

Opaque colony variants of *Streptococcus pyogenes*, group A, have been seen by light microscopy to produce long chains which were not disrupted by several different treatments which normally bring about cell separation, such as hot formamide extraction. When thin sections of the cells were examined by electron microscopy, the cocci were seen to be joined into elongated chains by exaggerated intercellular septa (Swanson and McCarty, 1969). The septa often
occupied the majority of a cell's circumference. Cell wall architecture and chemistry were not affected.

**Naturally Occurring Conditional Morphological Variants.** Organisms with a naturally occurring capacity to exhibit altered morphogenesis not only provide a system for studying control of growth and division but allow for a study of the environmental conditions which lead to the occurrence of this phenotype. One such genus, which has been studied extensively, is *Arthrobacter*. These organisms usually appear as Gram positive in the coccal form, Gram negative in the rod form, and convert from one form to the other during the growth of the culture. A review on this genus and procaryotic cells has been written by Clark (1971).

Rosan and Eisenberg (1973) and Eisenberg (1973) noted that strains of *Streptococcus sanguis*, when grown in the presence of a high partial pressure of oxygen exhibited a greatly altered morphology. The cells grown in the presence of oxygen were enlarged and contained many septal notches. Their growth was inhibited by oxygen and the cells rapidly lost viability. The rods appeared in late exponential phase cultures and their appearance could be delayed by diluting the culture in fresh medium before the exponential phase was attained. Rod formation could also be inhibited by the addition of catalase to the culture.

In 1924, Clark isolated a streptococcus from human dental carious lesions which exhibited rod-like forms in
old agar cultures. He attributed this change to the lowered pH of the old cultures, and named this organism *Streptococcus mutans*. Its most distinguishing features were its ability to produce a gelatinous substance in 5% sucrose and its ability to ferment mannitol and sorbitol. Studies during the last 15 years have concentrated on the antigenic, biochemical, and physiological characteristics of *S. mutans* as they pertain to initial adherence to smooth surfaces and the cariogenicity of the organism (see Fitzgerald *et al.* 1973). Although interest in this organism has increased over the last few years, only passing reference has been made to the change in morphology of *S. mutans* previously noted by Clarke. Coykendall *et al.* (1971), in describing the ultrastructure of the various serological groups of *S. mutans* noted that occasionally "frank rods" were observed in one of the cultures examined, but stated that these were not bacillary contaminants. No further work was done to study the nature of these rods or the conditions under which they were formed.

It is the purpose of this thesis to describe a strain of *S. mutans*, NCTC 10449, which exhibits conditional morphological aberrations. Since models for normal control of bacterial growth and division for both coccal and rod shaped organisms have been proposed (Higgins and Shockman, 1970; Burdett and Murray, 1974), it is hoped that the results
of this study will be useful in the examination of these models and that they might suggest further avenues of investigation.
II. MATERIALS AND METHODS

Organisms. *Streptococcus mutans* strains AHT, BHT, and 10449 were obtained from Dr. I. Shklair, Great Lakes Naval Training Center, Great Lakes, Illinois. Strain OMZ 176 was obtained from Dr. A. Bleiweis, University of Florida, Gainesville, Florida.

Maintenance of cultures. Stock cultures were maintained in fluid thioglycollate to which 2% calcium carbonate (CaCO₃) was added. These were kept refrigerated and transferred weekly. In addition, lyophilized stocks of each culture were kept under vacuum.

Culture media. Cultures were grown in caries active medium (Wittenberger and London, personal communication) which contains 0.5% yeast extract (Difco Laboratories, Detroit, Michigan), 0.5% tryptone (Difco Laboratories, Detroit, Michigan), 1% K₂HPO₄ and either 0.5% glucose (CAMG), 0.5% mannitol (CAMM) or 0.5% sorbitol (CAMS) (Sigma Chemical Co., St. Louis, Missouri). The media were adjusted to pH 7.0 with 9N HCl and autoclaved. For determination of viable counts, brain heart infusion agar (Difco Laboratories, Detroit, Michigan) supplemented to contain 0.5% glucose was used. The purity of cultures after each experiment was determined by streaking for isolation on sheep blood agar (BBL, Cockeysville, Maryland) and examining for a single characteristic colony type.
Culture technique. Inocula were grown for 12-14 hours in fluid thioglycollate broth (BBL, Cockeysville, Maryland) with 2% CaCO₃. The inoculum was mixed vigorously to obtain an even suspension of cells. Four ml of this suspension was inoculated into 200 ml of culture medium in a Spinner flask (Kontes Glass Co., Vineland, N. J.) (Fig. 1). The side arm of the flask was covered with a number 20 Morton closure (Bellco, Vineland, N. J.). The top of the flask was sealed with a size 6 rubber stopper through which a cotton plugged 2 ml pipette was inserted. The pipette tip was no more than 1 cm from the bottom of the flask. A mixture of 95% nitrogen plus 5% carbon dioxide (N₂:CO₂) or 95% oxygen plus 5% carbon dioxide (O₂:CO₂) (Liquid Carbonic Co., Chicago, Illinois), or air, using an aquarium air pump, was bubbled through the medium. The flow rates of the gases were controlled using a gas flow meter (Dwyer Mfg., Michigan City, Indiana) at a rate of 54 l/hr as determined by water displacement from a graduate cylinder. The relative oxygen in solution was determined using a Clark oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio) coupled to a Bausch and Lomb VOM 7 Chart recorder (Bausch and Lomb, Rochester, New York). Cultures were incubated at 37 C in a water bath.

Quantitation of growth. Growth was quantitated using three methods: turbidity measurements, direct cell counts,
Fig. 1. Schematic diagram of apparatus used for culture growth. A. compressed gas input; B. cotton plugged 2 ml pipette; C. size 6 rubber stopper; D. number 20 Morton closure; E. Spinner flask; F. culture medium (CAMG or CAMM).
and viable counts. Turbidity was measured in a Klett Sum-
merson colorimeter equipped with a #66 filter. All Klett
readings were corrected for deviation from Beer's law due
to light scattering. Direct cell counts were made using
a Petroff-Hausser counting chamber (C. A. Hausser and Sons,
microscope (Carl Zeiss Inc., New York, New York) fitted
with a Neofluor PH2 40X objective and a 2X intermediate
objective was used to view the cells. Each chain was
counted as a single unit. Viable counts, [the number of
colony forming units (CFU) per ml of culture], were deter-
mined by spreading 0.1 ml of appropriate dilutions in
duplicate on brain heart infusion agar supplemented to
contain 0.5% glucose (BHIG). Dilutions were made in sterile
distilled water. Plates were incubated in a candle jar
for 24-48 hours. The pH was determined at one hr intervals
during the growth of the culture. A one ml sample was
removed and the pH was measured using a Corning model 12
research pH meter (Corning Science Products, Corning,
New York) fitted with a combination electrode (Sargent
Welch, Chicago, Illinois).

Phase contrast microscopy. Culture samples were
examined using either an Olympus (Olympus Optical Co.,
Tokyo, Japan) or a Zeiss phase contrast microscope and
recorded photographically with an Olympus PM-10-M camera
using Kodak Panatomic X film (Eastman Kodak Co., Rochester,
New York). For determining cell diameter, an ocular micrometer was placed in the 6.5X lens of the camera and calibrated using a micrometer calibrated to 0.01 mm (American Optical Corp., Buffalo, New York).

Preparation of thin sections for electron microscopy. Cells were fixed with 2% osmium tetroxide in Kellenberger buffer (Kellenberger et al., 1959) overnight at 25 C and then treated with uranyl acetate for one hour. After dehydration through a graded acetone series the cells were embedded in Spurr's low viscosity medium (Spurr, 1969) (Electron Microscopy Sciences, Fort Washington, Pennsylvania) and polymerized at 65 C overnight. Sections were cut on an LKB 4800A ultramicrotome (LKB-Produkter AB, Stockholm, Sweden) using a diamond knife (E. I. duPont de Nemours, Wilmington, Delaware). They were mounted on 300 mesh copper grids (Ernest F. Fullam, Inc., Schenectady, New York), poststained with uranyl acetate and lead citrate (Reynolds, 1963) and viewed with a Zeiss EM9S electron microscope operating at 75kV. Results were recorded photographically using electron microscope film (Eastman Kodak Co., Rochester, New York).

Cell volume distribution. Cell volumes were determined on cells fixed in 10% formalin using a modified Coulter electronic particle size analyzer system (amplifier and multichannel analyzer from Nuclear Chicago Corp., Chicago,
Illinois). A 30 μ aperture was used. The cells were briefly sonicated to disrupt chains using a Blackstone ultrasonic probe (Blackstone Ultrasonics, Jamestown, New York). At intervals during the sonication process, the sample was monitored by phase contrast microscopy to assure that cells were not broken. When maximal chain disruption, without cell breakage was obtained, pictures were taken of the cells and culture samples were diluted into 0.1N HCl.

Cell concentrations were such that the total number of particles counted was less than $10^4$. At these concentrations the best size distribution can be obtained (H. Kubitschek, personal communication). Volume scales were calibrated with standard polystyrene latex microspheres in singlets, doublets, and triplets of 1.1 μm diameter per singlet (Kubitschek, 1968).
III. RESULTS

Determination of growth conditions

a. Oxygen in solution. A Clarke oxygen electrode was used to determine the relative amount of oxygen in solution under the culture conditions employed. It was important to determine the length of time required to either completely saturate the medium with oxygen or to completely remove the oxygen from solution. As can be seen in Fig. 2, oxygen saturation was achieved within six minutes. This concentration was calculated from theoretical considerations to be 1180 μM at 25 C. Similarly, when shifting from an atmosphere of oxygen to an atmosphere of nitrogen, oxygen was completely removed from solution within six minutes. This was substantiated by the absence of further reduction upon the addition of sodium dithionite, which chemically removes oxygen from solution. Only 15-20% of the saturation with 95% oxygen was achieved by bubbling air through the medium. Since air is 21% oxygen, the maximum oxygen saturation of a solution bubbled with air would be 262 μM at 25 C.

b. Correlation of turbidity with chain number. Three techniques were chosen for use in this study to measure cell growth: viable counts, direct counts using a Petroff Hauser counting chamber, and increase in the turbidity of a broth culture as measured with a Klett-Summerson colorimeter. As can be seen in Fig. 3, in nitrogen grown cultures,
Fig. 2. Relative amounts of oxygen in solution in CAMG measured with a Clark oxygen electrode. The shifts in atmosphere are: \( \text{--- } \text{N}_2:\text{CO}_2 \to \text{O}_2:\text{CO}_2; \)
\( \text{---- } \text{O}_2:\text{CO}_2 \to \text{N}_2:\text{CO}_2; \) \( \text{.... } \text{N}_2:\text{CO}_2 \to \text{air}; \) \( \text{.... } \text{air} \to \text{O}_2:\text{CO}_2. \) Arrows indicate addition of sodium dithionite.
Fig. 3. Standard chart illustrating correlation between either viable or direct chain counts and correct Klett units (CKU). Symbols: O—O viable counts; O—O direct counts.
a linear correlation was observed between culture turbidity and either viable or direct counts up to 100 corrected Klett units (CKU). When the culture was inoculated as described previously, an initial turbidity of 4 CKU was routinely obtained. This corresponded to $10^7$ chains/ml as determined by direct count.

**Effect of 95% oxygen on *S. mutans***

The effect of 95% oxygen on the growth and morphology of strains representative of the four subspecies of *S. mutans* was determined (Fig. 4, Table 1). Strain AHT (subspecies *cricetus*, serotype a) had a turbidity doubling time of 84 min when grown in N$_2$:CO$_2$. There was no significant increase in turbidity when the cultures were grown in O$_2$:CO$_2$. In both cases, the cells appeared as chains of streptococci. Strain BHT (subspecies *rattus*, serotype b) had a turbidity doubling time of 72 min grown in both N$_2$:CO$_2$ and O$_2$:CO$_2$. Under both conditions the cells appeared as normal chains of streptococci.

Strain 10449 (subspecies *mutans*, serotype c) showed a turbidity doubling time of 42 min when grown on a N$_2$:CO$_2$ atmosphere. These cells exhibited a normal morphology. In contrast with this, cultures grown in O$_2$:CO$_2$ contained morphologically aberrant cells as revealed by phase contrast microscopy (Fig. 4, inset A). The turbidity doubling time of the O$_2$:CO$_2$ grown cultures was 84 minutes. The final
Table 1. Growth characteristics of *S. mutans* species

<table>
<thead>
<tr>
<th>Strain</th>
<th>Subspecies</th>
<th>Serotype&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Doubling Time&lt;sup&gt;2&lt;/sup&gt; (min)</th>
<th>Morphology</th>
</tr>
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<tr>
<td></td>
<td></td>
<td></td>
<td>N&lt;sub&gt;2&lt;/sub&gt;:CO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>O&lt;sub&gt;2&lt;/sub&gt;:CO&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>AHT</td>
<td><em>cricetus</em></td>
<td>a</td>
<td>84 no growth</td>
<td>cocci;</td>
</tr>
<tr>
<td>BHT</td>
<td><em>rattus</em></td>
<td>b</td>
<td>72</td>
<td>cocci;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>chains</td>
</tr>
<tr>
<td>10449</td>
<td><em>mutans</em></td>
<td>c</td>
<td>42</td>
<td>cocci;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>chains aberrant</td>
</tr>
<tr>
<td>OMZ 176</td>
<td><em>sobrinus</em></td>
<td>d</td>
<td>72</td>
<td>cocci;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>chains</td>
</tr>
</tbody>
</table>

1. Bratthall, 1970
2. Determined turbidometrically
Fig. 4. Growth curves or representative strains of the four subspecies of *S. mutans* in CAMG. Inserts show typical cells from $O_2:C0_2$ cultures of 10449 and OMZ 176. Symbols: $O--O N_2:C0_2; -- O_2:C0_2.$
strain tested, OMZ 176 (subspecies sobrinus, serotype d) exhibited a normal morphology on a N<sub>2</sub>:CO<sub>2</sub> atmosphere and the turbidity doubling time was 72 min. When grown in O<sub>2</sub>:CO<sub>2</sub>, some short rods were seen, but the percentage of aberrant cells and the degree of aberration were not as striking as that seen with strain 10449 (Fig. 4, inset B). Turbidity doubling time for the O<sub>2</sub>:CO<sub>2</sub> culture was 108 min.

Strain 10449 was chosen for further study for the following reasons: 1) rapid doubling time in N<sub>2</sub>:CO<sub>2</sub>; 2) retarded but measurable growth rate in O<sub>2</sub>:CO<sub>2</sub>; 3) large percentage of abnormal cells in O<sub>2</sub>:CO<sub>2</sub> cultures.

Effect of carbohydrate on O<sub>2</sub>:CO<sub>2</sub> and N<sub>2</sub>:CO<sub>2</sub> cultures of strain 10449

a. Glucose. The growth pattern of both N<sub>2</sub>:CO<sub>2</sub> and O<sub>2</sub>:CO<sub>2</sub> CAMG cultures as determined by measurement of turbidity increase has been described in the previous section. During the growth of the organisms in N<sub>2</sub>:CO<sub>2</sub>, the pH of the culture medium dropped from 6.8 to less than 5.0 over the 8 to 9 hour growth period (Fig. 5A). During the time of incubation the colony forming units (CFU) increased from 1.1 x 10<sup>6</sup> to 5.0 x 10<sup>9</sup> per ml. This is equivalent to 12 generations (Fig. 5B). Direct counts increased from 2 x 10<sup>7</sup> to 9 x 10<sup>9</sup> chains/ml (Fig. 5A).

The turbidity of the O<sub>2</sub>:CO<sub>2</sub> cultures was more variable. Usually the turbidity increased for more than 14 hours,
Fig. 5. Growth curves of $N_2:CO_2$ CAMG cultures of \textit{S. mutans} 10449. Symbols: A. $\square$—$\square$ pH; $\circ$—$\circ$ direct counts (chains/ml); B. $\triangle$—$\triangle$ viable counts (colony forming units/ml). The average chain length was 6-8 cocci/chain.
however, occasionally it was seen to decrease after 9-10 hours of growth. Both colony forming units and direct counts appeared to increase at about the same rate as the turbidity of the culture (Fig. 6A and B) while the pH of the culture dropped slightly more than one pH unit over a 9-10 hour growth period (Fig. 6A).

Cells grown in N₂:CO₂ atmosphere in CAMG appeared to be normal chains of streptococci by phase contrast microscopy (Fig. 7A) with an average chain length of 6 to 8 cocci per chain. In cultures grown in a N₂:CO₂ atmosphere without agitation, the chain lengths were greater than 50 cocci per chain. The lesser number of cells per chain in the agitated cultures was probably due to chain breakage in the cultures in which the gas was bubbled through the medium during the course of growth.

Thin sections of the N₂:CO₂ cells revealed a wall with an inner electron dense and an outer electron lucent layer. The cytoplasm appeared uniform and the nucleus could be seen in most sections. Mesosomes were prominent in many sections (Fig. 7B). These observations are in accord with those of Coykendall et al. (1971).

Morphologically aberrant cells were observed from the beginning of exponential phase in the O₂:CO₂ cells grown in CAMG (Fig. 8A). Rods were the most common of the aberrant forms seen. Some of these had narrow ends with swollen
Fig. 6. Growth curves of $O_2:CO_2$ CAMG cultures of *S. mutans* 10449. Symbols: A. □—□ pH; ○—○ direct counts (chains/ml); B. △—△ viable counts (colony forming units/ml).
Fig. 7. *S. mutans* 10449 in N₂:CO₂ CAMG culture

A. Phase contrast micrograph

B. Electron micrograph of thin section
Fig. 8. *S. mutans* 10449 in $O_2:CO_2$ CAMG culture

A. Phase contrast micrograph
B and C. Electron micrographs of thin sections
centers. Swan-necked cells, with a large swollen end, a long "neck" and a smaller rounded end were sometimes observed. Rods or swans might be found in a chain with small round cells. It was not possible to determine if these contained any nuclear material. A striking feature of these aberrant cells was that there appeared to be symmetry within a chain. A configuration such as two swan-necked cells "tail to tail" flanked on either side by a small coccus or by two equivalent length rods was not uncommon (Fig. 8A).

Examination of thin sections by electron microscopy revealed rods with multiple initiated septa (Fig. 8B). The positions of the septa were usually such that completion of septation would have resulted in chains of cocci of normal dimensions. Infrequently, two pairs of initiated septa were seen closer together than would allow for formation of normal coccal forms upon completion of septation. Some cells had the same shape as the swan-necked cells seen by phase contrast microscopy. A thick septum or an electron dense structure could be seen close to the small end (Fig. 8C). The walls appeared normal although occasional thickened septa were noted.

b. Mannitol. The turbidity of the N<sub>2</sub>:CO<sub>2</sub> CAMM cultures increased at a rate comparable to the CAMG cultures up to a turbidity of 35-45 CKU (Fig. 9). At this point the turbidity doubling time decreased and continued at the new rate until the stationary phase was reached. A similar
Fig. 9. Growth curves of *S. mutans* 10449 in CAMM cultures. Symbols: 0—0 \( \text{N}_2: \text{CO}_2 \) atmosphere; □—□ \( \text{O}_2: \text{CO}_2 \) atmosphere. Phase contrast micrographs of the cultures at times indicated by the arrows.

a. \( \text{N}_2: \text{CO}_2 \) 3 hour culture; b. \( \text{N}_2: \text{CO}_2 \) 8 hour culture; c. \( \text{O}_2: \text{CO}_2 \) 3 hour culture; d. \( \text{O}_2: \text{CO}_2 \) 8 hour culture.
decrease was seen in the viable and direct chain counts of these cultures (Fig. 10). Although the shapes of the curves remained the same, a great deal of variability in viable counts was seen from culture to culture. The pH of the culture, over the 10 to 11 hr period of growth decreased from 6.8 to 5.5 or below (Fig. 10A).

A similar phenomenon was seen in the growth pattern of the O₂:CO₂ grown cells (Fig. 9). The rate of turbidity increase paralleled that of the N₂:CO₂ cultures, but at 25-35 CKU the rate of turbidity increase slowed down. As was the case in the N₂:CO₂ cultures, the viable and direct counts showed a similar pattern of increase. The pH drop during the growth period was less than 1 pH unit (Fig. 11A).

Cultures grown in a N₂:CO₂ atmosphere appeared to be normal streptococci during early exponential growth (Fig. 9a). At a culture turbidity of 35-45 CKU the cells started to increase in size. By late exponential phase the diameter of the cells appeared, by phase contrast microscopy, to be twice that of the glucose grown cells (Fig. 9b). The same increase was seen when sorbitol was used as a carbohydrate source. When thin sections of late exponential cells from the CAMM culture were examined by electron microscopy, very few exceptionally large cells were noted (Fig. 12A). Thin sections of cells from these cultures were observed to contain small, round, electron dense inclusions
Fig. 10. Growth curves of N$_2$:CO$_2$ CAMM cultures of *S. mutans* 10449.  
A. □—□ pH; ○—○ direct counts (chains/ml)  
B. △—△ viable counts (colony forming units/ml).
Fig. 11. Growth curves of O₂:C₅O₂ CAMM cultures of *S. mutans* 10449. A. □—□pH; O—o direct counts (chains/ml); B. △—△ viable counts (colony forming units/ml).
Fig. 12. Electron micrographs of *S. mutans* 10449 in CAMM cultures. A. $N_2:CO_2$ atmosphere; B. $O_2:CO_2$ atmosphere. Wedge shaped septa (a, b) and thickened multilayered wall (c) can be seen.
throughout their cytoplasm. Otherwise, the cells appeared like those of \( \text{N}_2:\text{CO}_2 \) CAMG cultures. Aberrant cells appeared in the \( \text{O}_2:\text{CO}_2 \) CAMM cultures in early exponential phase of growth (Fig. 9c). These appeared similar to those observed in glucose cultures. As culture turbidity increased beyond 25-35 CKU, the cells appeared much larger than comparable glucose grown cells (Fig. 9d). Examination of thin sections of these organisms revealed a greater degree of aberration than seen in glucose grown cells (Fig. 12B). Many cells in these sections exhibited extremely thick wall profiles. Incomplete septa appeared as wedge shaped rather than the relatively long and narrow septa observed in the \( \text{O}_2:\text{CO}_2 \) CAMG cultures. In addition, the positioning of the septa was sometimes displaced. Septal invaginations were observed which were not in direct opposition to one another across the diameter of the cells. Odd numbers of septal invaginations were also observed. Completed septa were often extremely thick and sometimes "S" shaped (data not shown). A large amount of electron dense inclusion material could be seen throughout the cytoplasm of the cells.

**Cell Volume Distributions**

The diameter of the cocci in the \( \text{N}_2:\text{CO}_2 \) CAMM cultures appeared to be twice that of cells in \( \text{N}_2:\text{CO}_2 \) CAMG cultures as measured on photomicrographs. If this difference were a real size difference, then the volume of the CAMM cells
would be eight times greater than that of the CAMG cells. Samples of representative cultures in both CAMM and CAMG were fixed 10% formalin and briefly sonicated to disrupt chains. Immediately following disruption, the volume distribution for each sample was determined. The four distributions appear virtually identical. Since, even after sonication, there were still short chains, the mean cell number per chain was determined by direct counting from phase contrast micrographs taken at the time the distributions were determined.

Table 2 lists the mean particle volume as obtained from the volume distributions, the mean chain length and the volume per cell for each of the samples. Although a difference was noted between the volume per cell of the various samples, in no case was there a difference approaching the theoretical eight fold difference in volume predicted on the basis of phase contrast micrographs of these suspensions. Due to the sampling technique used, it was not possible to determine if the differences seen were statistically significant.

Aberrant forms in the absence of oxygen

Hydrogen peroxide (H$_2$O$_2$). The effect of various concentrations H$_2$O$_2$ on mid-exponential N$_2$:CO$_2$ CAMG cultures can be seen in Fig. 13. Samples of the culture were
Table 2. Volume distributions mean cell volumes of selected samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total Particles Counted</th>
<th>Mean Particle Volume (μm³)</th>
<th>Mean Chain Length and standard deviation</th>
<th>Volume/cell ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>N₂:CO₂</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAMG</td>
<td>11335</td>
<td>0.98</td>
<td>1.61 ± 0.1ᵃ</td>
<td>0.61</td>
</tr>
<tr>
<td>8 hour</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O₂:CO₂</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAMG</td>
<td>9521</td>
<td>1.05</td>
<td>1.22 ± 0.5ᵇ</td>
<td>0.86</td>
</tr>
<tr>
<td>7 hour</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N₂:CO₂</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAMM</td>
<td>10421</td>
<td>1.01</td>
<td>1.40 ± 0.1ᵃ</td>
<td>0.72</td>
</tr>
<tr>
<td>7 hour</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O₂:CO₂</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAMM</td>
<td>9631</td>
<td>1.11</td>
<td>1.60 ± 0.5ᵇ</td>
<td>0.69</td>
</tr>
<tr>
<td>7 hour</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ᵃ500 chains counted  
ᵇ80 chains counted  
ᶜdetermined by direct counts of cells per chain from photomicrographs.
Fig. 13. Growth curves of \textit{S. mutans} 10449 in N$_2$:CO$_2$ CAMG with varying concentrations of H$_2$O$_2$. Insert is phase contrast micrograph of cells in CAMG with $10^{-3}$M H$_2$O$_2$. Arrow indicates time of addition of H$_2$O$_2$. Symbols: $\circ$—N$_2$:CO$_2$ culture; $\circ$—O$_2$:CO$_2$ culture; $\Delta$—CAMG added; $O$—$10^{-4}$M H$_2$O$_2$; $10^{-3}$M H$_2$O$_2$; $\Delta$—$10^{-2}$M H$_2$O$_2$. 
removed at a turbidity of 40 CKU and H₂O₂ was added to give final concentrations from 10⁻² to 10⁻⁴ M. All cultures were incubated in an atmosphere of N₂:CO₂. A control culture, incubated in O₂:CO₂, was grown simultaneously.

Samples grown with no H₂O₂ or in 10⁻⁴ M H₂O₂ showed the same growth patterns. The increase in turbidity paralleled that of the parent culture. Phase microscopic examination of the cultures indicated that they were composed of normal chains of streptococci. When H₂O₂ was added to a final concentration of 10⁻² M, no further increase in culture turbidity was seen and the cells remained normal. At a final concentration of 10⁻³ M H₂O₂, the turbidity doubling time of the culture was increased as compared to the control. Microscopically, these cells exhibited morphologically aberrant cells, particularly rod shaped organisms similar to those seen in the O₂:CO₂ cultures.

Ethanol. The effect of ethanol on N₂:CO₂ CAMG cultures was also investigated (Fig. 14). Final concentrations of ethanol from 0.2 M to 0.02 M were added to culture samples taken from an exponential phase culture at 40 CKU. The control sample, to which only CAMG was added, and the sample with 0.02 M ethanol showed virtually the same growth pattern. The increase in turbidity paralleled that of the parent culture. Microscopically, these cells exhibited typical streptococcal morphology. Ethanol addition to a final
Fig. 14. Growth curves of *S. mutans* 10449 in N$\textsubscript{2}$:CO$\textsubscript{2}$ CAMG with varying concentrations of ethanol. Insert is phase contrast micrograph of cells in CAMG with 0.1 M ethanol. Arrow indicates time of addition of ethanol. Symbols: O—O N$\textsubscript{2}$:CO$\textsubscript{2}$ culture; □—□ O$\textsubscript{2}$:CO$\textsubscript{2}$ culture; △—△ CAMG added; O—O 0.02 M ethanol; □—□ 0.1 M ethanol; △—△ 0.2 M ethanol.
concentration of 0.2M concentration of 0.1M were not totally inhibited, but had a much longer turbidity doubling time than the control. These cells exhibited morphological aberrations including a number of rods.

Reversion of rods to cocci

It was of interest to determine whether the change from coccal to rod form was a reversible phenomenon. A 4 ml sample from a 10 hr O₂:CO₂ CAMG culture was inoculated into 200 ml of CAMG with an atmosphere of N₂:CO₂. The resulting growth curve and phase contrast micrographs of the cultures can be seen in Fig. 15. During the two hour lag period the cells were predominantly rod shaped. A few coccal cells, some phase light cells and some cell fragments were seen. At hour 3, a large increase in turbidity was observed. The culture appeared to be composed almost entirely of normal chains of streptococci with very few rods. After the first hour of growth, the turbidity increased with a normal doubling time and reached stationary phase at the same maximum turbidity seen with N₂:CO₂ CAMG grown cells from a thioglycollate inoculum.
Fig. 15. Growth curve *S. mutans* 10449 $O_2$:CO$_2$ to N$_2$:CO$_2$ shift. Four ml of a 10 hour $O_2$:CO$_2$ culture was inoculated into 200 ml of CAMG on a N$_2$:CO$_2$ atmosphere at time 0. A. Phase contrast micrograph of culture at time 0; B. Phase contrast micrograph of culture at 3 hours.
IV. DISCUSSION

The main purpose of this study was to examine the unusual morphological changes in *S. mutans* as originally described by Clarke in 1924 and to determine environmental factors which reproducibly effect this change. Strain 10449, and three other strains of *S. mutans* were grown under conditions of high oxygen tension (Fig. 3). Two of these strains, AHT and BHT showed no morphological changes in O$_2$:CO$_2$. Strain AHT was unable to grow under high oxygen tension, while BHT exhibited normal growth. Strain OMZ 176 did show some rod forms, but strain 10449 showed the greatest degree of aberration and had the most rapid doubling time in N$_2$:CO$_2$ atmosphere. It was thus considered the best candidate for further study.

In choosing appropriate measures of growth, several difficulties inherent to *S. mutans* were encountered. Strains of *S. mutans* have been shown to accumulate an internal polysaccharide (DiPersio *et al.*, 1974). It was thought that, because of this accumulation, dry weight determination would not be an accurate measure of cell growth in a batch culture. Turbidity, as measured with a Klett-Summerson colorimeter, direct counts using a Petroff Hauser counting chamber, and viable counts were chosen as independent measures of growth.
For taking direct counts, it was decided to count chains per ml rather than cells per ml. The reasons for this were two-fold. The sizes of the organisms were such that counting individual cells within a chain, and differentiating cells which had completed septation from those which were still in the process of dividing would be sufficiently difficult that accurate counts would not be achieved. In addition, a meaningful comparison of viable and direct counts would require both to be made either on a single cell per ml or on a chain per ml basis. Chains could not be broken to uniform single cell units without affecting viability. These problems indicated that measurement of chains per ml and of colony forming units without attempts to break chains should give the most comparable counts. However, these measurements limit the interpretations of the data. Equivalence of viable and direct counts indicates only that there is one viable unit per chain and can show parallel increases or decreases in viable counts, direct counts and turbidity over time.

During the course of the work, another problem arose which would further complicate interpretation of viable and direct counts. Repeated determinations of viable and direct counts showed viable counts to be ten fold lower than direct counts. This discrepancy could not be reduced by performing all dilutions and viable counts using
CAMG and CAMG agar. Cells diluted for determination of viable counts were observed, microscopically, to clump, giving rise to occasional groups of from 10 to 100 chains or more. When plated onto BHIG agar, two colony sizes were frequently seen on a single plate. Since the culture was not contaminated, these larger colonies probably arose from plating clumps of organisms. This clumping would account for the discrepancies noted and places further limitation on the interpretation of the viable counts.

Despite the problems encountered in quantitation of growth, the coccus to rod transformation of *S. mutans* 10449 has a number of characteristics which make it an excellent candidate for further study. Aberrant forms can be induced by a single change in the environment, an increase in the amount of oxygen in the atmosphere. The pH change of the medium did not correlate with the appearance of aberrant cells. Rods appeared in the O$_2$:CO$_2$ grown cells over a range of pH from 6.8 to 5.5 (Fig. 5), while no rods were seen in N$_2$:CO$_2$ grown cultures over the same pH range (Fig. 4).

The occurrence of rods and other non-coccal forms seems to be a division dependent phenomenon. Aberrant cells appeared from the beginning of exponential growth in all carbon sources studied. Only those strains of *S. mutans* which exhibited a sensitivity to oxygen, but not a total
inhibition of growth, developed morphologically abnormal cells. When hydrogen peroxide and ethanol were tested for their ability to induce aberration in cell shape, only those concentrations which were growth inhibitory but sublethal were effective (Figs. 13, 14). Thus it appears that some division processes are occurring but a part of the total division mechanism has been altered.

Ultrastructural examination of the oxygen grown cells suggests that the alteration in the division process had occurred after septation had been initiated (Fig. 7B and C). Cells occasionally possessed incorrectly placed septa. Cell shape, too, seemed to have been altered in some cases.

The studies by Rosan and Eisenberg on S. sanguis implicated peroxides as a causative agent of morphological changes since the cells retained normal shape in the presence of catalase. Hydrogen peroxide can be formed by a variety of reactions, catalyzed by oxidases which use oxygen as the final acceptor of a pair of electrons. These are usually FAD enzymes with the reaction proceeding by one of the following (where M is the substrate, En is the enzyme):

\[
\text{MH}_2 + \text{EnFAD} \rightleftharpoons \text{M} + \text{EnFADH}_2
\]

\[
\text{EnFADH}_2 + \text{O}_2 \rightleftharpoons \text{EnFAD} + \text{H}_2\text{O}_2
\]

or

\[
\text{MH}_2 + \text{EnFAD} \rightleftharpoons \text{EnFADH}^-\text{MH}^-
\]

\[
\text{EnFADH}^-\text{MH} + \text{O}_2 \rightleftharpoons \text{EnFAD} + \text{M} + \text{H}_2\text{O}_2
\]
Organisms such as streptococci, which do not produce catalase, thus have no way to remove the \( \text{H}_2\text{O}_2 \) formed by either of the above mechanisms. Under conditions favoring these reactions, \( \text{H}_2\text{O}_2 \) would be expected to increase in concentration and might bring about the morphological changes seen (Fig. 7). If \( \text{H}_2\text{O}_2 \) is involved in the division altering process, then addition of external \( \text{H}_2\text{O}_2 \), at sublethal concentrations, should induce the formation of morphologically aberrant cells. This was found to be the case (Fig. 13).

The carbohydrate source seems to affect the degree of aberration seen. Mannitol grown cells exhibited a much more extreme alteration in morphology than glucose grown cells (Fig. 11). The additional aberration seen when the organisms are grown in mannitol may be due to an end product of mannitol catabolism not formed when glucose is used as a carbon source. The pathway by which \( S. \text{mutans} \) utilizes mannitol has been reported by Brown and Wittenberger (1973) to be:

\[
\text{mannitol} \rightarrow \text{mannitol-1-PO}_4 \xrightarrow{\text{NAD} \quad \text{NADH}_2} \text{fructose-6-PO}_4
\]

An NAD dependent mannitol-1-PO\(_4\) dehydrogenase has been shown to be involved in the reaction. The extra \( \text{NADH}_2 \) produced per mole of mannitol utilized is reoxidized by an alcoholic fermentation which results in the formation of ethanol. The exact enzymatic steps in this fermentation are unknown. They may be similar to pathways in other
organisms which involve reducing acetaldehyde, a decarboxylation product of pyruvic acid, to ethanol by means of an NAD linked alcohol dehydrogenase (Brown and Patterson, 1973). When exogenous ethanol was added to anaerobic glucose cultures aberrant forms were seen. The concentration of ethanol necessary to induce the change was ten times higher than that which could have been accumulated if all the mannitol in the medium had been metabolized and yielded stoichiometric amounts of ethanol. This would account for the fact that anaerobic cultures grown in mannitol failed to exhibit any aberrant forms. The amount of ethanol may have been sufficient to cause the additional degree of aberration seen in the O₂:CO₂ mannitol cultures (Fig. 11).

Cells grown in mannitol exhibited another interesting phenomenon. Both N₂:CO₂ and O₂:CO₂ cultures exhibited a two-fold increase in cell diameter during exponential phase when examined by phase contrast microscopy which would imply an eight-fold increase in cell volume. When cell volume was measured using a Coulter electronic particle size analyzer. The data indicated that there may have been a slight increase in volume. This increase, however, did not approach the expected 800% increase. Examination of thin sections of these cells revealed electron dense cytoplasmic inclusions not seen in glucose grown cells. It is probably the accumulation of this material that causes
the cells to appear enlarged when viewed by phase contrast microscopy.

Comparison of the three different measures of culture growth indicate that the O₂:CO₂ cultures maintained viability for at least 12 hrs of growth (Fig. 5). Occasionally cultures grown beyond that time began to lyse, though continued slow growth for 15 hrs or more was often seen. Since the cells in the O₂:CO₂ were essentially all aberrant forms from the beginning of exponential growth, it can be concluded that these cells still retain the capacity, however altered, to grow and divide.

When 10 hr O₂:CO₂ grown cells were inoculated into fresh medium and incubated in a N₂:CO₂ atmosphere, the culture grew as normal chains of streptococci. No normal streptococci were seen in the inoculum, yet when examined at the first increase in culture turbidity, essentially all the cells observed had streptococcal morphology (Fig. 15). This can only be explained if the rod forms could revert to streptococcal chains. Even if 5% of the culture consisted of cocci (which on the basis of microscopic examination would be a high estimate), and it was assumed that only cocci were viable, the culture could only have had 17% cocci after 80 minutes of exponential growth. Even after seven doubling periods, 280 minutes, 13% of the cells would have still been aberrant forms. Since the number of coccal cells
seen in the inoculum was much less than 5% of the total culture, the results seen could not be explained in this manner. A more likely explanation is that the "blocked septa" commonly seen in the rod shaped cells were not permanently inhibited, and, upon removal of the oxygen, could complete septation. This would result in the early appearance of chains of cocci.

A model for streptococcal wall growth and division has been proposed based on considerable experimental evidence (Higgins and Shockman, 1970). According to the model, cocci increase their surface area by the construction and separation of cross walls. Both peripheral wall and cross wall are derived from peptidoglycan synthesized at, or near, the site of cross wall formation. This peptidoglycan layer is split in two by the action of autolytic enzymes localized near the site of synthesis. This splitting gives rise to peripheral wall. In addition to crosswall and peripheral wall formation, newly synthesized peptidoglycan can become involved in a third process, wall thickening. The fraction of the total peptidoglycan synthesized by the cell which is utilized in these three processes depends on:

(i) rate of wall elongation
(ii) rate of wall thickening
(iii) rate of peeling apart of newly synthesized wall into two layers of peripheral wall
The shape and size of the organism is governed by the relative rates of i and iii such that anything which affects those rates would result in a change in shape and size of the cell. In particular:

"Since the peripheral wall is made by splitting apart of the two layers of cross wall, inhibition of splitting would prevent peripheral wall extension and favor cross wall closure. Increased splitting, on the other hand, would result in peripheral wall extension at the price of cross wall closure." (Higgins and Shockman, 1971)

Autolytic activity is thought to be involved in the control of the rate of wall splitting. In *Streptococcus faecalis* the autolysin, an N-acetyl muramidase, is known to have both a latent and an active form. A mechanism which would control synthesis of autolysin, local concentrations of active autolysin, or interconversion of latent and active forms of autolysin would be involved in control of cell division and cell shape. Thompson (1971) hypothesized that autolytic activity may specifically influence cell shape in a manner analogous to the action of a lathe. The shape of the end of the cell would be determined by the relative rates of longitudinal wall formation and constriction of the diameter of the peptidoglycan septum by a combination of annular wall synthesis and autolytic activity. Since, in a coccal organism, longitudinal wall synthesis is governed by the amount of autolytic activity,
changes in autolytic activity could control end shape as well as determine the amount of longitudinal wall.

Assuming that the current models describe a general mechanism for control of growth and division in streptococci, the following explanation for the phenomena described in *S. mutans* 10449 can be proposed. The morphological abnormalities seen in *S. mutans* 10449 when grown under high oxygen tension are due to altered autolytic activity. This may be a result of direct action of molecular oxygen, though it appears probable that an oxygen radical or a metabolic product of oxygen consumption which the organism is incapable of removing is more directly responsible. Hydrogen peroxide, or perhaps a superoxide radical may be involved. The alteration results in an increase in autolytic activity relative to wall synthesis. This increased activity could account for the aberrant shapes seen. Increased autolytic activity would favor peripheral wall formation such that initiated septa would remain incomplete. Any further wall synthesized would be split, yielding lateral wall. Swollen cells might be a result of overall weakening of peptidoglycan structure. Other alterations in the relative rates and sites of synthesis and autolysis might lead to a variety of cell shapes such as tapered ends or very small cells.
Production of aberrant forms by such a mechanism would be expected to be growth dependent. It would also be very sensitive to the amount of increase in autolytic activity since too great an increase would result in lysis of the cell. This mechanism would not affect viability as long as autolysis has not been too extensive. Upon removal of the autolysin activating factor the cells could resume normal septation and growth.

Although the proposed mechanism is highly speculative, it is consistent with the data, consistent with currently accepted models, and could be tested experimentally. A streptococcal species, such as *S. mutans* 10449, in which a conditional morphological aberration can be reproducibly induced under well defined conditions, which maintains viability in the aberrant form over a relatively long period of time, and which retains the capacity to undergo normal cell division should be extremely useful in examining and testing models for control of cell growth and division in streptococci.
V. SUMMARY

*Streptococcus mutans*, strain 10449, was found to exhibit an altered morphology when grown under conditions of high oxygen tension. Cultures grown in oxygen had a longer generation time than those grown in a nitrogen atmosphere. When examined by phase contrast microscopy, organisms grown in a complex medium with glucose as a carbon source appeared as rods, often with swollen areas or swan necked ends. Chains of aberrant cells often appeared symmetrical. Ultrastructural examination of the oxygen grown cells revealed several incomplete septa along the length of the rod. Other bizarre septal conformation were seen. Addition of hydrogen peroxide to glucose cultures grown in nitrogen mimicked the growth pattern and morphology of oxygen grown cells.

When mannitol was used as a carbon source rather than glucose oxygen grown cultures still contained rod shaped organisms. The generation times of both the oxygen and nitrogen grown mannitol cultures decreased after 5-6 generations, after which the diameter of the cells appeared to double. A similar phenomenon was seen using sorbitol as a carbon source. Despite this apparent increase, measurements of cell volume using an electronic particle size analyzer failed to reveal a corresponding increase in volume. Examination of thin sections by electron microscopy revealed
a few abnormally large cells. Small electron dense inclusion bodies were seen throughout the cytoplasm of the nitrogen-grown cells. The oxygen-grown cells were grossly abnormal. Thickened, multilayered areas of wall, wedge shaped septa, and odd numbers of septal invaginations were seen. When ethanol, an endproduct of mannitol and sorbitol catabolism, was added to glucose cultures growing in a nitrogen atmosphere, rods, but not enlarged cells, were seen.

The oxygen grown cultures appear to be viable up to 12 hours after inoculation. Rods from cultures grown in oxygen for 10 hours appear to revert to coccal form upon dilution to fresh medium in a nitrogen atmosphere. These coccal cells subsequently exhibited a normal generation time.

A possible explanation of the phenomena seen, based on current models of streptococcal growth and division and the role of autolytic enzymes in these processes, has been proposed. The use of this system for the study of streptococcal growth and division is discussed.
VI. References


Van de Putte, P., J. Van Dillewijn and A. Rorsch. 1964. The selection of mutants of *E. coli* with impaired cell division at elevated temperature. Mutation Res. 1:121-128.

The thesis submitted by Ruth G. Emyanitoff has been read and approved by the members of the Advisory Committee listed below.

The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the thesis is now given final approval with reference to content and form.

The thesis is therefore accepted in partial fulfillment of the requirements for the degree of Master of Science.

May 6, 1973
Date

Signature of Advisory Committee Director

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