STUDIES OF THE DENTAL PLAQUE AND THE PHYSIOLOGY OF THE ORAL LACTOBACILLI

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DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of the Ohio State University

By

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The Ohio State University

1953

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ACKNOWLEDGMENT

I wish to thank the Procter and Gamble Company for their financial assistance and Dr. Chester I. Randles for his guidance in the research and in preparing this manuscript. I am especially indebted to Craig, Melinda, and Rosemary, for their sacrifices while I pursued this degree; and to my wife, Rosemary, for her work on this dissertation.

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A great many theories on the causes of dental caries have been advanced over a period of several hundred years. Although most of them have been discarded, there does not exist, even today, a universal acceptance of one theory. Most investigators, however, appear to be in accord with those who relate the production of carious lesions to the action of acid formed in very close proximity to the surface of the tooth. This does not restrict the etiology of caries to one factor alone, for a great multiplicity of physical, chemical, and biological factors will influence the formation of acid and its neutralization in the oral cavity. Carious lesions are commonly observed as well localized entities usually on the areas of the teeth which are protected from the self cleansing action of the mouth. There must then be a localization of the acid and a resistance to the immediate neutralization or removal of this acid. A collection of acid producing microorganisms adhering to the surface of the tooth and at least partially protected from the washing and buffering action of the saliva would satisfy these requirements. Such collections have been observed and have been called plaques.

Caries immune individuals have also been observed to possess these plaques. The availability of plaques
from both caries immune and caries susceptible individuals presented material for the comparison of the two. However, most of the work was an attempt to obtain a satisfactory evaluation of the flora of the susceptible plaque. Attempts to investigate the physiological activity of the plaque as a whole were unsuccessful because of the limited amount of plaque material available. Plaques can be artificially produced but, without a study of natural plaques, one can never be assured that these artificial specimens possess the properties of those obtained from the oral cavity.

In addition to the study of the plaque, the physiology of the lactobacilli and factors affecting their growth and biochemical activity were investigated. Lactobacillus counts on salivary specimens have become generally accepted as a good indicator of caries activity. Although these organisms are not claimed as the sole bacterial agent in the carious process the nature of their distribution made it seem advisable to study the nutritional requirements and metabolic activities of these oral bacilli. Armed with basic information of this nature one might proceed more readily to understand the reasons for their correlation with caries activity and their possible role, if any, in the caries process.
LITERATURE REVIEW

The literature on dental caries has become far too voluminous for anyone to attempt to review it, or perhaps even to list it all. In addition the scope of research has so broadened that research on many phases of the problem has no direct bearing on other investigations. For example, most of the work on fluorides and other caries inhibitors has no direct relationship to this investigation. Reviews of portions of the literature on the broad subject of dental caries have been made by Graber (1947) and Fosdick (1950). The concepts of many of those conducting research on the nature of the carious process and its control are presented in the symposium edited by Baslick (1943). A review of some of the more recent research on the carious process is presented by Nuckolls et al (1952).

It thus becomes necessary to restrict this review to a partial mention of literature more directly related to a study of the dental plaque and of the lactobacilli. Work on the oral bacteria had been greatly stimulated by the writings of Miller (1990) concerning his attempts to apply Koch's postulates to the caries process. Miller's work also stimulated a great search for the microorganism responsible for the production of caries, a search which is still going on to this day. The list of or-
ganisms isolated from saliva, from plaques and from decaying teeth runs the whole gamut of bacteria and has even been extended to the viruses (Turner et al., 1951).

Most of the work since the 1920's has been concentrated on the more potent acid producers isolated from the oral cavity. Streptococci and lactobacilli have received most of the attention. Streptococci have been consistently isolated in large numbers both from saliva and from plaques. Attention was focused on the lactobacilli through the observations of a good correlation between Lactobacillus counts and caries incidence and/or activity. Bunting and Farmerlee (1925) had cultured lactobacilli from all 33 cases of beginning caries which they studied but had found these organisms in only 3 of 19 caries-immune individuals. Bunting, in collaboration with a great many other investigators, went on to investigate this correlation in thousands of cases and found that it held in a majority of cases. This work led Bunting (1931) to accept Bacillus acidophilus (Lactobacillus acidophilus) as the causative agent in caries. While this view is not held today, the Lactobacillus count is still widely used as a caries index.

This early work on the lactobacilli stimulated a search for a good medium for the quantitative determination of the organism. The lactobacilli were found to grow poorly if at all on ordinary culture media and Kulp and White
(1932) had developed a tomato juice containing medium which gave them fairly consistent counts of *Lactobacillus acidophilus*. While this medium has been modified to some extent, a tomato juice agar is still widely employed in the determination of *Lactobacillus* counts, usually according to the Hadley (1933) technic.

All of this early work described these counts as *Lactobacillus acidophilus* (or other synonyms) counts and the concept that all oral lactobacilli are simply variants of *Lactobacillus acidophilus* became widely accepted. Different types were described according to the amount of acid produced but these were merely described as variants of this one species. Curran, Rogers, and Whittier (1933), however, believed that a majority of their strains were *Lactobacillus casei*. On the basis of their studies they concluded that the oral lactobacilli were not of one species, and that they were not usually *L. acidophilus*.

No exhaustive attempts were made toward the identification of oral lactobacilli until 1950. The first edition of *Bergey's Manual* (Bergey et al., 1923) had divided the genus into homo- and heterofermentative groups but no one had even separated the oral lactobacilli to this extent. In the sixth edition of *Bergey's Manual* (Breed et al., 1948) these two groups are further subdivided on the basis of optimum growth temperature and optical rotation of the lactic acid produced from carbo-
hydrate, with but little use of fermentation reactions. These characteristics alone do not provide for a clear-cut separation of all the oral species which have subsequently been described. Fermentation reactions were not utilized by earlier investigators because of the difficulty of obtaining a basal medium which would support good growth of all lactobacilli. In nutritionally inadequate media great variations were observed in the fermentation reactions and there appeared to be no constancy of fermentation patterns.

The first real attempts at the differentiation of oral lactobacilli were reported by Tilden and Svec (1960) and by Rogosa, Mitchell, and Fitzgerald (1960). Early work by the Rogosa group employed basal media which were not easily prepared, but during the course of their studies they developed a highly selective medium, yet one which satisfied the nutritional requirements of most if not all the lactobacilli (Rogosa, Mitchell, and Wiseman, 1951). This medium was then employed, with modifications, in their recently reported work on the species differentiation of the oral lactobacilli (Rogosa et al, 1953). Utilizing this medium they observed only a very limited number of variations in the characteristics of the organisms tested. They pointed out that the great variability of the lactobacilli reported by other investigators can be readily produced in nutritionally inadequate, poorly buffered, or improperly prepared media.
Armed with a good, easily prepared basal medium to which the desired carbon and energy source may be added, investigators should be able to make even greater strides forward in their study of the physiology of the oral lactobacilli. This basic information may then very well lead to a better understanding of the role of the Lactobacillus in oral environment.

While the lactobacilli have been associated with perhaps a major portion of the bacteriological research on caries, other acidogenic flora of the oral cavity have not been disregarded. Hundreds of references could be quoted to list a great many possible caries producers or associations of microorganisms capable of rapidly producing relatively large quantities of acid. It seemed much more desirable, however, to restrict this investigation and its literature review to the one genus which has shown some correlation with caries activity. This correlation was especially borne out by the results of Green (1953) obtained on another phase of this research project. Green observed a very significant difference in numbers of lactobacilli when comparing the salivary flora of caries susceptible to that of caries immune subjects.

Bacteriological investigations of the dental plaque have been far less extensive than those conducted on saliva despite the fact that they were described early
In the research on dental caries. Williams (1397) microscopically observed a dense and adhesive mass of fungi on the enamel surface of sectioned teeth. He postulated that acid produced by the bacteria affected the enamel at the point of juncture of plaque and enamel. Miller (1902) concluded from his observations that only in exceptional cases was caries ever initiated without an overlying plaque and that even these exceptions may have been due to the temporary absence of the plaque at the time of observation.

The problem of just what holds the bacteria together in a compact mass and cements this quite firmly to the enamel is still not satisfactorily answered today. Kirk (1910) suggested plaque formation as a result of coagulation of salivary mucin by the bacterially produced acid. However acid production in the free saliva probably never lead to concentrations sufficient to produce mucin precipitation. In addition Lothrop (1912) showed that acid in a concentration capable of dissolving enamel precipitates mucin in a flocculent non-adhesive form, and thus acid should be detrimental to a "mucinous" plaque. The possible role of mucin, or some altered form of mucin, as an initiator of plaque attachment is still debated today. Other theories have been proposed to explain plaque attachment and its structural integrity. Dietz (1943) suggests a role of the homogenous material of the
plaques, which he believes consists of degraded proteinaceous fractions of bacteria. Bannever et al (1951) suggest that the Actinomyces are responsible for the structural framework of the plaque and its attachment to the tooth.

Acid production by plaques has been clearly established both by in vitro and in vivo technics. Probably the best method employed to demonstrate acid production is the application of an electrode directly to the plaque. This was devised by Stephan (1940) for use with a small diameter antimony electrode. Later, Stephan (1944) showed that cases with high caries activity gave the lowest pH values and Stralfors (1948) observed a correlation of low pH values with high lactobacillus counts.

The problem of evaluating the flora of the dental plaque has not been satisfactorily solved. Kligler (1915) found bacterial concentrations of about 500 million organisms per mg. of plaque material obtained from areas of beginning caries. Bunting and Palmerlee (1925) found that plaques from caries-free subjects seldom contained lactobacilli while lactobacilli were readily cultivable from caries-active subjects. These results were identical with their observations on the lactobacilli in the saliva of the two groups. This correlation between the presence of lactobacilli in the plaque and the appearance of caries has been both confirmed and denied by subsequent
workers. Perhaps a major cause of disagreement has been the dividing of subjects into caries-active and caries-free groups so that an overlapping of the two has occurred.

Several technics have been employed to disperse the organisms from the plaque so that microscopic and plate counts could be conducted. Kligler (1915) merely shook the plaque in 10 ml. water in a small glass vial. Howitt and Fleming (1950) modified the technic by introducing glass beads to aid in breaking up the plaque. These investigators found streptococci and gram negative diplococci (Neisseria) to be by far the most prevalent forms. The lactobacilli comprised only about one thousandth part of the total flora cultivated.

Several modifications of a technic for the trituration of plaque material between two closely fitting glass surfaces are reported (e.g. Huntz and Miller, 1943; Stralfors, 1950). Stralfors (1950) employed both the shaking with glass beads and trituration between two ground glass surfaces in his performances of counts of plaque organisms. He observed, as did the earlier investigators, a positive correlation between the presence of lactobacilli and the occurrence of caries, but also noted that the lactobacilli constituted only a very small part of the total flora. The streptococci and gram negative diplococci made up the major portion of the micro-
organisms both by plate and by direct microscopic counts.

Thus the status of the dental plaque in the literature seems to be: 1) it definitely exists, 2) it is capable of producing relatively large amounts of acid and a correspondingly low pH, 3) it is largely composed of microorganisms, 4) the factor(s) responsible for its structural integrity and rather firm attachment to the tooth are unknown, 5) the significance, from a caries production standpoint, of any individual representative of its bacterial population is much in doubt.
MATERIALS AND METHODS

The bacteriological portion of this research project on dental caries was divided at its onset into a study of the plaque and a study of saliva. The results of the investigations conducted on the salivary aspects were the subject of the dissertation of Green (1953). Studies on the plaque are presented as one portion of this paper, while the other portion is concerned with physiological studies of some of the lactobacilli characterized by Green.

The caries susceptible individuals available to this study were, for the most part, elementary school children showing varying degrees but definite signs of caries activity past and present. The caries immune individuals were of varying age levels. They showed no evidence of ever having had caries activity, and at the time of observation did not have any dental caries detectable by complete clinical examination, including X-ray photography.

I. Investigation of the dental plaque.

Collection of dental plaques. Plaques were collected by the dentist or dental technician conducting the clinical study. Specimens from caries susceptibles were collected at about 9:30 A.M. No data were obtained on prior food intake or prior brushing of the teeth. Samples were usually removed from areas of the molars which were some-
what protected from the normal cleansing action of the mouth. The visible accumulation to be picked was washed by a moderately forceful stream of water and then picked and placed in a small sterilized plastic tube containing distilled water. After the work had progressed a few weeks the washing process was eliminated. Usually three samples were furnished each collection day and any investigation of them was completed by six hours after the collection time. By the time some of the fundamental details incident to the study had been completed the immune individuals available for collection of plaques had become extremely scarce and only a few caries immune specimens were obtained.

**Microscopic observations.** Microscopic observations were made on a portion of the plaque specimens, using both stained and unstained preparations. Plaques were observed in the intact form and after tearing them apart as much as possible with an inoculating needle.

**Attempts to disperse the flora of the plaque.** The first attempts to break up the clumps of plaque material utilized the technic of shaking with glass beads. The plaque specimen in its 2 ml of water was transferred to a sterile 25 ml Erlenmeyer flask containing about 15 glass beads. No attempt was made to determine the quantity of plaque material used as it was decided that the relative proportions of organisms would be just as significant as
counts based on a unit amount of plaque material. After adding 3 ml of sterile water the flask and contents were vigorously agitated for periods up to 15 minutes. It was observed that an oscillation of the flask which caused the beads to roll vigorously was probably as effective as a shaking procedure. No microscopic or plate counts were conducted since the main effect seemed to be a reduction to a smaller clump size accompanied by a freeing of many coccal and but few rod or filamentous forms. It was felt that the procedure would be favorable to the possible false impression that the flora consisted predominately of cocci. While the rod or filamentous forms might not have been as numerous as the cocci, there were considerable numbers of them visible in the microscopic observations of treated and untreated plaques. They were usually in a tangled mass much too dense to permit any accuracy by microscopic counting and certainly none by plate counting techniques.

The possibility that mucoprotein material of the saliva or bacterial debris might play a role in holding the plaque together suggested the application of proteolytic enzymes to plaque specimens. Papain (Difco) and trypsin (Armour) were added to the collection tubes. The amounts added were not determined but even in very high concentration no apparent dispersal of plaque material was obtained and the clumps were no more susceptible to
disintegration by the glass bead technic than untreated plaques.

The construction of a suitable all glass homogenizer from two concentric glass tubes was undertaken. Tubes were matched so that the outside diameter of the inner tubes were just slightly larger than the inside diameter of the outer member of the pair. The tubes were ground together using finely powdered alumina (Fisher Scientific Co.) as the abrasive. Considerable breakage was encountered both from vibration and from the heat produced, and although reduced grinding speeds severely prolonged the process they were found to be necessary. When the product was finally obtained it was found that the clearance was too great in the bottom of the apparatus to give any significant effect as a homogenizer of plaques. Grinding was repeated after shrinkage of the bottom portion of the outer tube by heating it in the flame of a Fisher burner. The resulting homogenizer still had too large a clearance to be effective. The alumina was replaced in the procedure by finely powdered pumice and a homogenizer was finally obtained which gave good breakage of clumps in two old plaque specimens which had been stored in the refrigerator for several days. At this time collection of plaques was discontinued and it was not possible to actually evaluate the apparatus.

While the construction of the homogenizer was in
progress several attempts were made to disintegrate plaque clumps through the application of ultrasonic vibrations. Ultrasonic oscillators were made available through the courtesy of the Battelle Memorial Institute. The first test was carried out by placing the plaque material from one collection tube and about 50 ml water in a specially designed tube with thin plastic windows at each end. The tube and its contents were irradiated in an oil bath by the ultrasonic vibrations produced in the crystal layer at the bottom of the bath. Irradiation was continued for two periods of five minutes each. The liquid took on a noticeable opaqueness characteristic of bacterial suspensions. Gram stained smears showed both bacilli and cocci but rod shaped forms were not culturable on tomato juice agar at pH 5, blood agar, or tryptone glucose extract agar. Several repeated irradiations of new specimens with a reduction of current input to the oscillator were conducted. Current input as low as 75 milliamperes and exposure times as short as ten seconds gave only partial breakup of clumps and lactobacilli were still not culturable on tomato juice agar plates. Because of the difficulty in using the equipment regularly it was not possible to conduct a whole series of tests which might possibly have lead to a general successfulness of the procedure.

Growth of organisms from a plaque inoculum. Plaque specimens were inoculated into tomato juice broth with an
initial pH of 4, 5, 6 or 7. Tomato juice agar plates were inoculated and gram-stained smears were prepared after 1, 2 and 5 days incubation at 37 C. Preliminary investigations of lactobacilli isolated from these plates showed Thunberg activities of the same nature as the lactobacilli isolated from saliva and included in the results section of this paper.

II. Investigation of the lactobacilli.

Lactobacillus cultures. Most of the cultures were isolated from saliva and the details of their isolation are described in the dissertation of Green (1953). Not all of the characteristics used in Bergey's Manual (Breed et al., 1943) to differentiate this genus from other genera were determined. Thus, Lactobacillus in this paper refers to gram-positive rods isolated from small, non-pigmented colonies developing on pH 6 tomato juice agar plates incubated aerobically at 37 C. While these characteristics do not restrict isolations to a single genus, most of the cultures had other characteristics of the genus.

Stock cultures were maintained in tomato juice broth during the early part of this investigation. Rough strains grew slowly and often poorly in this medium. Shockley (1953) observed very good growth of these strains following the addition of 0.05% tween 80 (polyoxyethylene sorbitan monooleate) to the tomato juice broth. This tween 80 tomato juice was then used for all cultures. With the
initiation of adaptation studies they were carried in glucose broth prepared according to the formula for the modified lactobacillus selection medium (Rogosa et al., 1953). Fresh stock cultures were prepared after two weeks storage in the cold room. Even this relatively short interval of storage resulted in the loss of two rough cultures. Several other rough cultures were difficult to subculture on the first transfer after storage. No smooth cultures appeared to be affected. The rough and smooth classifications refer to the colonial morphology observed on primary isolation. Only eighteen cultures were replated in this laboratory (nine rough, nine smooth) and one rough culture definitely produced smooth colonies after maintenance. This one rough strain had been isolated from an immune and Green (1953) had found this type only rarely in immune individuals.

**Media for culturing lactobacilli.** Media supplemented with tomato juice have become widely employed in the isolation and subsequent cultivation of lactobacilli. The medium used by Green (1953) utilized the filtrate from whole canned tomatoes. The medium used in this laboratory was prepared from canned tomato juice. While salt had been added in the commercial production of the tomato juice no apparent effect on the growth of the organisms was observed. The tomato juice was centrifuged to remove most of the pulp and the decantate was passed through filter
paper to further clarify it. The tomato juice medium was then prepared from this filtrate and had the following composition: 400 ml tomato juice filtrate, 10 g peptone (Difco), 10 g peptonized mild (Difco), 600 ml distilled water. Broth for maintaining stock cultures was adjusted to pH 7 with sodium hydroxide. Tomato juice agar was prepared at pH 5 and 7 by adding the appropriate amount of sodium hydroxide and 2 per cent Bacto-agar (Difco). The commercial tomato juice agar (Difco) gave poor results. A great many cultures grew poorly if at all on this medium.

The appearance of the article by Rogosa et al. (1953) indicated the possibility of growing the lactobacilli in an easily prepared medium containing only one carbohydrate or polyhydric alcohol. This medium was used in their studies of the fermentation reactions of the oral lactobacilli and was modified from the selective medium (SL agar) developed earlier (Rogosa et al., 1951). In this investigation the indicator was eliminated and the resulting medium had the following composition: 1 per cent trypticase (Baltimore Biological Laboratory), 0.5 per cent yeast extract (Difco), 0.6 per cent monobasic potassium phosphate (analytical reagent, Mallinckrodt), 0.2 per cent ammonium citrate (analytical reagent, Mallinckrodt), 1.5 per cent sodium acetate (analytical reagent, Mallinckrodt), 0.1 per cent tween 80 (polyoxyethylene sorbitan monoleate), 0.00575 per cent magnesium sulfate (C. P.,
Baker), 0.012 per cent manganese sulfate (C. P., Baker), 0.0034 per cent ferrous sulfate (C. P., Baker), 1.0 per cent carbohydrate or polyhydric alcohol (C.P., Pfanstiehl). Glucose was used in preparing the broth for stock cultures. The medium was sterilized by a modified autoclaving technique. The temperature gage was elevated to 115 C. as rapidly as possible, the steam supply was terminated immediately, and the pressure was then allowed to return to atmospheric pressure with the exhaust valve closed. Good growth of both rough and smooth strains was possible with this medium containing glucose.

Thunberg dehydrogenase studies. The technic employed was essentially that described by Burris (Umbreit, Burris, and Stauffer, 1949). The tubes used in this investigation were designed for use in the Klett-Summerson photoelectric colorimeter. They were consequently smaller than those described by Burris, measuring only 1.5 x 15 cm, and the quantities of materials listed were reduced to 1 ml \( \frac{1}{15} \) phosphate buffer at pH 7.0, 1 ml 1:10,000 methylene blue, 1 ml \( \frac{1}{50} \) substrate, and 1 ml cell suspension.

All cells used in the Thunberg experiments were grown in tomato juice broth with 0.05 per cent tween 30 added. After 13 to 24 hours growth at 37 C., the cells were harvested by centrifugation, washed twice by centrifugation from distilled water, and resuspended in distilled
water. Turbidity was measured in the Beckman Model B spectrophotometer at a wave length setting of 560 millimicroons. The suspensions used were adjusted so that a 1 to 10 dilution gave a 16 per cent transmittance reading.

The other reagents were placed in the tube with the cells in the sidearm cap. After assembling, with lanolin in the ground glass joint, the tubes were evacuating for three minutes by water pump and placed in the 37 C. water bath. After five minutes, for temperature equilibration, the cells were added from the sidearm and the time required for complete reduction of the methylene blue was recorded.

The use of the pH meter to determine rates of acid production. The pH meter was used very successfully by Stephan and hennens (1947) in a series of studies on the rate of acid production by pure cultures of oral microorganisms. They emphasized the desirability of using rather heavy cell suspensions. While the concentration of cells used in this investigation was reduced greatly from theirs, it was found that a much higher concentration was necessary here than in the Thunberg experiments. With slow rates of acid production the fluctuations of the pH meter were equal in magnitude to the pH change produced by fermentation for short lengths of time.

Cells were grown in broth prepared according to the formula of the modified Lactobacillus selection medium
(Rogosa et al., 1953). Several carbohydrates and polyhydric alcohols were employed. Incubation of two 100 ml portions of broth at 37 C. yielded more than enough cells for the experiments. Cells were harvested by centrifugation from distilled water. Washed cells were resuspended in sufficient distilled water that a 1 to 10 dilution gave about 1.1 per cent transmittance of 550 millimicron light. For greater accuracy in adjusting the turbidity, the 100 per cent transmittance setting with distilled water was made at sensitivity position one on the Beckman Model B spectrophotometer, and the diluted cell suspension was observed at position four. A reading of 35 per cent at this setting is equivalent to 1.1 per cent transmittance.

The fermentations were carried out in open 50 ml beakers. The reaction mixture consisted of 1 ml cell suspension, 1 ml 10 per cent (or 2 per cent) substrate, 0.5 ml M/15 phosphate buffer pH 7, and 7.5 ml distilled water. Phosphate buffer was found necessary to stabilize the pH at the start and was also added to satisfy all requirements for inorganic phosphate. After appropriate intervals of incubation at room temperature (about 30 C.), the pH was measured by means of the Beckman Model H-2 pH meter equipped with glass and calomel electrodes. The data are plotted in the results section.

Oxygen uptake and acid production measurements by the Warburg manometric technic. The procedure followed
was essentially that described in the book by Umbreit, Burris, and Stauffer (1949). Methylene blue was added to the flasks when oxygen consumption was measured since the lactobacilli were found to possess only poor capabilities for the transfer of hydrogen to molecular oxygen. For oxygen uptake the flasks received 0.5 ml M/15 phosphate buffer at pH 7, and 0.5 ml cell suspension. The center well contained 0.2 ml 10 per cent KOH. The methylene blue was added to the sidearm (0.1 ml 1 to 10,000 dilution) along with varying amounts of substrate and water, if necessary, to bring the fluid content of the sidearm to 0.5 ml. Various cell concentrations were employed. A period of equilibration was observed to permit the temperature of the flask and its contents to reach that of the 37 C. water bath. The sidearm contents were flushed into the flask and readings were made and recorded at appropriate intervals.

For the determination of acid production modifications are necessary. The elimination of the potassium hydroxide and the replacement of the phosphate buffer by 0.5 ml 1.8 M/100 bicarbonate solution permits one to measure the carbon dioxide evolved from the bicarbonate, as acid is produced by the fermentative action of the cells. A pH of 7 was obtained, with this concentration of bicarbonate, by replacing the air in the flasks with a gas mixture containing 5 per cent carbon dioxide and
95 per cent nitrogen. Replacement of the air was accomplished by a ten minute passage of the gas mixture through the flasks. The gas was forced at a slow rate through the flasks in the 37 C water bath with the shaking apparatus in operation. The flasks were then closed and part of the excess pressure released after a period of equilibration. The sidearm contents (0.5 ml M/50 substrate) were washed into the flask and readings were taken and recorded. After conversion of observed pressure changes to volume changes, by application of the flask constants, the microliters carbon dioxide produced were plotted against elapsed time. No correction was made for carbon dioxide produced by decarboxylative metabolism. The strain employed (no. 3) was homofermentative and one preliminary experiment indicated a negligible amount of carbon dioxide production during the fermentation of glucose. Acid production from endogenous materials was also negligible (less than five microliters carbon dioxide from bicarbonate) and was neither plotted nor subtracted.

Attempts to isolate intermediates in the dissimilation of the polyhydric alcohols. Using resting cell suspensions of a Pseudomonas, Sebek and Randles (1952) were able to isolate and identify the fructose formed from mannitol and sorbose formed from sorbitol. A similar technic was employed in this investigation. A suspension of washed, sorbitol grown cells was added to three flasks
containing 100 ml two per cent sorbitol, dulcitol, and mannitol, respectively. Calcium carbonate was added to a concentration of 0.5 per cent. After ten hours incubation at 37 C. the cells were removed by centrifugation. No reducing sugar test was given by any of the combinations either before or after concentrating the liquid to a small volume (10 ml).

With sorbitol as substrate and a suspension of mannitol grown cells, and extremely faint reducing sugar test was given by the concentrated liquid portion. No attempt was made to isolate this trace substance.

**Cell free extracts.** Three attempts were made to grind strain 8 by the McIlwain (1943) technic and thus obtain activity in the absence of cells. After 24 hours growth in the presence of sorbitol, the cells were harvested and placed in a cold mortar. Finely powdered aluminum oxide (C. P. baker) was added and grinding with the pestle was carried out in the cold room. The mixture in the mortar was kept fairly dry by the addition of aluminum oxide as grinding proceeded. Extractions with distilled water or phosphate buffer gave solutions of only very slight opacity and the only activity obtained was a very slight dissimilation of glucose. Thus the technic did not appear applicable to a study of polyhydric alcohol dissimilation by this organism.
RESULTS

I. INVESTIGATION OF THE DENTAL PLAQUE

The dental plaque is credited with providing those factors necessary for caries production. It might be suspected that individuals immune to caries would be devoid of plaques or would possess plaques with different characteristics. While there has been reference to the comparison of plaques from caries-free and caries-active individuals, the feeling has existed that many of these caries-free groups were not actually caries-free but merely quantitatively less caries active. Thus it seemed pertinent to strive for a comparative study on plaques obtained from the caries-active and caries-immune individuals available for this study, since the clinical examination of those immunes revealed no evidence of past or present caries activity.

Although only a few plaques from caries immunes were made available for study, macroscopic and microscopic observations of them presented no discernible differences from the plaques of caries-active individuals. The very adhesive nature of the plaque and the firmness with which it is held together were well displayed during the preparation of specimens for microscopic study. Once the plaque material was attached to the wire of an inoculating needle, or loop, it was often difficult to transfer it to
any other surface or fluid. It became attached much more readily to slightly rough surfaces, such as an inoculating wire, than to the smooth surface of a glass slide, and was often picked up in an intact form during any attempts to smear it on the slide. No true smears were ever made although it was sometimes possible to break the plaque into smaller clumps and at the same time achieve a partial smear of the coccal forms of the plaque.

Specimens of plaque material varied greatly in size and in structural integrity. Usually very large clumps of material were fairly readily resolved into small clumps which attained the properties described above. The samples were white to grayish-white in color and of no definite shape. Frequently a stepwise effect was noted on the surface of specimens disturbed as little as possible and viewed with relatively low magnification (100x). This suggested the laying down of successive layers of plaque material but no studies were made either to verify or disprove this suggestion. Under higher magnification (970x) most clumps appeared as dark masses because of their opaqueness to light. Views of the edges of these clumps or of very small aggregates showed extremely tangled masses of filaments, with cocci filling much of the space in between. While resolution of individual forms was usually quite difficult, at least some of the filamentous forms appeared to consist of chains of bacilli and not
actually long individual filaments. The great majority of the cocci were gram-positive although a few gram-negative forms were visible in most fields. The cocci were found much more frequently free of the clumps suggesting a much greater ease of dissociation from the plaque material.

Attempts to disperse the microorganisms of the plaque did not give satisfying results. While Stralfors (1950) reports several tables of bacterial counts obtained from suspensions of plaque material he emphasizes the fact that "the greatest difficulty encountered in determining the numbers of bacteria in a material of this kind is the breaking up of the clumps of bacteria."

All the technics employed gave some dispersion of the plaque. Shaking with glass beads seem the most inefficient of the methods employed and even after fifteen minutes treatment many clumps remained. These clumps were too dense to permit any microscopic counting. The clumps appeared to be relatively free of coccal forms so that any plate counts or direct microscopic counts made on material of this nature might be expected to reveal a preponderance of cocci. Accordingly no counts were made.

Proteolytic enzymes gave little if any disintegration of the plaque and used in conjunction with the glass beads were little if any better than shaking with glass beads alone. The first homogenizers constructed by grinding two
concentric tubes together gave only slightly better disintegration of clumps. An homogenizer with a very tightly fitting inner tube was finally constructed by using fine pumice as a grinding agent. On one plaque specimen it gave a good homogenous suspension of microorganisms but at this time the collection of plaques was discontinued and material for the comparison of plaques from caries-immune and caries-active individuals was no longer available.

The ultrasonic treatment gave some promise of success since the material could be reduced to an almost complete freedom from clumps. However, bacilli observed on direct microscopic observation of smears prepared from these suspensions could not be cultivated with any regularity on tryptone glucose extract agar (Difco), blood agar, or on pH 5 tomato juice agar plates. With very short exposure times (10 to 15 seconds), and current input to the vibrating crystals as low as 75 milliamperes, only a partial disaggregation of clumps was achieved and any counts attempted would be of no validity.

Colonies of streptococci appeared after inoculating blood or tryptone glucose extract agar plates regardless of the severity of ultrasonic treatment. A few colonies of other coccoid forms were observed. No quantitative counts are reported since the object was to find a technique suitable for the comparison of the bacterial flora
from caries-immune plaques to the flora from caries-susceptible plaques and these treatments did not give suitable suspensions for such evaluation. A few tissue cells and structureless debris were found in most specimens.

When plaque specimens were inoculated into tomato juice broth at pH 6 or 7 streptococci were almost invariably the organisms found to be growing profusely for the first day. From 24 to 48 hours after inoculation bacilli resembling the lactobacilli began to appear more abundantly in smears made from the broths. After prolonged incubation smears gave all indications of being pure or nearly pure cultures of lactobacilli. When tomato juice broth at pH 5 was inoculated there was occasionally an initial growth of streptococci but usually the only organisms growing out were the lactobacilli.

Some of the cultures of both streptococci and lactobacilli growing out of the plaque specimens presented a very clumped type growth. Clumps were observed adhering to the walls of the flask or tube in several instances. This observation of a clumping tendency was later noticed with most lactobacilli isolated from rough colonies and grown in tomato juice broth. While the adherence to the glass surface was not strong, these observations indicate a possibility that streptococci or lactobacilli might play some role in holding the plaque together and to the surface of areas of the tooth isolated from the normal
cleansing action of the mouth. No immune plaques were made available at the time of these crude checks on the fate of plaque organisms when placed in a medium suitable for the growth of at least part of the flora.

Attempts to study the physiology of the plaques through a modified Taunberg technic with glucose substrate gave no reduction of methylene blue. With larger plaque samples it is conceivable that demonstrable activity could be attained. Attempts to show acid production were subject to the same difficulty, for while traces of acid could be detected in the tubes, the process was much too slow for any good comparison of the activity of one plaque to that of another. The use of an indicator to detect acid production is admittedly a rather crude technic and attempts to run the test on a semi-micro scale with very small volumes of fluid were subject to great experimental error. The color of the indicator was very difficult to control with such small volumes and color changes due to the adding of the components of the test were prevalent.

II. INVESTIGATION OF THE ORAL LACTOBACILLI

General physiological characteristics: The studies of Green (1953) showed that the saliva of immunes contained few in any lactobacilli. They were prevalent in varying but relatively high numbers in the saliva of caries active individuals. Thus it seemed that clues to this state of caries immunity, which exists in an estimated two per cent
of the population, might be attainable through a study of the physiology of the lactobacilli isolated by Groen. Subsequent correlation of this basic information with the distribution of characteristics of the lactobacilli might then serve to clarify the relationship of the lactobacilli to caries production.

Early investigations of the physiology of the lactobacilli were conducted by the Warburg direct method for measuring oxygen uptake. It immediately became apparent that this technique was of doubtful value in studying the metabolism of this microaerophilic to anaerobic group of organisms. Only a very few microliters of oxygen were consumed in the dissimilation of glucose. With the addition of methylene blue to the Warburg flasks the oxygen uptake could be increased. With some strains the values approached the theoretical amount of oxygen uptake required for the oxidation of glucose to the pyruvate level.

This use of methylene blue is comparable to a Thunberg technic modified so that the amount of oxygen required to reoxidize the methylene blue is measured. It was felt that the actual Thunberg technic could be more easily applied to the study of a relatively large number of cultures. While this technic can be made more quantitative through the colorimetric determination of the extent of methylene blue reduction, only times for complete reduction of the methylene blue were recorded for this
experiment. This was believed sufficient for the purposes of a broad classification of activity.

Tables I and II show the results of a study conducted on *Lactobacillus* strains isolated from saliva. Colonial morphology, source, and manner of isolation present the basis for the separation of the organisms. Only one rough strain from an immune was utilized in this study. Rough strains occur only rarely in these individuals.

The most obvious correlation existing in these data is the lack of sorbitol activity by all rough strains while only three of eleven smooth strains failed to reduce the methylene blue in the presence of sorbitol. In addition to being related to colonial morphology, this lack of activity is also associated largely with the susceptible state to which these rough strains are almost entirely restricted. Possible double selections of the same strain from a given individual may exist with strains 3 and 5, 32 and 33, 2 and 4, 36 and 37. The possible co-identity in the last two cases has no effect on the correlation but it is interesting to note that strains 3 and 32 are smooth while 5 and 33 are rough strains. Strains 3 and 32 are the two susceptible smooth strains showing the lack of sorbitol activity. It appears that colonial morphology may not always be a good criterion for the determination of strain differences. Fermentative characteristics have been reported to be very stable for the
# Table I

**DEHYDROGENASE ACTIVITY OF SALIVARY LACTOBACILLI HAVING A SMOOTH COLONIAL MORPHOLOGY ON PRIMARY ISOLATION**

<table>
<thead>
<tr>
<th>Strain No.</th>
<th>Source (a)</th>
<th>Primary Isolation (b)</th>
<th>Substrates (c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>S-2</td>
<td>a</td>
<td>17 min.</td>
</tr>
<tr>
<td>7</td>
<td>S-2</td>
<td>an</td>
<td>7 min.</td>
</tr>
<tr>
<td>32</td>
<td>S-5</td>
<td>an</td>
<td>Partial</td>
</tr>
<tr>
<td>2</td>
<td>S-21</td>
<td>a</td>
<td>4</td>
</tr>
<tr>
<td>4</td>
<td>S-21</td>
<td>an</td>
<td>8</td>
</tr>
<tr>
<td>17</td>
<td>I-16</td>
<td>a</td>
<td>23</td>
</tr>
<tr>
<td>19</td>
<td>I-16</td>
<td>an</td>
<td>42</td>
</tr>
<tr>
<td>20</td>
<td>I-18</td>
<td>an</td>
<td>21</td>
</tr>
<tr>
<td>21</td>
<td>I-18</td>
<td>an</td>
<td>24</td>
</tr>
<tr>
<td>22</td>
<td>I-20</td>
<td>an</td>
<td>32</td>
</tr>
</tbody>
</table>

(a) *S*-caries susceptible, *I*-caries immune

(b) *a*-isolated aerobically, *an*-isolated anaerobically

(c) Thunberg tubes received 2 ml M/15 phosphate buffer at pH 7, 1 ml 1:10,000 methylene blue, 2 ml M/50 substrate, 1 ml cell suspension (turbidity adjusted to reading of about 200 on Klett-Summerson photoelectric colorimeter for 1:10 dilution).

(d) Minutes for complete reduction of methylene blue (-, no reduction in 1 hr.) (partial, partial reduction of methylene blue after 1 hr.). No reduction in any endogenous tubes.
<table>
<thead>
<tr>
<th>Strain No.</th>
<th>Source</th>
<th>Primary Isolation</th>
<th>Glucose</th>
<th>Mannitol</th>
<th>Sorbitol</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-21</td>
<td>a</td>
<td>3 min.</td>
<td>27 min.</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>S-4</td>
<td>an</td>
<td>4</td>
<td>24</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>S-5</td>
<td>a</td>
<td>4</td>
<td>17</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>S-5</td>
<td>an</td>
<td>5</td>
<td>Partial</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>S-11</td>
<td>a</td>
<td>7</td>
<td>20</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>S-11</td>
<td>an</td>
<td>8</td>
<td>57</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>S-21</td>
<td>a</td>
<td>6</td>
<td>12</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>S-21</td>
<td>an</td>
<td>6</td>
<td>Partial</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>S-21</td>
<td>an</td>
<td>8</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>I-20</td>
<td>an</td>
<td>7</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

The footnotes of Table I also apply to this table.
lactobacilli (Rogosa et al., 1953) and sorbitol is indicated as a valuable substrate for the classification of oral *Lactobacillus* species into two large groups.

Strains 23 and 37 were the only ones showing no mannitol activity and all strains attacked glucose readily. The time required for the reduction of methylene blue with fructose as substrate was very nearly the same as with glucose for all these strains. Thus, sorbitol was the only substrate which was of value in a broad separation of the strains. Results to be shown later indicate the adaptive nature of the attack on sorbitol and mannitol so it seems that tomato juice broth may offer the materials necessary for this adaptation.

Later in the project another group of salivary lactobacilli was furnished for investigation. These organisms were equally distributed with respect to the caries activity of the subjects and the colonial morphology observed on primary isolation. However, part of the cultures were from stocks which had been maintained in tomato juice broth for some time. The correlation between roughness of colony and lack of activity on sorbitol is not as apparent in the bar graphs of figure 1 as it was in tables I and II. Only three of these eight rough strains failed to act on sorbitol. Since these strains were isolated by Green later in his work on the problem it is possible that he had now selected strains
Figure 1. Thunberg dehydrogenase activity of a group of salivary lactobacilli. Tubes received 1 ml 1:10,000 methylene blue, 1 ml M/15 phosphate buffer pH 7.0, 1 ml M/50 substrate, 1 ml cell suspension (1:10 dilution gave 16 per cent light transmission at 550 nm). No endogenous reduction in 1 hr.
which would not have been classified as rough in his earlier observations. Tilden and Svec (1952) found the rough colonial type to be characteristic of heterofermentative species and of homofermentative *Lactobacillus acidophilus*. While colonies of the other homofermentative species of oral lactobacilli were observed to be rough on occasion, they might be regarded as smooth if compared to the typical colonies of the aforementioned group. Thus one selecting definitely rough strains would choose essentially heterfermentative species and *L. acidophilus*. None of these species of oral lactobacilli dissimilated sorbitol in the study made by Rogosa *et al.* (1953). If rough colonies of the other oral homofermenters were included in a rough classification, then rough strains as a group would include sorbitol fermenters. Thus it seems that the first supply of rough organisms may have consisted entirely of the *L. acidophilus*-heterofermentative group while the second supply contained rough colonies of the homo-fermentative group. Limited fermentation tests showed that only strains 9, 17, and 18 of this second group could possibly be of the heterofermentative-*L. acidophilus* group.

From a quantitative standpoint, the rate of methylene blue reduction shown in figure 1 is slower with strains from caries susceptible than with those from caries immune individuals. This generalization holds for all three substrates although the differences with glucose are not
great, and immune rough strain nine is an exception. Later studies showed that Thunberg activity cannot be quantitatively correlated with acid production, thus this slower Thunberg activity does not necessarily imply a lower acido-sensitivity of susceptible strains.

Attempts to relate ability to grow at a low pH with the colonial morphology of the lactobacilli gave no evidence of such correlation. Only number 17 of 16 strains listed in figure 1 failed to grow in tomato juice broth with an initial pH of 4.2. Strains 9, 16, 17, and 18 did not grow at pH 3.8 and strains 11, 12, 21, and 22 grew only slightly at this pH. Thus it appears that the positive correlation is between inability to grow at the lower pH and inability or slowness of acting on sorbitol, a characteristic which appears to be more positively correlated with the caries susceptible or immune distribution of the strains. It thus appears that lactobacilli from caries-susceptible individuals are, in general, less aciduric and have lower dissimilative powers than lactobacilli from caries-immune individuals. This is especially true of the rough type which is almost restricted to the caries-susceptibles.

Carbohydrate and polyhydric alcohol dissimilation by the lactobacilli: In order to pursue an investigation of factors affecting the distribution of the lactobacilli it was considered necessary to investigate basic metabolic
patterns and tools or technics which might be readily applicable to the study of these organisms. Strain number eight of figure 1 was used for a major portion of this study. This strain from a caries-immune individual gave the most rapid reduction of methylene blue with the substrates previously tested by the Thunberg technic. Stock cultures of the strains listed in figure 1 were maintained during the remainder of these investigations in the modified Lactobacillus selection medium used by Aogosa et al. (1953) for the repetition of their fermentation tests. Glucose was added to a concentration of one per cent and the indicator was omitted. Adaptation of lactobacilli was studied by using the same medium but with glucose replaced by the desired substrate.

Figures 2 and 3 show the type of results obtainable through application of pH measurements to the study of the physiology of the lactobacilli. While the results are not all that could be desired they show that rates of substrate dissimilation can be readily determined in the absence of more elaborate equipment, such as the Warburg apparatus. The deviation from linearity in the early part of the curves was also shown in a standard curve obtained by adding known concentrations of lactic acid to the system. The leveling at the extremes was also characteristic of the addition of lactic acid and represents the minimum pH attainable.
Figure 2. pH changes produced by Lactobacillus strain S in open beakers receiving 1 ml 10% substrate, 0.5 ml M/15 phosphate buffer at pH 7, 1 ml cell suspension, 7.5 ml water. (1:10 dilution of cell suspension gave about 1 per cent light transmission at 550 m\textmu).
Figure 3. pH changes produced by Lactobacillus strain 8 in open beakers receiving 1 ml 2% substrate, 0.5 ml 0.15 M phosphate buffer at pH 7, 1 ml cell suspension, 7.5 ml water. (1:10 dilution of cell suspension gave about 1 percent light transmission at 550 mJ).
A rough idea of the extent of the conversion of glucose to acid was obtained through application of this pH measurement technique. In 10 ml of 1 per cent glucose the pH was lowered to a value equivalent to adding 81 per cent of the theoretical amount of lactic acid which could be formed by the complete conversion of glucose to lactic acid. In the presence of smaller amounts of glucose the per cent conversion would probably be even higher. Thus it seems that the dissimilation of carbohydrate by this organism is directed almost entirely along one pathway leading to lactic acid.

The sorbitol activity in all curves except the one for sorbitol grown cells represents the production of acid from the trace of reducing sugar present in the commercial product. Sorbitol grown cells produced acid more rapidly from sorbitol and mannitol, while mannitol grown cells gave only a very slow attack on sorbitol, which became evident after the thirty minute period. The rate of acid production from mannose is almost the same as from glucose regardless of the substrate present during growth. Acid production from fructose became equally rapid only with fructose grown cells. The possible hexose intermediate in the dissimilation of sorbitol or mannitol is not indicated by the data. According to the principles of simultaneous adaptation sorbose is not an intermediate. Since activity on the other hexoses is so rapid in all instances, with
the large concentration of cells employed, no conclusions can be drawn as to their presence or absence from the sorbitol and mannitol dissimilative pathways.

In an effort to obtain more definite information, acid production was followed by measuring the evolution of carbon dioxide from bicarbonate buffer in the Warburg apparatus. A more dilute cell suspension is applicable to this technic and the results of figure 4 more vividly illustrate the interrelationships among the three six-carbon polyhydric alcohols than did the pH studies. Sorbitol grown cells dissimilate all three alcohols rapidly. Dulcitol grown cells show rapid activity on only sorbitol and dulcitol, while mannitol grown cells are equally effective only on mannitol. The mannitol dissimilation by dulcitol grown cells undoubtedly represents the constitutive activity similarly observed in the pH studies reported in figures 2 and 3. The sorbitol activity by mannitol grown cells is very slow but did continue at the same rate over a three hour period of observation. The significance of this low activity is not immediately apparent and the point will again be brought up in connection with the discussion of later results. Certainly this is not a result of acid produced from the reducing sugar inherent in the sorbitol, for the pH studies indicate that only about 10 microliters of CO₂ would be evolved by this acid.

The interrelationships among the alcohols suggested
Figure 4. Acid production from poly-alcohols by Lactobacillus strain 8. Measured by carbon dioxide production from bicarbonate buffer in Warburg flasks. pH 7, 10 μmoles substrate, 0.5 ml of cell suspension which in 1:10 dilution gave 5 per cent light transmission at 550 μ. No endogenous acid production.
an investigation of the possible hexose intermediates. Although considerable constitutive activity is possessed by the organisms it was believed that some valuable information could be attained through observations on the simultaneous adaptation to the alcohol and the hexose.

Glucose, mannose, fructose, and galactose appeared to offer the greatest possibilities as intermediates. Figure 5 presents data which show the interrelationships existing among these hexoses. Acid production from galactose is very poor with all three types of cells. However, four successive periods of growth in galactose medium did not yield cells with an appreciably greater activity.

Only fructose grown cells gave a rapid dissimilation of fructose. Glucose and mannose were rapidly converted to acid by all three types of cells. Thus it appeared that analysis by the principles of simultaneous adaptation might yield some information concerning the first intermediate formed in the dissimilation of sorbitol, mannitol and dulcitol.

Figure 6 represents the results obtained following growth in the presence of these three polyhydric alcohols. Sorbitol and mannitol grown cells produced acid more rapidly from fructose than the glucose and mannose grown cells. While the activity is not as rapid as that of fructose grown cells there would seem to be at least some
Figure 5. Acid production by Lactobacillus strain 3. Measured as carbon dioxide production from bicarbonate buffer in Warburg flasks at pH 7 with 10 μmoles substrate, 0.5 ml cell suspension which in 1:10 dilution gave 30 per cent light transmission at 550 nm. No endogenous acid production.
Figure 6. Acid production by Lactobacillus strain 8. Measured as carbon dioxide production from bicarbonate buffer in Warburg flasks at pH 7 with 10 μ moles substrate, 0.5 ml cell suspension which in 1:10 dilution gave 30 per cent light transmission at 550 μμ. No endogenous acid production.
indication for the conversion of sorbitol and mannitol to fructose prior to its further dissimulation. The situation with dulcitol grown cells is difficult to analyze. It appears that none of the four hexoses is directly involved in the fermentation of this alcohol. Previous attempts to obtain more rapid activity on galactose by growth in the presence of this hexose were not successful, however, and it would thus seem that galactose is not completely eliminated as a possibility. If sorbitol and mannitol are oxidized to fructose one might expect the oxidation of dulcitol to a ketose. The ketose related to dulcitol is tagatose and none was available for use as substrate in this study.

All attempts to supplement these results through isolation of the hexose intermediates were unsuccessful. As most of the previous results indicated, the rate of dissimilation of alcohols was considerably slower than that of the hexoses. Any hexose formed would be immediately converted to acid, even by cells adapted to the alcohol and not especially increased in activity on the hexose. This is well illustrated by the fact that sorbitol grown cells produced acid as rapidly from dulcitol as from sorbitol although one would not expect sorbitol grown cells to be particularly adapted to the first intermediate in the dissimilation of dulcitol.

Inhibition of the further breakdown of hexose was
attempted but no success was achieved. Concentrations of fluoride, octyl alcohol, or copper sulfate sufficient to inhibit acid production from glucose were evidently inhibitory to the dehydrogenases for the polyhydric alcohols.

No reducing sugar was detectable after incubation of washed cells with these alcohols in the presence of 0.1M sodium fluoride, 0.03M octyl alcohol, or 0.001M copper sulfate. A study with the pH meter showed that these concentrations of fluoride and copper inhibited acid production from glucose and from the alcohols. Fluoride at a concentration of 0.01M acid production from glucose with the high cell concentration used in the experiment (1:10 dilution of cells gave 2 per cent transmission of light with a wave length of 550 m).  

It is possible that better answers to many of the questions could be obtained through a study employing cell free extracts of this organism. However, several attempts to grind the organism by the McIlwain (1948) technique gave extracts with little or no activity. Shockley (1953) observed that lactobacilli having the growth and centrifugation characteristics of this strain & were refractory to grinding in the numerous attempts which he conducted to obtain cell free extracts active on the polyhydric alcohols. He employed other grinding techniques with no greater success on organisms of this type. Heavy growths of these refractory strains became ropy after settling.
out of broth but could be resuspended fairly readily. After two to three washings the cells were very difficult to spin down in the centrifuge.

One preliminary study of a rough strain was conducted by the technique of measuring acid production with the pH meter. The relatively poor activity, in comparison with the smooth strain 8 just described, is shown in table III. The strain, number 24, was not used in any of the previously reported work. It gave reduction of methylene blue in two minutes with glucose as the substrate in Thunberg studies. It had slow Thunberg activity with mannitol (21 minutes for reduction of the methylene blue) and none with sorbitol as substrates. The rapid rate of methylene blue reduction in the Thunberg thus does not correlate with the acidogenicity of a lactobacillus.

It should be noted that this organism possesses activity on this substrate under the same conditions. While this does not constitute proof, it gives further indication of the heterofermentative nature of this organism, a rough strain with Thunberg activity characteristic of most of the rough strains studied.
### TABLE III

ACID PRODUCTION BY LACTOBACILLUS STRAIN 24,
A STRAIN SHOWING ROUGH COLONIAL MORPHOLOGY

<table>
<thead>
<tr>
<th>Substrate</th>
<th>pH after time elapsed of:</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>60 min.</td>
<td>100 min.</td>
<td>17½ hrs.</td>
</tr>
<tr>
<td>Endogenous</td>
<td>7.2</td>
<td>7.1</td>
<td>7.1</td>
<td>7.1</td>
</tr>
<tr>
<td>Glucose</td>
<td>7.2</td>
<td>6.5</td>
<td>6.3</td>
<td>4.1</td>
</tr>
<tr>
<td>Fructose</td>
<td>7.2</td>
<td>6.85</td>
<td>6.8</td>
<td>4.85</td>
</tr>
<tr>
<td>Ribose</td>
<td>7.2</td>
<td>6.85</td>
<td>6.75</td>
<td>4.25</td>
</tr>
</tbody>
</table>

(1) pH values with mannose, sorbose, orabinoose, sorbitol, and mannitol were the same as the endogenous.
DISCUSSION

At the beginning of this investigation several fundamental problems existed concerning the dental plaque and the bacterial flora of the plaque and saliva. It was accepted that, although acid decalcification may not be the only factor in the production of a carious lesion of the enamel, the proponents of acid as the primary force in dental caries have most of the facts in their favor. Practically coexistent with the acid decalcification theory is the concept that the plaque is the site of acid production, and that it confines this acid at the tooth surface in concentrations sufficient to drop the pH below the level required for the solubilizing of the hydroxyapatite of the enamel (pH 5.5 to 5.0).

The few studies of the flora of the plaque have been consistent in reporting high concentrations of microorganisms, and in finding streptococci as the most abundant of the acidogenic flora. Although Stralfors (1950) concluded that the lactobacilli constitute only a very small part of the total flora, he confirmed the observations of a few others that the lactobacilli were more abundant in the plaques of persons with caries than in those who were caries-free. Streptococci were about half a million times more abundant than lactobacilli in his studies, yet were found equally abundant in both caries-free and caries-
active subjects.

What then is responsible for the lower pH in a caries-active plaque? What is the significance of the correlation between small numbers of lactobacilli (or none) and freedom from caries? These questions were raised in addition to the long prevailing one concerning the factor or factors responsible for the union of the plaque with the tooth and its resistance to disintegration.

This investigation has certainly not answered these questions but has yielded observations which further fortify the already strong demand for an answer to some of them. The plaque can certainly be said to be very resistant to disintegration. Direct microscopic observation of the clumps of material revealed a tangled mass of filaments with cocci filling the spaces between them. Ennever et al. (1951) suggested a possible role of the Actinomyces in the attachment and structural integrity of the plaque. However, a part of the filaments observed in this investigation gave the appearance of tangled chains of lactobacilli. Lactobacilli, and especially those with rough colonial morphology, produced clumps of tangled chains when pure cultures were grown in tomato juice broth. Some of these clumps became attached to the glass wall of the tube or flask. While the union was not as firm as the attachment of plaque to tooth, conditions in the oral cavity might even permit a role of lactobacilli in plaque
formation. A few cultures of streptococci presented the same characteristics in broth culture. Possibly any organism tending to grow in tangled chains or filaments, and/or clumped by virtue of some external material of the cell, can play a role in the formation of the plaque.

Do the lactobacilli exert such an influence? They do not appear to be essential to the formation of a plaque for they were absent or were present in only limited numbers in caries-free individuals. Their presence in greater numbers in the plaques of caries-active subjects, however, might indicate a role in maintaining a thicker plaque. The importance of a thicker plaque was stressed by Stralfors (1950). He formulated an acid production diffusion theory based on experimental observations. The acid concentration at the junction of plaque and tooth was found to be directly proportional to the square of the plaque thickness (in addition to being directly proportional to the diffusion coefficient for the acid). While he neglected the role of acid destruction by the plaque flora, this would serve to strengthen the significance of a thick plaque. The dissimilation of organic acids is largely an aerobic process and would be greatly restricted in the anaerobic or weakly aerobic portions of a thick plaque.

Are the lactobacilli, even in the plaques of caries-active subjects, actually too few in numbers to significantly
contribute to the structure or activity of the plaque? From observations made during this investigation it appears that previously reported studies might easily be weighted in favor of the coccal forms. The cocci were much more easily dispersed during the various techniques employed in this laboratory. The use of ultrasonic vibrations gave dispersed preparations in which cocci, bacilli and debris were observed. However, only the cocci appeared to be cultivable suggesting that the rod-shaped organisms were more susceptible to destruction or to being rendered non-viable. Thus mild techniques tended to leave bacilli in a clumped form and more severe treatment probably reduced their numbers. This may be responsible for the observation of Stralfors (1950) that the lactobacilli constitute only a very small part of the total flora. His microscopic counts of "rods" were of the order of 10 million per mg plaque material yet he reports very few if any "rods" by plate counts on blood, tomato juice, and gentian violet agars. With saliva, however, the microscopic counts of "rods" were only about 20 times the plate counts. A suitable method for more accurately determining the flora of the plaque is needed.

Of course, it is entirely possible that the lactobacilli play little if any role in the carious process. It may be quite significant that lactobacilli can be counted on pH 5 tomato juice agar without much interference
from other genera. This also happens to be near the pH hypothesized as the maximum at which enamel decalcification can occur. Perhaps the attainment of carious conditions established selective conditions to about the same degree, and lactobacilli entering the oral cavity find an enviroment in the caries-active plaque which greatly favors them in the competition for energy and assimilable materials.

In an attempt to resolve the problem it seemed advisable to concentrate on a search for basic information which might be utilized to explain their association with the carious process. An investigation of the nutrient requirements and metabolic activities of the oral lactobacilli was conducted to furnish the foundation on which to build future studies of this problem.

It is well known that the nutrient requirements of the lactobacilli are extremely complex. Most of the media employed in the very early studies were insufficient for the growth of all the species which have subsequently been isolated from the oral cavity. The addition of tomato juice produced media which more satisfactorily provided the required nutrients. Although the many oral species which have been described will grow well on the conventional tomato juice agar (Kulp and White, 1932), all oral isolates were described as variants of \textit{L. acidophilus}. This idea was perpetuated despite scattered reports which indicated
the existence of several species. In fact as far back as 1933, Curran et al. (1933) observed: ".... the lactobacilli occurring in carious teeth are not of one species and are not usually of the acidophilus type." Not until recently (Tilden and Svec, 1952; Rogosa et al., 1953) were the oral species rigorously differentiated and identified. The former authors found that the colonial types, which had been described as variants of *L. acidophilus* (Hadley and Bunting, 1932), were somewhat characteristic of the individual species. Rough colonies appeared to be typical for the growth of the heterofermentative species and *L. acidophilus*. They found that separation of the species was often very difficult, requiring many replatings before the fermentation reactions became stabilized. Thus the reports of variability in colonial morphology and fermentation reactions may have been partially the result of a very slight and difficultly detectable contamination of one species with a second. In fact Rogosa et al. (1953) found variation in fermentation reactions to be a very rare occurrence, if strains were carefully isolated and fermentation reactions were carried out in properly prepared and nutritionally adequate media. These authors pointed out that although colonial types are distinctive for each species on a standard medium, changes in colony type are relatively easy to produce by transfer to a new environment, and these changes do not imply a change in the
fermentative pattern of the organism.

Thus colonial morphology would appear to be extremely unreliable in the classification of the organisms unless the environment could be very carefully controlled. Even then the distinction between rough and smooth is often very poor since the rough colony of one species may be smoother than the smooth colony of a typically rough organism. The ultimate goal would then be the identification of the species. While Bergey's Manual (Breed et al., 1948) makes very little use of the fermentation reactions as a basis for species differentiation, Rogosa et al. (1953) found the fermentative capabilities of the organisms to be very applicable.

The first group of salivary lactobacilli studied in this investigation showed a definite correlation between inability to dehydrogenate sorbitol and roughness of colonial morphology. The heterofermentative oral lactobacilli and *L. acidophilus* do not attack sorbitol and typically produce rough colonies. Thus early differentiation of isolates on the basis of colonial morphology seemed to separate them into heterofermentative-*L. acidophilus* and homofermentative groups.

A group of isolates obtained later in the investigation, however, did not show this correlation as well. Five of eight rough organisms in this second group possessed sorbitol activity indicating a selection of rough
homofermentative organisms. This undoubtedly resulted from designating as rough, colonies which bordered between the rough and smooth characteristics used for the first group. The colony usually formed by these sorbitol fermenting lactobacilli is very smooth and the rough form might well pass for smooth if the line were not drawn too finely, or would be designated rough by a very restrictive classification. This further emphasizes the desirability of determining the species, or at least certain fundamental fermentation reactions, in classifying the lactobacilli.

With this in mind, the physiology of one homofermentative strain was studied more thoroughly with the idea of formulating a basis for future investigation of the various types of lactobacilli isolated from the oral cavity. Since preliminary investigations had shown the adaptive nature of the activity on some substrates, it seemed that some knowledge of fermentative pathways might possibly be gained by discovering a simultaneous adaptation to one of the possible intermediates in the dissimilative scheme.

The first problem to be solved was the choice of the best method for studying the physiology of the lactobacilli. The Warburg direct method for measuring oxygen uptake did not appear to be too reliable. Oxygen uptake with most strains was small but could be increased by the addition of methylene blue. The use of methylene blue, however, did not seem to be desirable. Although the net
effect is an increase in oxygen uptake, the action on each of the enzymatic components of the system is unknown. Certainly the normal transfer of hydrogen has been affected, with perhaps a greater alteration of some hydrogen transferring systems than others. This might well lead to a false impression concerning the relative rates of attack on two different substrates. In addition, methylene blue has been shown to be toxic and thus might also inhibit some activities more than others.

The determination of rates of acid production by pH measurements had been reported by Stephan and Hemmens (1947). The application of pH measurement to this investigation yielded essentially the same clues to the metabolic pathways as those obtained when measuring acid production by the Warburg technic, although existing differences in rates of dissimilation were less evident in the former. The rates were probably more nearly equal in the pH measurements because of the heavier cell suspensions which were necessary to reduce the per cent error caused by meter fluctuations.

The manometric measurement of carbon dioxide released from bicarbonate buffer by the action of the acid produced gave the best results. Using this technic several interesting relationships were observed in the dissimilation of several hexoses and polyhydric alcohols. The rates of acid production from mannose and glucose were
high and essentially the same with all resting cell sus-
pensions of strain 8 which were tested. Thus it appears
that the activity on these two substrates is constitutive,
and that alterations in the molecules necessary to reach
some common configuration in the glycolytic scheme are pro-
duced by the action of the same enzymes or enzymes acting
at the same rate. Thus in figure 7 the mannohexokinase and
hexokinase enzymes may be identical, as may phosphomannose
isomerase and oxoisomerase (phosphohexose isomerase). The
phosphomannose isomerase and oxoisomerase activities have
been indicated as inseparable in reports on attempts to
purify the enzymes. Thus it is not known whether glucose-
6-phosphate or fructose-6-phosphate is the first inter-
mediate formed in the conversion of mannose-6-phosphate,
since an equilibrium mixture of the two is always found.
Chemical studies employing deuterium labeling show that
in alkali isomerization, fructose is intermediate between
glucose and mannose (Topper and Stetten, 1951). Thus, if
biological activity can be correlated, as it can in many
instances, fructose-6-phosphate is formed first from man-
nose-6-phosphate.

The possibility of glucose and mannose (or the same
derivative of each) being activated by the same enzyme is
indicated by the report of phosphoglucosomutase activity in
the reversible conversion of mannose-1-phosphate to man-
nose-6-phosphate (Leloir, 1951).
Figure 7. Tentative scheme for hexose and polyhydric alcohol dissimilation by *Lactobacillus* strain 8 (probably *L. casei*).
There appears to be only slight constitutive activity on fructose which may represent a limited ability of hexokinase to phosphorylate fructose. After growth in the presence of fructose the activity is considerably increased suggesting the formation of a distinct fructohexokinase. The galactose dissimilation appeared to be constitutive in nature and was very slow in all cases, even in one experiment conducted with galactose grown cells. The mechanism of its conversion as listed in figure 7 was not shown in this investigation but has been demonstrated in yeast. (See review by Leloir and Cardini, 1953). The conversion of galactose-1-phosphate to glucose-1-phosphate appears to be a true Walden inversion at the fourth carbon atom without any splitting and reconstitution of the molecule (Topper and Stetten, 1951). It has been shown to be reversible and in the absence of subsequent activity an equilibrium mixture of about 75% glucose phosphate and 25% galactose phosphate was formed. The enzyme has also been definitely demonstrated in mammalian liver.

The studies on the dissimilation of the polyhydric alcohols yielded several interesting relationships. A small amount of mannitol activity was possessed by all types of cells studied. The rate of mannitol dissimilation was significantly increased by growing cells in the presence of mannitol or sorbitol but not dulcitol.
Sorbitol grown cells were adapted to all three alcohols, and cells grown in dulcitol broth showed adaptation to dulcitol and sorbitol but not mannitol. The range of activities on these alcohols can be related to the positions of the hydroxyl groups on the asymmetric carbon atoms. In figure 8 the wavy lines are drawn to indicate the center of the molecules. It may be seen that both ends of the dulcitol molecule have hydroxyl groups in a trans-position relative to the adjacent hydroxyl. This configuration occurs again only in the upper half of the sorbitol molecule, and it was found that dulcitol-grown cells had adapted to only these two of the three alcohols. Sorbitol has both the trans-arrangement and a cis-arrangement and, indeed, sorbitol grown cells were adapted to all three alcohols. Mannitol grown cells, in addition to dissimilating mannitol, would be expected to attack sorbitol at the lower (cis-) end of the molecule. However, mannitol grown cells produced acid from sorbitol at an extremely slow rate. The absence of activity on L-sorbose and L-gulose suggested that perhaps these sugars were actually being formed but were not further dissimilated. However, no reducing sugars were detectable following the incubation of mannitol-cells in a sorbitol solution.

Another interesting aspect of this cross adaptation is the mannitol dehydrogenase activity exhibited by sorbitol grown cells. It was expected that the ability of sor-
Figure 8. Configurations of the three polyhydric alcohols, a list of the cells active on them, and a list of the hexose produced if dehydrogenation were to occur at carbons 1, 2, 5, or 6.
bitol grown cells to attack mannitol was a result of these cells having attacked the cis-end of the sorbitol molecule. The resulting sorbose or gulose should be detectable since neither of these substrates was attacked. However, no reducing sugars were detected after incubating sorbitol grown cells in a sorbitol solution. Thus it appears that this might possibly be a demonstration of enzymatic adaptation without activation of the substrate molecule which induced this adaptation.

Having established the basic interrelationships existing among the hexoses and among the polyhydric alcohols studied, there remained the problem of determining the hexose intermediates in the dissimilation of the alcohols. Sorbitol and mannitol grown cells showed a higher rate of acid production from fructose than had glucose grown or mannose grown. While the rate was lower than that of fructose grown cells, it seemed significant enough to suspect fructose as the hexose produced by the dehydrogenation of sorbitol and mannitol. Ketoses have been reported as the usual hexoses formed by the oxidation of polyhydric alcohols and aldose formation evidently is quite rare. While this does not constitute proof for the same point of attack by all microorganisms, it does establish a precedent for attack at carbon 2 and 5. Thus, in the scheme of figure 7, the dissimilation of sorbitol and mannitol is tentatively pictured with fructose as the first
The nature of dulcitol dissimilation is more speculative. Simultaneous adaptation to galactose could not be demonstrated with dulcitol grown cells. However, galactose grown cells produced acid from galactose at essentially the same, very slow rate. Therefore, galactose is not eliminated as a possible intermediate. In view of the comments made on ketose formation, we would expect d-tagatose formation here. Both these possibilities for the dissimilation are shown in figure 7. A search of the literature failed to disclose information on biochemical studies of tagatose. For it to enter the glycolytic scheme there must be an inversion at carbon 4. Therefore the hypothetical chain of events is listed in figure 7 as being similar to the steps which have been shown for galactose fermentation by yeast. Another possibility would be a path through the galactose series of compounds, probably following phosphorylation at position 6 of the tagatose molecule. No tagatose was available for study during this investigation.

The mechanisms for the dissimilation of carbohydrates and related substrates are by no means completely characterized. While certain general patterns are followed, this investigation of a *Lactobacillus* discloses the continuing need for information concerning the individual reactions of the sequence.
SUMMARY

Several technics were employed in an effort to evaluate the bacterial flora of the dental plaque. None of the methods appeared to give an equivalent treatment to all types of microorganisms and it was suggested that numbers of lactobacilli in plaque material may actually be higher than has been reported in the literature.

The ability of some lactobacilli to form rather tenacious clumps in broth culture suggested a possible contribution to plaque structure. However, the aciduric nature of the lactobacilli just as strongly suggested a selection of lactobacilli by the acidic conditions already existing in the plaque.

The physiology of the lactobacilli was studied to obtain basic information which could possibly be applied to explain the nature of their distribution. Colonial morphology could be correlated with dehydrogenase activity for one group but not for a second group of oral lactobacilli isolated at a later date. A possible explanation for this difference was discussed.

Determination of oxygen uptake showed a poor ability of the lactobacilli to couple oxidation reactions with molecular oxygen. Oxygen uptake was increased with methylene blue.

The rate of acid production was much slower from
polyhydric alcohols than from hexoses with one homofermentative Lactobacillus. Glucose and mannose were dissimilated at an equal and rapid rate by all types of cells. Adaptation to fructose was observed with fructose, sorbitol, or mannitol grown cells. These results suggested fructose as the product of sorbitol or mannitol dehydrogenation.

The ketose suspected from the dehydrogenation of dulcitol (D-tagatose) was not studied. All attempts to isolate the hexose formed in the dissimilation of the polyhydric alcohols were unsuccessful.

Cross adaptation among the polyhydric alcohols could be correlated with the molecular configuration of these substrates. Adaptation to one end of the sorbitol molecule without activity of this adaptive enzyme on the sorbitol was possibly indicated.

Attempts to produce active cell free enzyme preparations were unsuccessful.

One study of a heterofermentative lactobacillus showed a very slow rate of acid production in comparison to the homofermentative strain.
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