A STUDY OF THE ORAL ANAEROBIC GRAM-NEGATIVE COCCI, AND THEIR POSSIBLE ASSOCIATION WITH DENTAL DISEASE

by

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I. INTRODUCTION

Our knowledge of the complex mass of bacteria comprising the oral flora is fragmentary; some groups have been precisely described, whilst others have scarcely been studied. Although the anaerobic gram-negative cocci occur in very large numbers in this flora, they are a poorly defined group. Little is known of their interrelationship with other groups of bacteria in the mouth, and their possible association with oral diseases amounts to little more than conjecture. The literature pertaining to these organisms, though not extensive, is confusing and contradictory. A clarification will be attempted by considering in turn: the literature relating to the morphology and tinctorial properties of these organisms, the descriptions of their physiological characteristics, the serological studies, and finally the reports of their alleged association with various diseases.

Until comparatively recent times most authors were in agreement with the original description, by Veillon and Zuber[1] in 1898, of the morphology and tinctorial properties of these anaerobes. These workers described them as minute gram-negative cocci growing in masses, pairs, and occasional short chains. Appropriately, because of their predominant growth in masses and their very small size (diameter less than 0.5 micron), Veillon and Zuber suggested the name Staphylococcus parvulus. Foubert and Douglas[2] in 1948 stated that these organisms retained the gram stain in young cultures. Ennever[3] in 1951 confirmed

this observation. However, Langford, Faber, and Pelczar[4] in 1950 reported that none of their own strains or stock strains received from other workers, including Foubert, retained the gram stain even in young cultures. These workers described these organisms as growing predominantly as diplococci, and suggested they be classified with the anaerobic Neisseria. Hare, Wildy, Billett, and Twort[5] in 1952, who are the most recent workers to attempt a classification of the anaerobic cocci, again described them as gram-negative cocci growing mainly in masses and pairs, with occasional short chains. This description is used in the current (6th) edition of Bergey's "Manual of Determinative Bacteriology"[6] to characterize the genus Veillonella. As described in this manual the two genera, Neisseria and Veillonella, form the family Neisseriaceae. The Neisseria are described as growing mainly as pairs with flattened adjoining sides, whereas the Veillonella grow predominantly as masses. The Neisseria are larger, being on average one micron in diameter which is twice that of the Veillonella. The Neisseria are mainly aerobes though some anaerobic species are described; the Veillonella are strict anaerobes. The generic name Veillonella was suggested by Prévot[7] in 1933 as the result of his extensive study and review of the anaerobic cocci.

Subdivision of the Veillonella into species is entirely on the basis of their physiological characteristics. It follows the scheme outlined by Prévot[7]. The old Staphylococcus parvulus of Veillon and Zuber[1], now known as Veillonella parvula after Prévot[7], is the type species for the genus. It is said to ferment glucose with production of

acid and gas, to reduce nitrates to nitrites, and to produce hydrogen sulphide from polypeptides. Occasionally, it may weakly hemolyze blood and produce small amounts of indole. Three years after Veillon and Zuber's original report, Lewkowicz[8] in 1901 isolated a similar organism from the mouth of a nine day old infant. He found that his organism did not ferment glucose, did not produce hydrogen sulphide, and was unable to reduce nitrates to nitrites. Because of these physiological differences, Lewkowicz named this organism Micrococcus gazogenes alcalescens anaerobius. This quadrinomial is invalid under the rules of bacterial nomenclature; therefore, when Hall and Howitt[9] in 1925 found their isolates to have the same biochemistry as the organism described by Lewkowicz, they suggested the name Micrococcus gazogenes. Under the scheme proposed by Prévot[7], Lewkowicz's organism becomes the type for the second species in the genus and is renamed Veillonella gazogenes. The criteria suggested by Prévot for species differentiation appear unsatisfactory when the findings of recent studies are considered, but many of the older investigators doubted that there was any real difference between Staphylococcus parvulus and Micrococcus gazogenes. For example, Holman and Krock[10] in 1923, who were the first to isolate organisms of this type in the United States, reported their strains to be Micrococcus gazogenes. Branham[11] in 1928 collected a strain of Staphylococcus parvulus from Holman, and a strain of Micrococcus gazogenes from Hall. She found that neither strain fermented glucose, both strains produced hydrogen sulphide and reduced nitrates to nitrites, and furthermore that they cross agglutinated at a titer of 1:20,000. Langford,

Faber, and Pelczar[4] examining strains of Veillonella parvula and Veillonella gazogenes, supplied by Prévot, again found that neither strain fermented any sugars and both produced hydrogen sulphide and reduced nitrates to nitrites. Morris[12] in 1954 reported that some of his strains seemed to be Veillonella parvula, some Veillonella gazogenes, but most of them were intermediate in physiological characteristics. Apart from Morris[12], all other workers in recent years including Foubert and Douglas[2], Langford, Faber, and Pelczar[4], Hare, Wildy, Billett, and Twort[5] have found that the anaerobic gram-negative cocci do not ferment carbohydrates but do produce hydrogen sulphide and reduce nitrates to nitrites. Thus, all strains studied in recent times can be considered either as Veillonella parvula which have lost the power to ferment sugars, or as Veillonella gazogenes having acquired the ability to produce hydrogen sulphide and reduce nitrates to nitrites.

The discovery by Foubert and Douglas[13] in 1948 that the Veillonella were able to metabolize lactate aroused much interest in the biochemistry of this group. These two workers, as previously mentioned, considered these organisms to be gram-positive in young cultures. This fact, coupled with their biochemical findings, led them to suggest that these organisms be reclassified under the genus Micrococcus with the species name lactilyticus. Most of the investigators studying the biochemistry of these bacteria have adopted the name Micrococcus lactilyticus. Those investigating pathogenicity, or taxonomy, have tended to retain the name Veillonella, but Hare, Wildy, Billett, and Twort[5]

prefer to regard them as Anaerobic Cocci Group V in their classification. Working with strains isolated from the rumen of sheep, Johns[14,15] in 1951 showed that lactate was fermented to propionate via pyruvate, oxalacetate, malate, fumarate, and succinate. Fermentation did not take place in the absence of carbon dioxide, and the amount of propionic acid formed was related to the carbon dioxide concentration. He also noted the dehydrogenase activity of these organisms. This was further studied by Whitely and Ordal[16] in 1955 who found the enzyme to be soluble and to require a particulate extract of the organisms to effect the reduction of methylene blue. Whitely and Douglas[17] in 1951 also reported their findings on the metabolism of purines by this group. They found that hypoxanthine oxidized to equivalent amounts of xanthine and hydrogen, a previously unknown method of hydrogen production by bacteria. Wildy and Hare[18] in 1953 found that the salts of fatty acids did not alter the metabolism or morphology of the Veillonella, whereas other anaerobic cocci were affected. Hare, Wildy, Billett, and Twort[5] showed that the group metabolized citrate and tartrate as well as the other organic acids already mentioned. They also found that the Veillonella were resistant to streptomycin and were the only anaerobic cocci susceptible to polymyxin. Lackey and Fitzgerald[19] in 1955 confirmed this point in their studies of antibiotic sensitivity. This knowledge of the biochemistry and antibiotic sensitivity of the Veillonella has led to the development of selective media. Douglas[20] in 1950 described a simple medium consisting of sodium lactate and yeast extract. Following his studies on the nutritional requirements of the Veillonella, Rogosa[21]

in 1956 described a highly selective medium which contained trypticase, thioglycollate, basic fuchsin and streptomycin in addition to the ingredients employed by Douglas[20].

The serology of the Veillonella has not been given much attention. In the course of their studies on the causes of pyorrhea in convicts in San Quentin prison, Hall and Howitt[9] did make a serological study of their isolates. They found that their strains readily produced high agglutinin titers in rabbits when the usual techniques were employed. Their strains appeared to be serologically homologous. Twenty-two of twenty-four strains studied fell into one serological group; the remaining two strains constituted a second group. They also tested the agglutinin titer of eighteen human sera against strains of Veillonella isolated from the same individuals. They found that nine of the individuals had agglutinins, though the highest titer recorded was 1:40. The subjects with the highest titers also had the most healthy gingival condition, whereas the group with no titer had the poorest. These authors suggested that the presence of agglutinins to Veillonella in the serum of an individual might constitute an immunity mechanism to periodontal disease. Hemmens and Harrison[22] in 1942 found that the sera they produced in rabbits was strain specific and of no value for purposes of classification.

By use of their respective selective media, both Douglas[20] and Rogosa[21] were able to show that the Veillonella occur in enormous numbers in the mouths of humans and many laboratory animals. Earlier investigators, unaware of this prolific occurrence in the normal oral

flora, isolated the organism from various disease states and suggested a cause and effect relationship. The isolation of Veillonella from cases of scarlet fever and measles by Thomson[23] in 1923, and Branham's[24] isolations from cases of influenza in 1926 are examples. A great many similar reports can be found in the older literature. In more recent times, Loewe, Rosenblatt, and Alture-Werber[25] in 1946 reported a case of subacute bacterial endocarditis caused by Veillonella gazogenes, but their criteria for classifying the organism isolated as Veillonella are hardly admissable. McEntegart and Porterfield[26] in 1949 isolated the organism from the blood following dental extractions. Benstead[27] in 1950 has made isolates from bronchoscopy specimens. Hall and Howitt[9], Branham[11], and many other investigators have studied the pathogenicity of the Veillonella for laboratory animals. The results in the main indicated non-pathogenicity, but Howitt[28] in 1930 offered good evidence that massive injections may be lethal to rabbits and injection of smaller numbers may cause localized abscesses or purulent joint lesions. Hall and Howitt[9] were the first to suggest possible association of this group with dental disease. Their isolation and serological studies on prisoners in San Quentin prison have already been mentioned. Hemmens and Harrison[22] isolated Veillonella from the gingival crevices of seventy per cent of healthy mouths and from forty-eight per cent of diseased mouths. They found no difference in the percentage occurrence in the crevices of normal and diseased monkeys. When injected into the gingival papillae of monkeys, these organisms produced only a transient inflammation. Hemmens, Blayney, Bradel, and Harrison[29] in 1946 found

Veillonella in twenty per cent of plaques which eventually gave rise to a carious lesion and in those under which the enamel remained sound. Ennever[3] found the organism in all the plaques he investigated. Douglas[20] suggested that the Veillonella, by virtue of their ability to metabolize lactate to the salts of weaker acids, might constitute a protective factor with regard to dental caries. Bibby[30] in 1956 suggested that ways be sought of increasing the numbers of oral Veillonella, as this might enhance resistance to dental decay.

Three aspects of the oral Veillonella have been investigated and are reported in this thesis. First, an attempt has been made to clarify the taxonomy of the Veillonella by studying the tinctorial and biochemical properties of pure strains isolated from saliva specimens. Second, the possible connection between these organisms and dental caries has been appraised by establishing the quantitative occurrence of Veillonella in the salivas of caries active and caries inactive groups of children. Third, the possibility that a relatively high agglutinin titer to Veillonella organisms confers immunity to periodontal disease, has been investigated by measuring the individual titers of a periodontally healthy and a periodontally diseased group of adults.

II. MATERIALS AND METHODS

A. Materials

1. Saliva. Specimens of saliva were obtained by having the subjects chew a small pellet of paraffin wax for two to three minutes and expectorate into a screw-capped bottle. The specimens from the two groups of children used in the dental caries study were collected one to three hours after breakfast, transferred to the laboratory, and refrigerated until cultured. Veillonella counts were performed within eight hours, and Lactobacillus counts within twenty-four hours of collection. Other saliva specimens were collected at varying times and were plated immediately.

2. Blood Serum. Approximately 5 ml. of blood was obtained from the median basilic vein, by the usual technique, from all subjects in the periodontal study. The blood was placed in a 15 ml. centrifuge tube, allowed to clot at room temperature, and refrigerated overnight. The clot was loosened with a sterile glass rod and the tube centrifuged for fifteen minutes to obtain a clear serum. The serum was removed with a sterile pipette and stored in small sterile test tubes. One drop of one per cent solution of merthiolate in borax was added as preservative.

3. Media.

a. Douglas Lactate Medium[20]. This was made up as follows:

10 grams of Yeast Extract (Difco) was dissolved in 500 ml. of distilled water. To this was added 20 ml. of a fifty per cent solution of lactic acid. The solution was adjusted to pH of 7.4 with twenty per cent sodium hydroxide to provide the desired concentration of sodium lactate. Twenty grams of agar was added and the volume adjusted to one liter with distilled water. The agar was omitted if the lactate broth was desired.

b. Modified Douglas Lactate Agar. This was made as described above except the pH was adjusted to 8.8, and 10 ml. of a 1:5000 solution of brilliant green dye was added per liter, which gave a final concentration of 1:500,000 brilliant green in the medium.

c. Rogosa SL Agar (Difco).

d. Glucose Broth (1%).

e. Nitrate Broth (0.5% potassium nitrate)

f. Thioglycollate Broth (Difco). This medium was used for testing production of hydrogen sulphide and indole.

g. Nutrient Broth (Difco), Proteose Peptone No. 3 (Difco). These two media, with and without the addition of 0.5% l-argine hydrochloride, were used to test for ammonia production.

Except for the Rogosa SL agar which was not autoclaved, and the glucose broth which was autoclaved at ten pounds pressure for fifteen minutes, all media were autoclaved at fifteen pounds pressure (121 degrees centigrade) for fifteen minutes.

4. Stains and Reagents.

a. Fixative. Methanol Formalin Solution. This consisted of ninety parts of methyl alcohol and ten parts formalin.

b. Stains.

(i). Ammonium Oxalate Crystal Violet. Crystal violet 2.0 grams, ethyl alcohol (95%) 20 ml., ammonium oxalate 0.8 grams distilled water 80 ml.

(ii). Gram's Iodine. Iodine 1.0 gram, potassium iodide 2.0 grams, distilled water 300 ml.

(iii). Safranin. Safranin O (2.5% solution in 95% ethyl alcohol) 10 ml., distilled water 100 ml.

c. Decolorizing Solution. Ethyl alcohol (95%)

B. Methods

1. Veillonella Counts. The saliva specimens were shaken for five minutes on a Kahn mechanical shaker. From each specimen 0.5 ml. saliva was pipetted into a test tube containing 4.5 ml. of sterile water. After mixing, 0.5 ml. was conveyed to a second tube of 4.5 ml. sterile water, then likewise to a third tube. From this third dilution tube (1:1000), 0.1 ml. was inoculated onto a plate of modified Douglas lactate agar and spread over the surface by means of a sterile bent glass rod. Thus, the number of colonies arising on these plates represented

the number of Veillonella present in 0.0001 ml. of the original saliva specimen. After seventy-two hours incubation, the colonies were clearly visible. All the colonies occurring on a given plate were counted by means of an electric solenoid counter, one electrode of which was embedded in the agar and the other needle electrode of which completed the circuit by touching the individual colonies.

2. Lactobacillus Counts. The saliva specimens were shaken for five minutes on a Kahn mechanical shaker. From each specimen 0.5 ml. of saliva was pipetted into a tube containing 4.5 ml. sterile water. After mixing, 0.1 ml. was pipetted onto a plate of Rogosa SL agar and spread over the surface by means of a sterile bent glass rod. After four days incubation, plates were counted with the aid of a dissecting microscope (magnification x7), Frost counting card #4099, and the electric solenoid counter. The number of colonies per plate represented the number of lactobacilli present in 0.01 ml. of the original saliva.

3. Incubation. All counts, isolations, and biochemical tests were incubated under strictly anaerobic conditions. Standard Brewer anaerobic jars and specially adapted pressure cookers were used for this purpose. The pressure cookers had inlet and outlet valves sealed into the lid, and a sealed heating element with external connecting plug. These adapted pressure cookers enabled large numbers of petri plates or racks of test tubes to be incubated. Jars and cookers were exhausted by means of an electric vacuum pump to 565 mm. mercury below atmospheric pressure and then filled with a mixture of ninety per cent hydrogen and ten per cent carbon dioxide. In the case of the Brewer

jars, the procedure of exhausting and replacing with the hydrogen-carbon dioxide mixture was repeated twice. The technique differed slightly with the pressure cookers. With these, after the evacuation of air, the hydrogen-carbon dioxide gas was allowed to flow into the pressure cooker until the pressure within was slightly above atmospheric pressure. The outlet valve was then opened and the gas mixture flushed through the pressure cooker for thirty seconds. Both inlet and outlet valves were then shut simultaneously. This sealed the pressure cooker with an internal pressure of hydrogen-carbon dioxide gas a little above atmospheric pressure. Current was applied to the heaters in both types of jar for fifteen minutes to ignite the remaining oxygen with hydrogen. The temperature for all incubations was 37 degrees centigrade.

4. Isolation of Pure Strains. With the exception of a strain of Micrococcus lactilyticus (#221) kindly supplied by Dr. H. C. Douglas of the University of Washington, all strains of Veillonella in this study were isolated from specimens of saliva. Following primary isolation on modified Douglas lactate agar, two successive subcultures at forty-eight hour intervals were made on Douglas lactate agar from selected colonies. A third subculture was made to tubes of meat mash broth. These tubes were kept at room temperature for twenty-four hours and then held in the refrigerator until required. By the time the periodontal studies were in progress it was known that only one subculture onto Douglas lactate agar was necessary to obtain a pure strain. Therefore, after one subculture, two slants of Douglas lactate agar were inoculated and incubated for forty-eight hours to provide a sufficient

suspension of cells for agglutination tests.

5. Gram Stain. Smears were made on 22 x 40 mm. coverslips. These were fixed in methanol formalin for two minutes. The procedure for gram staining was exactly that described as the Hucker modification of the gram stain in the "Manual of Methods for the Pure Culture Study of Bacteria"(31). Staining jars were filled with fresh stains before each batch of coverslips was stained. Timing was by stop watch.

6. Biochemical Tests. These were incubated for 72 hours. Ability to ferment glucose was tested by measurement of pH using a pH meter. No change in pH, or an increased pH value was accepted as evidence that glucose was not fermented. Tubes inoculated with Veillonella and uninoculated control tubes were measured.

The tests for hydrogen sulphide, indole, and nitrite were those suggested in the "Manual of Methods for the Pure Culture Study of Bacteria"(31). Lead acetate paper strips were used for the detection of hydrogen sulphide, and paper strips impregnated with oxalic acid for the detection of indole.

To test for ammonia production, 0.5 ml. of Nessler's reagent was added to 0.5 ml. of culture filtrate. Development of a deep orange color indicated a positive test.

The test for catalase was as follows: one to two drops of a ten per cent solution of hydrogen peroxide were placed on a colony on the surface of an agar plate and observed with the aid of the dissecting microscope. Development of effervescence indicated a positive test.

7. Agglutinations. These were prepared in 13 x 100 mm. serological tubes arranged in series of twelve in wire racks. To each tube 0.5 ml. of 0.4% sodium chloride was added. An equal amount of serum was added to the first tube and, after mixing, 0.5 ml. transferred to the next tube. This procedure was repeated until eleven serial dilutions of the serum were prepared, the range of which was 1:2....1:2048. The twelfth tube served as the control. Antigen was prepared by washing the cells from two slants of Douglas lactate agar with 1.5 ml. of 0.4% sodium chloride for each tube. This provided about 1.5 ml. of suitable cell suspension, of which 0.1 ml. was placed in each tube in the series. The tubes were gently shaken and incubated overnight at 50-55 degrees centigrade. Readings were made in daylight with the aid of a hand lens, and agglutination was recorded as either positive or negative for a given tube. That is, anything less than the conventional (four plus) agglutination was recorded as negative.

III. RESULTS

1. Selective Medium. Early experience with Douglas lactate agar indicated that it was not sufficiently selective in respect to the Veillonella, since other organisms, particularly gram-positive rods and cocci, were able to form colonies on this medium under anaerobic conditions. To increase selectivity various modifications of the medium were tried. The pH was varied, or inhibitory substances such as sodium azide or dyes were incorporated in the medium. It was found that the Veillonella could grow within a pH range of 5.5 to 9.2. At pH values above 8.0 the selectivity of the medium was improved without affecting the growth of the Veillonella. A more important finding was that the Veillonella were able to grow in media which contained high concentrations of brilliant green dye (tetraethyl-diamino-triphenylmethane sulphate). Growth of Veillonella organisms occurred in Douglas lactate broth containing brilliant green at a concentration of 1:5000. These organisms were able to reduce brilliant green to its colorless leuco-form, presumably by their dehydrogenase activity. Broth cultures containing the dye at a concentration of 1:10,000 were completely decolorized after forty-eight hours incubation. The color did not return on exposure to air, but if a little of the broth was placed in a small U-tube and an electric current passed, color returned at the anode. It would appear that the reduced dye gave up an electron at the anode and was thereby oxidized to the original resonating colored form. Douglas lactate agar which

contained brilliant green at an optimal concentration of 1:500,000, and had a final pH of 8.2 to 8.4 after autoclaving, was found to be completely selective for Veillonella organisms occurring in saliva specimens. Gram-negative organisms grew readily on the medium; it was therefore of no value for isolating Veillonella from fecal specimens. Decolorization of the dye is seen frequently but not invariably in agar plates. When plates of this medium, which had been spread with saliva diluted 1:1000, were examined after forty-eight hours incubation, all the colonies consisted of gram-negative cocci. These colonies were circular, pulvinate, and had an entire margin. They were smooth and glistening, with a slight cream coloration. Size ranged from 0.5 mm. to 3.0 mm. depending on how crowded the colonies were on various parts of the plate. With age the colonies became umbonate and acquired a more pronounced yellowish coloration. Any deviation from the foregoing description was a certain indication that organisms other than Veillonella were growing symbiotically within the colony. Although these colonies still consisted primarily of Veillonella, gram-positive rods, yeasts, and large gram-negative or gram-positive diplococci could be demonstrated. These contaminated colonies had an irregular, rough, craggy appearance and were yellow-gray in color. These occurred only on crowded plates and obviously should be avoided for isolation of pure strains.

2. Gram Stain. The publications of Foubert and Douglas[2] and Ennever[3] indicated that the Veillonella were gram-positive in young cultures, but became gram-negative after four to ten hours of incubation. Other workers have been unable to confirm this. Bartholomew and Mittwer[32] in 1952

stated that before an organism be called gram-positive it should be able to withstand decolorization sufficient to render species of Neisseria gram-negative. The gram stain is an important differential criterion in the classification of bacteria. It is especially important in the case of the Veillonella because if these organisms do retain the gram stain in young cultures, then they must be placed in the genus Micrococcus. Therefore, since the result of the gram stain determines the genus to which these organisms may be assigned, the matter warranted further investigation. It was desired to inspect a given strain of Veillonella at each hour of its incubation for the first eight hours. To avoid opening an anaerobic jar at hourly intervals, thereby disturbing the incubation, eight Spray dishes were employed for each strain studied. Four strains of Veillonella isolated from saliva specimens and a strain of Micrococcus lactilyticus (#221) were studied in the following manner. Eight Spray dishes containing Douglas lactate agar were inoculated with 0.1 ml. of an eighteen hour culture of the selected organism and the inoculum spread over the surface of the agar with a sterile bent glass rod. The dishes were then placed in the rim of the cup and sealed with melted agar. The cup contained pyrogallic acid and sodium hydroxide separated by a small partition. When the agar had solidified, the pyrogallic acid and sodium hydroxide were mixed to provide anaerobic conditions. The dishes were incubated at 37 degrees centigrade. One dish was removed every hour from the time of initial incubation. Upon opening the dish, impression smears of the surface growth were made and fixed and stained as previously described. Tubes

of lactate broth were also inoculated at the same time as the Spray dishes and incubated in an anaerobic jar. The jar was opened at two hour intervals, one tube removed, and then anaerobic conditions produced again as quickly as possible by the method described. Cultures grown in the Spray dishes and in broth were found to be invariably gram-negative regardless of the age of culture. A simpler incubation procedure was adopted for the study of a further thirty strains of Veillonella. These were incubated in batches of ten plates and ten tubes in Brewer anaerobic jars. Gram stains were performed after three hours growth on Douglas lactate agar and in Douglas lactate broth. All smears proved to be gram-negative. Before each series of Veillonella smears were stained, a mixed smear of Neisseria catarrhalis and Bacillus subtilis was stained as a control. No evidence that the Veillonella were gram-positive, even in young cultures, was found in this study.

3. Biochemical Tests. The results of these tests were remarkably uniform. Ninety-six strains isolated from saliva specimens were studied. No strains fermented glucose. The pH of the glucose broth increased 0.1 to 0.2 pH units as compared with uninoculated control tubes. All strains produced hydrogen sulphide, reduced nitrates to nitrites, and were catalase positive. No strains produced indole; an indole producing strain of Escherichia coli was used as control. All strains produced ammonia from nutrient broth and peptones. The reaction with Nessler's reagent was markedly intensified when l-arginine hydrochloride was added to the medium. These results in general were in agreement with the findings of other investigators who have studied the Veillonella in recent years.

Apart from Morris[12], who found that some of his strains fermented glucose but did not produce hydrogen sulphide nor reduce nitrates, all other workers have found that all their strains were unable to ferment glucose or produce indole but were able to produce hydrogen sulphide and reduce nitrates to nitrites. Foubert and Douglas[2] found their strains to be catalase positive, which accords with the findings presented here, but Langford, Faber, and Pelczar[4], and Morris[12] reported their strains to be catalase negative.

4. Dental Caries. Two groups of children were used in this study, one group from the Washington State School of the Deaf, Vancouver, Washington, and the other from the Chemawa Indian School in Salem, Oregon. One-hundred and fifty children at each of these institutions were participating in a study which included evaluation of their caries activity. From this data it was known that the children at the Washington State School of the Deaf had a much higher caries activity than the Navajo Indian children at the Chemawa Indian School. Sixty of the Washington children with very high caries activity and sixty Indian children with very low caries activity were selected for study in this project. The ages of the sixty children from the Washington State School of the Deaf, calculated on the basis of the age last birthday at the first examination, ranged from seven to eighteen years with a mean age of 11.2 years. The ages of the Indian children could not be established with certainty, but ranged from eleven to eighteen years with a mean age of approximately fourteen years. Dental examinations were performed in September 1955 and September 1956; a different examiner was employed for each group.

Examinations were made with mouth mirror and explorer, using a dental spotlight for illumination. Posterior bite-wing radiograms, two films for each subject, were taken at each examination. For various reasons some children did not return to school after the summer vacation and as a result only fifty of the Washington State School of the Deaf group, and forty-five of the Navajo Indian group were available for the second examination. The caries incidence was calculated from the data obtained from these smaller groups. The findings of the dental examinations were calculated in terms of decayed, missing, and filled (DMF) surfaces. At each examination any surface which was decayed or filled, or decayed and filled, or missing, was counted as one DMF surface. The Veillonella and Lactobacillus counts were performed on single saliva specimens collected about half way through the study period (February or March 1956). As the Navajo Indian children were known to have unusually low Lactobacillus counts, their salivas were not diluted when these counts were performed; instead, 0.1 ml. of saliva was plated directly. The results of the Veillonella count, Lactobacillus count, and the dental examinations for each subject in the study are listed for comparison in Table I and Table II.

TABLE I

Comparison of Veillonella and Lactobacillus count with dental caries activity for children at the Washington State School of the Deaf.

Case Number	Veillonella Count x 10^{-6}	Lactobacillus Count x 10^{-5}	D.M.F. Surfaces Exam. 1	D.M.F. Surfaces Exam. 2	Caries Incidence D.M.F. Surfaces
5002	1.96	1.8	9	10	1
5005	0.98	0.056	19	27	8
5010	5.61	0.77	16	27	11
5018	16.98	0.304	14	20	6
5022	0.06	6.4	76	-	-
5028	7.24	0.73	11	16	5
5037	4.2	0.184	15	22	7
5039	1.88	0.39	37	49	12
5044	0.73	0.18	18	22	4
5046	6.42	1.46	15	-	-
5050	2.98	1.08	14	19	5
5051	1.34	2.02	38	41	3
5058	0.18	1.05	17	21	4
5059	13.8	9.32	21	28	7
5066	3.98	0.28	18	25	7
5083	2.84	8.62	41	58	17
5088	7.2	0.47	52	57	5
5093	6.0	0.4	9	13	4
5102	6.21	13.9	5	12	7
5103	2.61	0.56	10	13	3

TABLE I - continued

Case Number	Veillonella Count x 10^{-6}	Lactobacillus Count x 10^{-5}	D.M.F. Surfaces Exam. 1	D.M.F. Surfaces Exam. 2	Caries Incidence D.M.F. Surfaces
5111	13.4	13.6	10	16	6
5114	9.43	20.9	6	10	4
5115	4.97	0.093	6	9	3
5123	1.29	10.6	2	7	5
5124	3.41	0.56	31	37	6
5128	3.58	0.96	6	7	1
5137	0.76	0.17	38	40	2
5139	4.21	8.5	0	8	8
5141	8.7	18.8	13	14	1
5142	4.74	0.376	5	9	4
5146	1.06	3.04	1	4	3
5147	0.19	7.1	2	15	13
5149	0.51	1.68	11	18	7
5152	1.8	0.79	43	-	-
5154	9.56	0.382	4	7	3
5510	2.14	1.18	17	-	-
5511	1.76	9.1	18	-	-
5513	9.1	1.56	3	3	0
5532	1.36	0.45	39	44	5
5538	8.29	5.9	33	-	-
5541	0.48	7.1	65	67	2
5551	1.37	4.9	9	9	0
5553	0.31	5.2	37	42	5
5557	2.71	0.196	13	22	9

TABLE I - concluded

Case Number	Veillonella Count x 10^{-6}	Lactobacillus Count x 10^{-5}	D.M.F. Surfaces Exam. 1	D.M.F. Surfaces Exam. 2	Caries Incidence D.M.F. Surfaces
5558	1.68	0	6	15	9
5559	2.9	1.06	54	-	-
5567	4.47	0.163	13	-	-
5568	5.96	7.8	21	35	14
5570	0.43	0.042	14	13	0
5575	3.92	6.6	9	10	1
5578	0.15	0.67	4	6	2
5580	0.05	7.4	59	65	6
5584	6.65	0.66	5	9	4
5588	4.58	0.41	8	8	0
5590	8.67	18.3	4	-	-
5591	9.41	6.24	76	-	-
5602	4.53	9.7	8	9	1
5606	1.62	15.1	2	3	1
5607	5.16	5.6	13	13	0
5608	1.74	6.8	12	16	4
Mean	4.17	4.328	19.6	21.4	4.9

TABLE II

Comparison of Veillonella and Lactobacillus count with dental caries activity for children at the Chemawa Indian School.

Case Number	Veillonella Count x 10^{-6}	Lactobacillus Count	D.M.F. Surfaces Exam. 1	D.M.F. Surfaces Exam. 2	Caries Incidence D.M.F. Surfaces
1002	5.21	0	14	-	-
1003	2.82	0	2	4	2
1012	0.98	0	3	3	0
1013	0.78	0	6	6	0
1014	1.94	0	14	14	0
1015	3.62	0	4	4	0
1016	3.59	0	7	7	0
1019	0.72	0	2	2	0
1023	8.15	0	8	8	0
1025	2.43	0	15	15	0
1026	0.82	0	3	3	0
1032	9.83	0	18	21	3
1035	1.96	0	8	8	0
1046	8.51	0	6	8	2
1049	1.19	0	1	1	0
1055	6.04	0	10	-	-
1061	1.27	310	11	12	1
1063	5.92	0	2	3	1
1064	0.76	0	5	5	0
1066	6.41	0	5	5	0

TABLE II - continued

Case Number	Veillonella Count x 10^{-6}	Lactobacillus Count	D.M.F. Surfaces Exam. 1	D.M.F. Surfaces Exam. 2	Caries Incidence D.M.F. Surfaces
1067	1.06	0	4	-	-
1070	3.21	0	2	-	-
1072	1.11	0	5	5	0
1073	1.25	0	3	3	0
1074	0.87	0	3	-	-
1075	4.01	0	5	-	-
1080	6.93	0	4	4	0
1081	2.94	0	13	-	-
1086	4.41	360	6	6	0
1087	3.82	0	4	5	1
1091	2.18	0	1	-	-
1099	2.22	0	7	7	0
1100	0.99	0	2	-	-
1104	3.91	0	6	6	0
1106	5.72	0	8	8	0
1112	5.31	0	8	14	6
1114	3.31	0	16	-	-
2001	0.63	0	1	-	-
2002	3.78	0	0	0	0
2005	3.62	0	2	-	-
2006	1.40	0	0	2	2
2008	9.84	0	18	20	2
2011	11.40	0	8	8	0
2012	1.87	0	1	1	0

TABLE II - concluded

Case Number	Veillonella Count x 10^{-6}	Lactobacillus Count	D.M.F. Surfaces Exam. 1	D.M.F. Surfaces Exam. 2	Caries Incidence D.M.F. Surfaces
2017	10.04	0	0	0	0
2018	0.62	0	0	0	0
2020	7.30	0	10	13	3
2025	8.80	0	2	2	0
2034	1.05	0	0	0	0
2035	2.39	690	0	0	0
2036	4.67	0	6	6	0
2040	5.12	0	2	2	0
2041	7.31	0	5	7	2
2043	2.31	460	16	17	1
2044	2.71	1400	1	-	-
2048	2.96	0	2	-	-
2049	0.38	0	17	18	1
2050	3.20	0	2	2	0
2057	7.84	1900	2	-	-
2060	4.09	0	9	9	0
Mean	3.65	85.3	5.75	6.53	0.60

A summary of the data in Tables I and II is presented in Table III. This table reveals that although these two groups differed markedly in caries activity and Lactobacillus count, there was little difference in Veillonella count. The children from the Washington State School of the Deaf had high caries activity and high Lactobacillus counts, whereas the reverse was the case for the Navajo Indian children. The Veillonella counts were somewhat higher for the children from the Washington State School of the Deaf than for the Navajo Indian children. The variances of the Veillonella counts for each group differed significantly at the five per cent level when an F-test was applied. Therefore, a modified t-value calculated according to the recommendations of Stearman[33] in 1955 was employed for the t-test. The result of the t-test revealed that there was no statistically significant difference between mean Veillonella counts when tested at the five per cent level.

TABLE III

Summary and statistical analysis of data presented in Table I and Table II.

Washington State School of the Deaf

	Veillonella Counts x 10^{-6}	Lactobacillus Counts	Caries Incidence D.M.F. Surfaces
Mean	4.17	432,800	4.9
Range	16.93	2,090,000	17.0
Standard error of the mean	0.481	68,640	0.543

Chemawa Indian School

	Veillonella Counts x 10^{-6}	Lactobacillus Counts	Caries Incidence D.M.F. Surfaces
Mean	3.65	85	0.60
Range	11.02	1900	6.0
Standard error of the mean	0.353	41.3	0.178

5. Periodontal Disease. Early work with sera produced in rabbits had shown that to obtain reproducible results the agglutinations had to be incubated at 55 degrees centigrade. The concentration of sodium chloride in the electrolyte also had to be reduced to 0.4 per cent to eliminate spontaneous agglutination in the control tube. Two groups of adults were studied. Twelve female and twelve male patients undergoing treatment in the Department of Periodontia formed the study group. The control group consisted of staff members and students and also was made up of twelve females and twelve males. All subjects in the study group exhibited marked periodontal pocketing, whereas the control subjects had no periodontal pocketing and no previous history of gingivitis. Inevitably, the scarcity of older persons with healthy periodontal conditions led to a lower mean age in the control group than in the study group. The mean titer of the control group was slightly higher than the study group, but this difference was not statistically significant when tested at the five per cent level. The mean titer of females was higher than that for the males, though again the difference was not significant. These findings indicated that there was no relationship between periodontal disease and agglutinin titer to Veillonella. Table IV lists the age last birthday, sex, and agglutinin titer to Veillonella of each subject in the study.

TABLE IV

Comparison of agglutinin titer to Veillonella of a periodontally diseased group of adults and a periodontally healthy control group.

Study Group				Control Group			
Case No.	Age	Sex	Titer	Case No.	Age	Sex	Titer
P1	29	F	1:16	C1	26	F	1:32
P2	42	F	1:8	C2	23	F	1:64
P3	41	F	1:8	C3	23	F	1:8
P4	45	F	1:16	C4	42	F	1:32
P5	23	F	1:32	C5	25	F	1:32
P6	48	F	0	C6	26	F	1:8
P7	40	F	1:32	C7	38	F	1:2
P8	36	F	1:16	C8	32	F	1:32
P9	25	F	1:4	C9	40	F	1:8
P10	35	F	1:64	C10	40	F	1:8
P11	29	F	0	C11	47	F	1:64
P12	36	F	1:64	C12	42	F	1:16
P13	35	M	1:16	C13	30	M	1:8
P14	31	M	1:8	C14	26	M	1:16
P15	50	M	1:16	C15	34	M	1:4
P16	41	M	0	C16	30	M	0
P17	33	M	0	C17	35	M	1:32
P18	48	M	1:16	C18	36	M	1:64
P19	36	M	0	C19	34	M	1:4
P20	46	M	1:8	C20	29	M	1:16
P21	57	M	1:16	C21	32	M	0
P22	34	M	1:8	C22	31	M	1:2
P23	36	M	1:32	C23	30	M	1:32
P24	38	M	1:8	C24	53	M	1:8
Mean	38.0		1:16.2		33.3		1:20.5

IV. DISCUSSION

The development of highly selective media for the Veillonella renders their isolation a simple and rapid procedure; large numbers of pure strains can be readily obtained and studied. This should lead to more satisfactory taxonomical criteria for the genus Veillonella than are available at present. The medium used in this work has the disadvantage in that, while it is completely selective, it is not optimal. Only those Veillonella organisms able to tolerate the concentration of dye employed will be selected. The medium can be rendered more optimal only by the sacrifice of selectivity. In contrast, the media devised by Douglas[20] and Rogosa[21] are more optimal but less selective. The criterion of larger colony size, which is used with these media to distinguish the Veillonella from contaminants, is adequate only when the plates are sparsely seeded. With more crowded plates it becomes increasingly unreliable. The numbers of Veillonella occurring in saliva samples may vary by several millions, and some plates are certain to be crowded at a given dilution. If an incompletely selective but optimal medium is employed, several plates must be seeded over a range of dilutions of saliva to establish a total Veillonella count for a given saliva specimen. Although this method is undoubtedly more reliable than plating a single fixed dilution of saliva on a more selective less optimal medium, when large numbers of saliva specimens are to be cultured the latter method is preferable. This is

especially true where only the relative occurrence of Veillonella in saliva specimens is being studied. All the strains of Veillonella used in this study were isolated by means of a highly selective sub-optimal medium. Therefore, the results of the biochemical tests apply only to those Veillonella organisms which were able to grow at the concentration of brilliant green dye (1:500,000) used in the selective medium. The possibility remains that some organisms of this group, which were unable to grow at the concentration of dye employed, could differ in their biochemical characteristics.

The anaerobic gram-negative cocci classified as Veillonella constitute a valid genus. They are as well demarcated from other related groups of bacteria as most other accepted bacterial genera. The suggestion of Langford, Faber, and Pelczar[4] that the generic description should read "anaerobic gram-negative diplococci" should not be adopted, as practically all other workers describe their growth to be predominantly as masses of cocci. The suggested transfer of the Veillonella to the genus Micrococcus on the basis of being gram-positive in young cultures has little to recommend it. Only Foubert and Douglas[2] and Ennever[3], of all the workers who have studied the Veillonella, consider them to be gram-positive in young cultures. The work of Langford, Faber, and Pelczar[4], Morris[12], and that reported here, in which the gram reaction was especially studied, produced no evidence of gram-positivity in young cultures. Retention of the gram stain is a relative characteristic. There is no precise division between gram-positive and gram-negative organisms. All that can be done is to stain the organism in question

according to the methods recommended by established authorities in this field. The Veillonella, like the Neisseria, appear to be borderline in their gram staining characteristics. Therefore, variations in gram staining by different workers are to be expected. In view of the fact that most workers consider these organisms to be gram-negative, and since the reports of them being gram-positive apply only to very young cultures, the purposes of classification will best be served by accepting the anaerobic gram-negative cocci to be Veillonella. The classification of the Veillonella as Group V Anaerobic Cocci as adopted by Hare, Wildy, Billett, and Twort[5] seems undesirable, since all the other groups described by these workers consist of gram-positive organisms. The Veillonella show many of the features of other gram-negative organisms, notably in their antibiotic sensitivity and reaction to dyes. All the other anaerobic cocci retain the gram stain and in general have the characteristics of gram-positive organisms with respect to dyes and antibiotics. The present classification of the anaerobic gram-negative cocci as the second genus in the family Neisseriaceae with the generic name Veillonella should be retained.

The results of the biochemical tests show that none of the ninety-six strains studied could be classified as Veillonella parvula or Veillonella gazogenes. Their inability to ferment glucose excludes them being classed as Veillonella parvula, and the fact that they produce hydrogen sulphide and reduce nitrates prevents them being classified as Veillonella gazogenes. Most other workers in recent years have also been unable to classify their isolates in accordance with the currently

accepted criteria for species differentiation within the genus Veillonella. With regard to the fermentation of glucose, the following facts may have contributed to errors in interpretation:

a. Under anaerobic conditions the higher concentrations of carbon dioxide employed may, by formation of carbonic acid in the glucose broth, sufficiently lower the pH to change the indicator. Unless the broth is allowed to stand exposed to air for several hours before the pH is recorded, errors may result.

b. Although it is not possible to be certain, it would appear that some of the earlier investigators accepted the production of gas as evidence of carbohydrate fermentation. The Veillonella produce abundant gas from any medium which supports their growth. Hare, Wildy, Billett, and Twort[5] have clearly shown that gas production by Veillonella is not influenced by the presence of carbohydrates in the medium.

c. The reports of sugars favoring growth without the sugars being utilized by the organisms, can be explained on the basis that the sugars lower the oxidation-reduction potential to a level more favorable for the growth of strict anaerobes like the Veillonella.

Variable findings with regard to other biochemical tests such as hydrogen sulphide production and reduction of nitrates can be explained by differing technique or actual metabolic difference in the strains of Veillonella studied. However, reports of strains which do ferment glucose, or do not produce hydrogen sulphide or reduce nitrates must be regarded with skepticism as they are very few in number and other workers investigating the same strain have not been able to reproduce the original

results. If the present scheme of species differentiation within the genus Veillonella is retained, then a third species to include those strains which do not ferment glucose but do produce hydrogen sulphide and reduce nitrates to nitrites must be created. Veillonella oris might be considered a suitable name for this new species. The key to the species of the genus Veillonella would then read as follows:

I. Acid and gas from glucose.

Weakly hemolytic. . . . . .Veillonella parvula

II. Carbohydrates not attacked. Gas from peptones.

Non-hemolytic. Hydrogen sulphide and nitrites not produced. . . . . .Veillonella gazogenes

III. Carbohydrates not attacked. Gas from peptones.

Non-hemolytic. Hydrogen sulphide and nitrites produced. . . . . .Veillonella oris

This new species would include practically all the strains of Veillonella which have been reported; therefore, it is preferable to amend the description of one of the existing species. If the ability to ferment glucose is deleted from the description of Veillonella parvula, most known strains would become Veillonella parvula. Alternatively the description of Veillonella gazogenes could be amended so that organisms of this species are regarded as being able to produce hydrogen sulphide and reduce nitrates to nitrites. Such matters must be decided by an accepted judiciary committee. It is to be hoped that such decisions will be forthcoming in the near future. Other biochemical tests such

as ammonia production, catalase activity, and the ability to reduce brilliant green may prove to be valuable taxonomic criteria.

Dental caries activity does not appear to be related to the numbers of Veillonella organisms occurring in saliva specimens. The large difference in Lactobacillus counts of the present groups suggests, as do numerous other studies, that this organism is in some way concerned with dental caries. The Lactobacillus counts in this study were recorded to see if Veillonella and Lactobacillus counts were related; they clearly are not. The relationship between Lactobacillus count and dental caries is an incidental finding in this study and will not be discussed here. The racial difference of the groups and the use of different dental examiners, while undesirable, does not seriously impair the conclusion that Veillonella count and dental caries are unrelated.

Since the Veillonella occur in such large numbers in all saliva specimens, it would seem reasonable to assume that most dental plaques would be saturated with these organisms. Englander, Carter, and Fosdick[34] in 1956 have commented on the remarkable buffering capacity of the dental plaque. They also found that the fall in pH when sucrose was applied to the plaque was due almost entirely to the amounts of lactic acid formed. Thus, the Veillonella by virtue of their ability to metabolize the lactate ion, have been suggested as a very important factor in the ability of the dental plaque to buffer lactic acid. When the pH of a dental plaque falls below a pH of 5.5, this acidity is not only deleterious to the underlying enamel but also the important buffering capacity of the Veillonella organisms is eliminated.

As both the caries active and caries inactive groups in this study had large numbers of Veillonella in their salivas, the difference in caries activity cannot be explained on the basis of having more or less buffering in the plaque due to Veillonella metabolism. A possible explanation is that while caries active individuals may possess adequate numbers of Veillonella, the beneficial metabolism of these organisms is repeatedly halted by other factors which allow a pH of 5.5 and lower to develop in the dental plaque.

No association between periodontal disease and agglutinin titer to Veillonella was detected in this study. Hall and Howitt[9], of course, did not find anything more than suggestive evidence of an association in their work. All that can be said is that the suggestive findings of Hall and Howitt have been further investigated using larger numbers of subjects but no evidence of an association has been detected. Most subjects in the study had Veillonella agglutinins in their serum. These probably constitute normal agglutinins developed during many years of exposure to Veillonella. These organisms could gain access to the blood stream via any lesion of the oral cavity. The fact that parenteral exposure to a given organism in the mouth can be detected by examining the serum of an individual is not without interest. The type of investigation reported here represents another method of approach to the difficult problem of establishing the possible association between oral microorganisms and periodontal disease.

V. SUMMARY

A highly selective medium for the oral Veillonella has been developed. The use of this medium in the isolation and enumeration of these organisms has been described. Ninety-six strains of oral Veillonella have been obtained in pure culture and their biochemistry studied. No strains fermented glucose or produced indole. All strains produced hydrogen sulphide, ammonia, reduced nitrates to nitrites and were catalase positive. The significance of these findings with respect to species differentiation in the genus Veillonella is discussed. The gram stain reaction of thirty-four strains after three hours culture on agar and in broth have been studied. All strains were gram-negative. Veillonella counts have been compared with Lactobacillus counts and caries activity in caries active and caries inactive groups of children. Veillonella counts were not related to Lactobacillus counts or caries activity. The agglutinin titer to Veillonella has been measured in the blood serum of periodontally healthy and periodontally diseased adults. No difference in titers was detected.

VI. CONCLUSIONS

Consideration of the results of this study leads to the following conclusions:

1. Organisms of the genus Veillonella are gram-negative even in young cultures.
2. A new species in the genus Veillonella should be established to include those strains which do not ferment carbohydrates but do produce hydrogen sulphide and reduce nitrates to nitrites. The name Veillonella oris is suggested for this new species. Alternatively, the description of one of the existing species should be amended so as to include these strains.
3. Veillonella counts are not related either to Lactobacillus counts or dental caries activity.
4. There is no association between periodontal disease and agglutinin titer to Veillonella.

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