# SOME BACTERIOLOGICAL AND IMMUNOLOGICAL CHARACTERISTICS OF FUOSBACTERIUM POLYMORPHUM

by

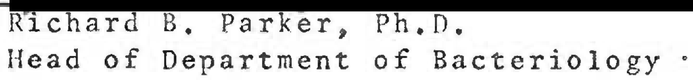
Steven Eric Berglund, B.A.

A Thesis

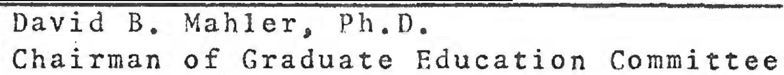
Presented to the Department of Bacteriology
and the Graduate Education Committee of the
University of Oregon Dental School
in partial fulfillment
of the requirements for the degree of
Master of Science
June, 1965

UNIVERSITY OF OREGON DENTAL SCHOOL LIBRARY
PORTLAND 1, OREGON

APPROVED



Richard B. Parker, Ph.D.
Head of Department of Bacteriology



David B. Mahler, Ph.D.
Chairman of Graduate Education Committee

ACKNOWLEDGEMENTS

The author wishes to thank Dr. Richard B. Parker for his encouragement and helpful advice during the course of this work.

Appreciation is also expressed to Miss Muriel Gregorius, Mrs. Shelby Mathias, and Mr. Wesley Bullock for their able technical assistance.

# TABLE OF CONTENTS

Page No.

I. Review of Literature . . . . . . . . . . . . . . . . . 4
II. Statement of Problem . . . . . . . . . . . . . . . . . 15
III. Materials and Methods . . . . . . . . . . . . . . . . 16
3.1 Isolation of The Organism . . . . . . . . . . . 16
3.2 Identification of The Organism . . . . . . . . 17
3.3 Mass Culture of The Organism . . . . . . . . . 17
3.4 Harvest of The Organism . . . . . . . . . . . . 18
3.5 Disruption of The Organism . . . . . . . . . . 19
3.6 Isolation of Cell Walls . . . . . . . . . . . . 20
3.7 Isolation of Test Antigens . . . . . . . . . . 20
3.7.1 Base Extractable Protein . . . . . . . . 20
3.7.2 Acid Extractable Protein . . . . . . . . 21
3.7.3 Polysaccharide . . . . . . . . . . . . . 21
3.7.4 Boivin Type Antigen . . . . . . . . . . 23
3.7.5 Water Extractable Antigen . . . . . . . 23
3.7.6 Culture Medium Control Antigen . . . . 24
3.7.7 Soluble Cytoplasm . . . . . . . . . . . 24
3.8 Production of Antisera . . . . . . . . . . . . 24
3.9 Gel Diffusion Procedure . . . . . . . . . . . . 26
3.10 Gross Chemical Analysis of Cells and Components . . . . 30
3.10.1 Nitrogen . . . . . . . . . . . . . . . 30
3.10.2 Phosphorus . . . . . . . . . . . . . . 30
3.10.3 Carbohydrate . . . . . . . . . . . . . 30
3.10.4 Lipid . . . . . . . . . . . . . . . . . 30
3.10.5 Cellular Production of Reducing Material . . . . 31
IV. Findings . . . . . . . . . . . . . . . . . . . . . . . 34
4.1 Some Observations on Cultural Characteristics of a Strain of Fusobacterium . . . . . . . . 34
4.1.1 Cultural Characteristics . . . . . . . . 34
4.1.2 Morphological Characteristics . . . . . 38
4.1.2.1 Colonial . . . . . . . . . . 38
4.1.2.2 Cellular . . . . . . . . . . 38
4.2 Metabolic Characteristics and Identification of the Organism . . . . . . . . . . . . . . . 42
4.3 Cell Wall Isolation . . . . . . . . . . . . . . 43
4.4 Results of Gross Chemical Analyses . . . . . . 49
4.5 Results of Gel Diffusion Analyses . . . . . . . 49
V. Discussion . . . . . . . . . . . . . . . . . . . . . . 50
5.1 Difficulties in Culture of the Organism . . . . 50
5.2 Morphology of *F. polymorphum* . . . . . . . . . 50
5.3 Fermentation Reactions . . . . . . . . . . . . . 53

TABLE OF CONTENTS (CONT'D)

Page No.

5.4 Isolation of Antigens . . . . . . . . . . . . . 55
5.5 Antigenic Analysis . . . . . . . . . . . . . . 58
5.6 Chemical Analyses . . . . . . . . . . . . . . . 61
VI. Summary . . . . . . . . . . . . . . . . . . . . . 64
VII. Conclusions . . . . . . . . . . . . . . . . . . . 65
VIII. Bibliography . . . . . . . . . . . . . . . . . . 66

# FIGURES AND TABLES

Figures Page No.

1. Rabbit inoculation schedule . . . . . . . . . . . . 25
2. Schematic of gel diffusion template . . . . . . . . 27
3. Schematic of gel diffusion analyses . . . . . . . . 29
4. Protocol for production of cellular reducing material . . . . 32
5. _F. polymorphum_ cultured on defined medium . . . . 37
6. _F. polymorphum_ cultured on thioglycollate medium . . . . 37
7. Electron micrograph of _F. polymorphum_ . . . . . . . 40
8. _F. polymorphum_ colony on blood agar . . . . . . . . 40
9. Results of fermentation and cellular by-product analyses . . . . . . . . . . . . . . . . . . . . . 42
10. Graph of reducing substanced produced by cellular metabolism . . . . . . . . . . . . . . . . . . . . 44
11. Electron micrograph of crude cell walls . . . . . . 46
12. Electron micrograph of cell walls contaminated with cytoplasmic bodies . . . . . . . . . . . . . . . . 46
13. Electron micrograph of clean cell walls . . . . . . 48
14. Gel diffusion plate showing presence and absence of precipitin . . . . . . . . . . . . . . . . . . . . 48

Tables

1. Cellular reducing substances . . . . . . . . . . . 44
2. Results of chemical analyses . . . . . . . . . . . 59
3. Results of gel diffusion analyses . . . . . . . . . 62

# I. REVIEW OF LITERATURE

According to Boe (3, p.1), members of the genus Fusobacterium were probably first noticed and described around 1880. Interest in these bacteria was precipitated by their constant presence both alone and with spirochaetes in such necrotizing lesions as noma, cellulitis and necrotizing ulcerative gingivitis. The bacteriology involved with these lesions was studied by Plaut (39), Vincent (54), Veillon (52) and others. The above lesions were termed fusospirochaetal by these workers because of the relatively constant bacteriologic picture they presented. It was recognized early by Babes (1) and Plaut (39) that fusobacteria and spirochaetes were present in normal healthy mouths. This information led Babes to conclude that fusospirochaetal lesions developed only after local tissue resistance had been reduced. The need for predisposing factors to allow initiation of such infections has been suggested by others. Factors such as trauma, existing disease, nutritional deficiencies and poor body hygiene have all been assigned significance in the initiation of

so-called fusospirochaetal disease (22, 46, 51). Plaut (39) felt that fusobacteria and spirochaetes were of pathologic significance because although they were present in the normal mouth, their number increased markedly in the early stages of certain necrotic diseases of the mouth. He further observed that such diseases were often contagious and that the bacterial flora of the lesion returned to a normal level upon restoration of tissue health. For these reasons he suggested that the fusobacteria and spirochaetes were of primary etiologic significance. Besides oral lesions, fatal septicemias, abscesses of the lung and brain as well as infections of the intestinal tract and genitalia have all had fusobacteria demonstrated as etiologic factors (39, 46, 49). Spirochaetes and other bacteria were often combined with the fusobacteria in such lesions.

The role of fusobacteria and spirochaete mixtures as a primary etiologic factor has long been questioned. Some workers feel that fusobacteria and spirochaetes are more likely secondary invaders of a preexisting lesion (6, 11, 27). The idea that fusobacteria alone or in mixtures with spirochaetes has the capability of initiating necrotic and/ or febrile responses, however, has been advanced by several investigators (13, 46, 49). Some of the evidence in favor of this concept is indeed interesting. Rosebury has shown

that the flora of certain inflammatory periodontal diseases is an overgrowth of the normal oral flora (42). Increased numbers of fusobacteria and spirochaetes as well as other organisms are present in slight marginal gingivitis and in severe necrotizing lesions. Treatment of such conditions with antibiotics often results in prompt clinical resolution of the disease.

Furthermore, it has been demonstrated that fuso-spirochaetal-like lesions can be induced in guinea pigs by injecting them subcutaneously with gingival scrapings from normal human mouths (42). These studies also show that only a few members of the normal oral flora are capable of inciting fusospirochaetal lesions in experimental animals. Recombination experiments indicate that two species of the genus *Bacteroides* (one of which was *Bacteroides melaninogenicus*), an anaerobic Gram negative rod, and a diphtheroid are the only essential bacteria needed to transmit necrotic abscesses in guinea pigs (27). Fusobacteria and spirochaetes were shown to be nonessential for transmission of the lesion. On the other hand, it has been pointed out that deaths have resulted from septicemias and abscesses of the brain and lung which were caused by fusobacteria. This indicates that some pathologic significance must be assigned to these organisms (16).

The role of endotoxins in mechanisms of necrotic lesions associated with fusobacteria had been suggested by several workers (3, 19). Demonstrations of endotoxin in fusobacteria (13) and the fact that both the purified endotoxin as well as the bacterial cell itself are capable of stimulating Shwartzman's reaction give additional impetus to the concept of pathologic factors associated with fusobacteria (3, 18).

It has been suggested that a synergistic mechanism between fusobacteria and spirochaetes exists in necrotic lesions. Abscesses which were caused in guinea pigs by fusobacteria underwent severe exacerbation when the animal was subsequently inoculated with spirochaetes. In the same study it was shown that fusobacteria undergo anachoresis i.e. localization and refuge whereas spirochaetes do not. Migration of fusobacteria into spirochaetal abscesses caused much more severe infections as indicated by increased necrotic response (18).

Early studies suggested that the etiology of fusospirochaetal infections was complex. The apparent synergistic action between fusobacteria and spirochaetes as well as the inability of early investigators (15) to demonstrate either exotoxin or endotoxins associated with the flora of such lesions led to many inconclusive results regarding the

etiology and pathologic processes acting in fusospirochaetal infections. The recently suggested role of endotoxins and localized hypersensitive reactions in such lesions is interesting. It also reveals new paths of investigation into an old problem.

Although most of the pioneering research efforts dealing with fusobacteria were concerned with pathogenic potentials and attempts at fulfilling Koch's postulates interest was eventually developed about the classification of these organisms and their bacteriologic characteristics. The first adequate description of a typical fusobacterium was submitted by Lewcowicz in 1901 (26). He observed that it was necessary to use a serum enriched media to support growth. The fact that these organisms were pleomorphic become evident when he cultured the cells *in vitro* after isolating them from a fusospirochaetal lesion. The short rod-shaped organism which had appeared in the lesion became a long filamentous form when cultured *in vitro*. It was also observed that the organism was Gram negative and that it grew only near 37° C.

In 1906 Tunnicliff (48) confirmed the work of Lewcowicz. She concluded that there must be several different species of *Fusobacterium* because many strains she had isolated presented different biochemical characteristics.

Because of the marked pleomorphism of such bacteria, she claimed that fusobacteria and spirochaetes were different forms of the same organism. Observations on apparently pure cultures of fusobacteria after 48 hours revealed the presence of spirochaetes.

In 1906 Muhlens and Hartmann (30) isolated pure cultures of both fusobacteria and spirochaetes. It was their opinion that the two organisms were distinctly separate and that they were in no way variants of the same parent.

The first attempts to classify Fusobacterium were made by Veszpremi (53). He thought it possible to form three groups based on morphological criteria. Later, however, other workers (37) concluded that because of the pleomorphism exhibited by these bacteria, morphology was not an adequate criterion for classification when used alone.

Krumwiede and Pratt (24) in 1913 made the first attempt at a systematic classification of Fusobacterium. They concluded again that morphology could not be used to differentiate these organisms even when grown under standard conditions. By utilizing biochemical criteria they were able to divide 15 strains into two groups. Group I fermented only glucose while Group II fermented both glucose and saccharose. All of their organisms were Gram negative,

required serum for growth, were nonmotile and produced strong odors when cultured.

In 1923 Knorr (23) attempted to resolve different fusobacteria on the basis of colonial and cellular morphology and cultural characteristics. He recognized three groups within the genus *Fusobacterium* which he named *Fusobacterium nucleatum*, *Fusobacterium polymorphum* and *Fusobacterium plauti-vincenti*.

Classification on morphological, biochemical and serological basis was attempted by Varney in 1927 (51). He felt four groups could be designated on morphologic grounds. Agglutination tests revealed that antisera would react only with its homologous species. He felt, however, that agglutination was a reliable test for these organisms but that some difficulty was experienced with spontaneous agglutination.

In 1927 Pratt (40) again confirmed the futility of using morphology alone as a criterion to separate different species of *Fusobacterium*. She attempted to use complement fixation procedures for such purposes. Although high titers were produced against fusobacteria, complement was fixed only by homologous antigens. She concluded that the bacteria must be a heterologous group.

Slanetz and Rettger (45) in 1933 conducted experiments to evaluate the criteria of morphology, biochemical reactions and serological techniques for classification. Four groups were formed from 53 species of isolated fusobacteria. Groups I and II could be resolved morphologically but not biochemically. Groups III and IV had their own biochemical characteristics but could not be separated morphologically from Group II. The agglutination tests used were designated as unreliable because of the difficulty with spontaneous agglutination of the cells.

On the other hand, Spaulding and Rettger (47) examined 80 strains of *Fusobacterium* and found agglutination tests suitable for classification. It was their opinion that serologic techniques generally agreed with categories established by morphological and biochemical criteria. As a result of their tests, two groups were suggested. Group I was composed of rods 5 microns or less in length. Group II was composed of longer cells which had rounded ends. Group I was inactive against carbohydrates, formed hydrogen sulfide and indole and reduced nitrates. Group II formed large amounts of acid but did not form indole, hydrogen sulfide or reduce nitrates. They concluded that the groups were different enough to warrant separating them into various genera. There were so many intermediate forms, however, that they did not suggest the division.

Later work by others suggests the formation of three species based on morphology, cultural and biochemical properties (15).

An exhaustive study of Fusobacterium by Boe (3) led him to conclude that Fusobacterium should be classed as a single genus. He felt that the pleomorphism and overlapping biochemical characteristics of so-called strains were so great that the criteria were unreliable for species resolution. The morphologic and biochemical picture however, was excellent for identification of the genus. Attempts to use agglutination, precipitin and complement fixation tests to resolve species were unsuccessful because of similarity between various isolates and the lack of correlation between morphology and biochemical behavior. Boe concluded that the antigenic structure of fusobacteria must be very complex.

Other features regarding fusobacteria in general were that serum and strict anaerobiosis were necessary upon isolation of such bacteria from their native environment. Upon continued culture the requirement for these two conditions relaxed to the point where serum was no longer needed and microaerophilia was adequate growth.

Recent workers express the feeling that only slight morphological differences resolve F. nucleatum and

F. polymorphum and, therefore, they should be combined into one species (36). Organisms of somewhat similar morphology but longer and more slender with increased acid forming capacities should be considered for placment in the genus Leptotrichia.

Information regarding the chemical composition of fusobacteria is almost nonexistent. A limited report of some amino acids and sugars contained in some fusobacteria has been presented (2). Some information regarding the composition of endotoxin is also available (9). Generally the bulk of information about the chemical composition of bacteria deals with Gram positive organisms. As has been pointed out by the Gram positive and Gram negative bacteria have marked compositional differences (12). Recent studies have shown that marked differences exist between cell walls of Gram negative and Gram positive bacteria (44). Methods for isolation of cell walls, which are numerous throughout the literature (44, p.4) have made this revelation possible. Chemical analyses of cell walls indicate a strong similarity between walls of different Gram positive bacteria. Minor qualitative differences in amino acids and sugars are said to have taxonomic significance (44, p.30). Similar information regarding Gram negative cell walls is limited. The walls of such organisms contain substantially more lipid

material than Gram positive walls (44, p.48). They are also unique in that they are said to have sulfur containing amino acids (2, p.466).

Isolation methods for cell walls applicable to Gram positive bacteria are generally not readily successful when applied to Gram negative organisms. Often each genus demands a specific isolation method (41). Isolated Gram negative cell walls can provide helpful information. An example is that the "O" antigen of Gram negative bacteria has been shown to reside in the cell wall rather than in the cell cytoplasm (28).

In summary, little is definitely known about fusobacteria as well as gram negative bacteria in general. The literature presents fusobacteria as a pleomorphic, anaerobic, Gram negative, nonmotile organism. Its pleomorphism appears to be dependent on cultural age, nutritional factors and environmental conditions. *Fusobacterium* as a genus are readily noted by the picture of morphology, cultural and biochemical properties. The pathogenicity of the organisms in some processes has been established. They are unique as pathogens because of the apparent need for predisposing factors acting on the host and the possible role played by endotoxins in pathogenesis.

## II. STATEMENT OF THE PROBLEM

This thesis is concerned with the isolation and study of a strain of oral fusobacteria. Its morphological, cultural and biochemical characteristics will be studied so that it might be identified according to Bergey's Manual of Determinative Bacteriology (5). A study designed to gain insight into the antigenic nature of fusobacteria as well as information regarding the chemical make up of the cell will also be conducted.

Realizing that a complex problem is best solved by initial study of component factors, it is felt that a study dealing with the antigenic nature of the bacterial cell and some of its component parts may provide data that will help throw light on the relationship of the fuso-spirochaetal complex to periodontal disease.

# III. MATERIALS AND METHODS

## 3.1 Isolation of the Organism

A strain of oral *Fusobacterium* was isolated from interproximal scrapings of mandibular molars in the mouth of a normal two year old child. Scrapings were immediately placed in a tube of freshly steamed thioglycollate medium enriched with 10% beef serum. The inoculated broth was then streaked on the selective medium of Omata and Disraely (34) and immediately placed in an atmosphere of 10% carbon dioxide and 90% hydrogen. The culture was incubated at 37° C. for 72 hours. Upon removal of the plates from the incubator, an isolated colony was selected. Part of this colony was subjected to Gram stain. The remaining portion of the colony was streaked on several blood agar plates and incubated as before. The procedure of selecting an isolated colony with subsequent transfer and streaking was repeated three times to insure selection of a single strain of organism. The isolated bacterium was then carried in stock cultures on either fluid thioglycollate broth or in the dehydrated state on 40% skim milk plus 10% beef serum. Cells which were carried in broth remained viable at least one month when contained in air tight tubes at 5° C. Lyophilized cultures have been found viable after two years.

Such cultures were best reconstituted on fluid thioglycollate medium enriched with 10% beef serum. Growth appeared after incubation for 72 hours in an atmosphere of 10% carbon dioxide and 90% hydrogen.

3.2 Identification of the Organism

The identification of this organism is based on criteria put forth in Bergey's Manual of Determinative Bacteriology (5). Procedures for evaluating morphological, cultural and biochemical features were obtained from the Manual of Microbiological Methods (38).

3.3 Mass Culture of the Organism

Cultures of the organism were grown on the potato extract agar of Slanetz and Rettgers (45). One hundred fifty ml of this medium was dispensed into Roux bottles of 500 ml capacity. Ten ml of a 12 hour culture was used to inoculate each vessel. The inoculated containers were placed in an atmosphere of 10% carbon dioxide and 90% hydrogen. Incubation proceeded at 37° C. for 72 hours. The Roux bottle inoculum was prepared by culturing cells in 100 ml dairy dilution bottles containing potato extract broth for 12 hours just prior to use.

### 3.4 Harvest of the Organism

Cells were removed from Roux bottles by flushing and sweeping the agar surface with cold distilled water and a glass rod. The harvest was kept at 5° C. during this procedure. Small pieces of agar were removed from the cells by filtering the mass through a fine wire screen. Centrifugation at a relative centrifugal force of 3,020 for 15 minutes easily sedimented the filtered cells. Centrifugation resulted in a pellet of bacteria. This was resuspended in 200 ml of cold distilled water by the action of a Servall Omnimixer*. The suspension was again centrifuged and resuspended as before. This procedure was repeated three times to effect removal of as much medium as possible which might have adhered to the cells. The entire procedure was carried out at 5° C.

### 3.5 Disruption of Organisms

A 300 ml capacity container attached to the Servall Omnimixer was the instrument used to obtain cell breakup. Freshly harvested and washed cells were suspended in 50 ml of cold distilled water. A volume of 100-200 mesh plastic beads** was added to the bacterial suspension until a

---

* Servall Omni Mixer, Ivan Servall, Norwalk, Conn.

** Polystyrene Divinylbenzene Copalmer Beads, Dow Chemical Co., Midland, Mich.

viscosity that approximated nondiluted glycerol was obtained. The entire apparatus was placed in a cold room and the mixing chamber was lowered into a pail of ice water. The mixer was allowed to operate for about one hour at a setting of 50 volts. Inspection of the disrupted cell mass by electron microscopy confirmed the completeness of breakup.

3.6 Isolation of Cell Walls

Freshly disrupted cells were immediately spun down in the cold at a relative centrifugal force of 30,900 for one hour in a Servall SS1 refrigerated centrifuge. The supernatant was decanted and the crude cell walls were resuspended in a solution of 10% sodium succinate. The resulting insoluble material was washed two additional times by repeating the above procedure. Three washes of one molar potassium chloride were then used to remove the sodium succinate and any remaining soluble cytoplasmic contaminants from the cell walls. Potassium chloride was removed from the walls by three applications of cold distilled water. The product was judged pure by electron microscopy. It was then lyophilized and stored in a desiccator over phosphorus pentoxide.

## 3.7 Isolation of Test Antigens

### 3.7.1 Base Extractable Protein

The method employed is a modification of that described by Kessler and Nickerson (21). Approximately one gram (dry weight) of freshly washed wet cells was suspended in 50 ml of cold 1 N sodium hydroxide. The mixture was treated in a 20,000 Kc sonorater* at 5° C. for one minute. The sonorated mass was then added to 400 ml of 1 N sodium hydroxide and stirred overnight in the cold. The suspension was then centrifuged at a relative centrifugal force of 34,800 for one hour. The supernatant was carefully decanted into a dialysis bag and placed in front of a fan until evaporation had reduced the volume to 200 ml. This volume was placed in a new dialysis bag overnight in cold running tap water. Protein material was then salted out by slowly saturating the protein solution with sodium chloride while maintaining a constant pH of 3.6. The saturated solution was centrifuged at a relative centrifugal force of 12,100 for 15 minutes. The precipitate was dissolved in distilled water and dialyzed overnight against running tap water. The protein was reprecipitated and again centrifuged. The material was dissolved in water and lyophilized. It then was stored over phosphorus pentoxide in a desiccator.

---

* Biosonic Model CF 21, Bronwill Scientific, Rochester, N.Y.

3.7.2 Acid Extractable Protein

The method employed is a modification of that conceived by Heidelberger and Kendall (14). One gram (dry weight) of freshly harvested and washed cells was suspended in 50 ml of cold 0.2 N sodium acetate buffered at pH 4.6. The suspension was then sonorated in the apparatus mentioned above for one minute. After sonic treatment the mass was added to 500 ml of cold buffer and stirred overnight in the cold room. The mass was centrifuged for one hour at a relative centrifugal force of 34,800. The supernatant was placed in a dialysis bag and the volume reduced in 200 ml by evaporation 5° C. The resulting 200 ml was dialyzed overnight against running tap water. The dialyzed material was then saturated with slow addition of aodium chloride while maintaining a constant pH of 3.6. The saturated solution was then centrifuged at a relative centrifugal force of 12,100 for 15 minutes. The precipitate was dissolved in distilled water and dialyzed against running tap water overnight. The protein was rep-recipitated, dialyzed and lyophilized. It was stored over phosphorus pentoxide.

3.7.3 Polysaccharide

Polysaccharide was isolated by a technique based on that described by Webster (55). One gram (dry weight) of freshly harvested and washed cells was suspended in 50 ml

of 1 N sodium hydroxide. The suspension was sonorated for one minute in the previously used Biosonic unit. After centrifugation of the sonorant at a relative centrifugal force of 34,800 for one hour, the supernatant was removed and added to a volume of 500 ml cold 1 N sodium hydroxide. This mixture was stirred overnight in the cold. The volume was then reduced to about 200 ml by evaporation at 5° C. The material was then dialyzed overnight against cold water to remove the sodium hydroxide solvent and inorganic salts. The volume was reduced to 50 ml by evaporation of 5° C. Fifty ml of cold 0.5 molar trichloroacetic acid was added to the extract and shaken. The mixture was allowed to stand for three hours in the refrigerator. The insoluble residue was removed by centrifugation at a relative centrifugal force of 3,020 for 15 minutes. The resulting supernatant was dialyzed to eliminate soluble salts and residual trichloroacetic acid. The supernatant was saturated with sodium chloride (35 gm/100 ml) and ethanol was added at room temperature with rapid stirring to a final concentration 7.5 gm/100 ml. The resulting precipitate was recovered by centrifugation for 15 minutes at a relative centrifugal force of 3,020. It was then dissolved in water and lyophilized. The polysaccharide was stored over phosphorus pentoxide.

3.7.4 Boivin Type Antigen

For isolation of this material the phenon-water technique of Westphal as described by Kabat was utilized (20). One gram (dry weight) of freshly harvested and washed cells was suspended in 20 ml cold water. This was added to 60 ml of cold 75% phenol-water mixture. The mass was agitated for 14 hours in the cold. Separation of the two-phase system was effected by centrifugation in the cold for 30 minutes at a relative centrifugal force of 3,020. The aqueous top phase was decanted and the lower phase was extracted three times with cold water. All aqueous extracts were then combined. The lipo-protein-polysaccharide complex was precipitated by the addition of six volumes of ethyl alcohol to the aqueous extractant. The precipitate was taken up in cold water, dialyzed overnight in the cold and again precipitated with 95% alcohol. The resulting precipitate was dialyzed again in the cold and then lyophilized.

3.7.5 Water Extractable Antigen

The basic technique utilized was described by Dudman and Wikinson (10). Two hundred twenty mg lyophilized cells were suspended in 15 ml water. The suspension was sealed in a glass tube and boiled at 100° C. for 30 minutes. The sediment was then removed by centrifugation at a relative centrifugal force of 12,100 for 15 minutes. The supernatant

was removed and lyophilized for future use.

### 3.7.6 Culture Medium Control Antigen

The potato extract medium utilized for culture Fusobacterium was used as a control antigen. Agar was omitted from the broth to allow adequate diffusion during precipitin test procedures.

### 3.7.7 Soluble Cytoplasm

Cytoplasm utilized for testing was recovered from suspensions of crude cell walls immediately after disruption by agitation in the Servall Omnimixer. Crude cell walls and cytoplasmic components were sedimented by centrifugation at a relative centrifugal force of 30,900 for one hour. The upper one half of the supernatant was siphoned off and stored at minus 20° C. until use.

## 3.8 Production of Antisera

Antisera was produced by 2 1/2 pound New Zealand white rabbits*. Four groups of animals were used. Each group contained two members. Twenty ml of blood was drawn from each animal by cardiac puncture to obtain initial titers. Intravenous and intramuscular injections of whole cells,

---

* New Zealand white rabbits were obtained from the Wm. Davis Rabbitry, 80 N.W. 341 Avenue, Hillsboro, Oregon. All animals were approximately five weeks of age and weighed about 2 1/2 pounds each.

cell walls, cell soluble cytoplasm and potato media were administered over a five-week period to each group respectively. The inoculation schedule is shown below. Three

Figure 1

Schedule of Animal Inoculations

Injection Route

| Time | Intra-Venous | Intra-Muscular |
|---|---|---|
| Initial | .04 mg | 1.0 mg |
| 1st week | .14 mg | 1.1 mg |
| 2nd week | .24 mg | 1.2 mg |
| 3rd week | .34 mg | 1.3 mg |
| 4th week | .44 mg | 1.4 mg |
| 5th week | .54 mg | 1.5 mg |

Each dose was dissolved overnight in distilled water and administered in a/ml aliquot

days after the last booster injection 30 ml blood was taken from each animal by cardiac puncture. The blood was placed in sterile tubes and refrigerated overnight. The cellular components were removed by centrifugation at a relative centrifugal force of 3,020 for 30 minutes. The resulting serums were pooled within groups and dispensed in 10 ml serum

bottles. The material was stored at minus 20° C. until used.

3.9 Gel Diffusion Procedure

The method used is the basic Ouchterlony procedure as described by Wilson and Pringle (58). Gel diffusion plates were prepared as follows. Glass slides 50 x 75 mm were coated with Siliclad[R]*. Templates for the diffusion patterns were constructed from 72 x 38 x 3 mm plexiglass. Holes which were to serve as antigen and serum reservoirs were placed as illustrated in Figure 2, p.27.

Center holes were 3/16" in diameter. The peripheral holes were 1/8" in diameter. The template was then coated with Siliclad[R]. Siliclad[R] processed slides and templates facilitated the adherence of agar to the glass and increased the ease with which the template could be removed from agar covered slides. Spacers consisting of two thicknesses of Homart[R] plastic electricians tape** were placed at the ends in the center of each template. The template was then positioned on the glass slide with the spacers against the glass. The gap provided by the plactic tape between the template and the slide was charged with the agar diffusion medium.

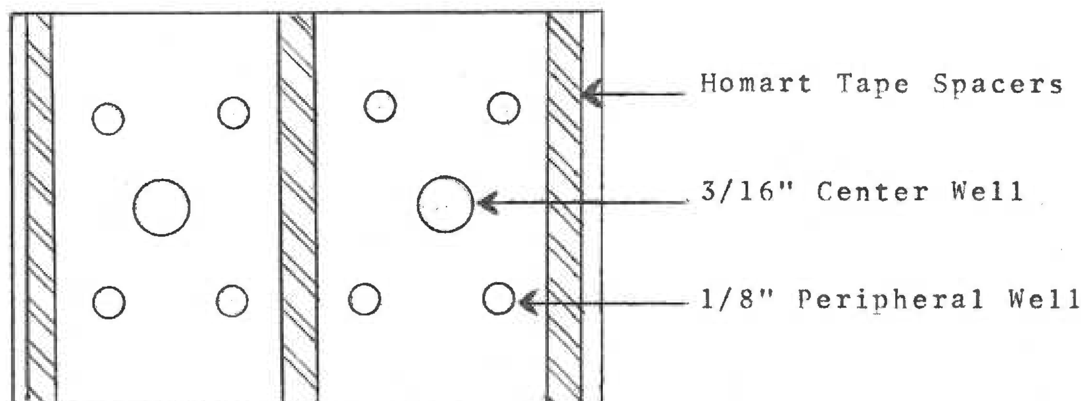
---

* Siliclad[R] is a silicone compound which can be purchased from Van Water and Rogers, Inc., Portland, Oregon.

** Homart[R] purchased from Sears Roebuck #34-5003.

Figure 2

SCHEMATIC OF TEMPLATE DESIGN




Template Constructed from 72 x 38 x 3 mm Plexi-Glass

A schematic of the template used with gell diffusion plates. Center wells were charged with nondiluted antisera. Peripheral wells contained test antigens.

The diffusion medium was composed of 0.8% Ionagar (Oxoid) plus 1% sodium azide. After the agar had been liquified, it was cooled to 65° C. and syringed into the diffusion plate with an eyedropper. The finished diffusion plates were stored in 100% humidity at 5° C. until used. Tests were conducted by charging the center wells with nondiluted antiserum and the surrounding wells with antigen in a concentration of 2 mg per ml. At total of 80 gel diffusion plates were used to test combinations of the four antisera against the seven test antigens. The test of single antisera is shown in Figure 3, p. 29.

The antigen control consists of challenging antisera with distilled water--the vehicle for all test antigens. Control sera which was taken from rabbits prior to initial inoculation was reacted with each test antigen. Figure 3, p.29, shows how control sera and antisera were reacted with each test antigen. Numbers represent different test antigens while letters represent antisera and control serums.

The charged diffusion plates were placed in 100% humidity at 5° C. Plates were examined for precipitin lines every 12 hours. When the diffusion plates exhibited maximum numbers of bands, they were removed from the refrigerator. The templates were removed and the agar covered slides were

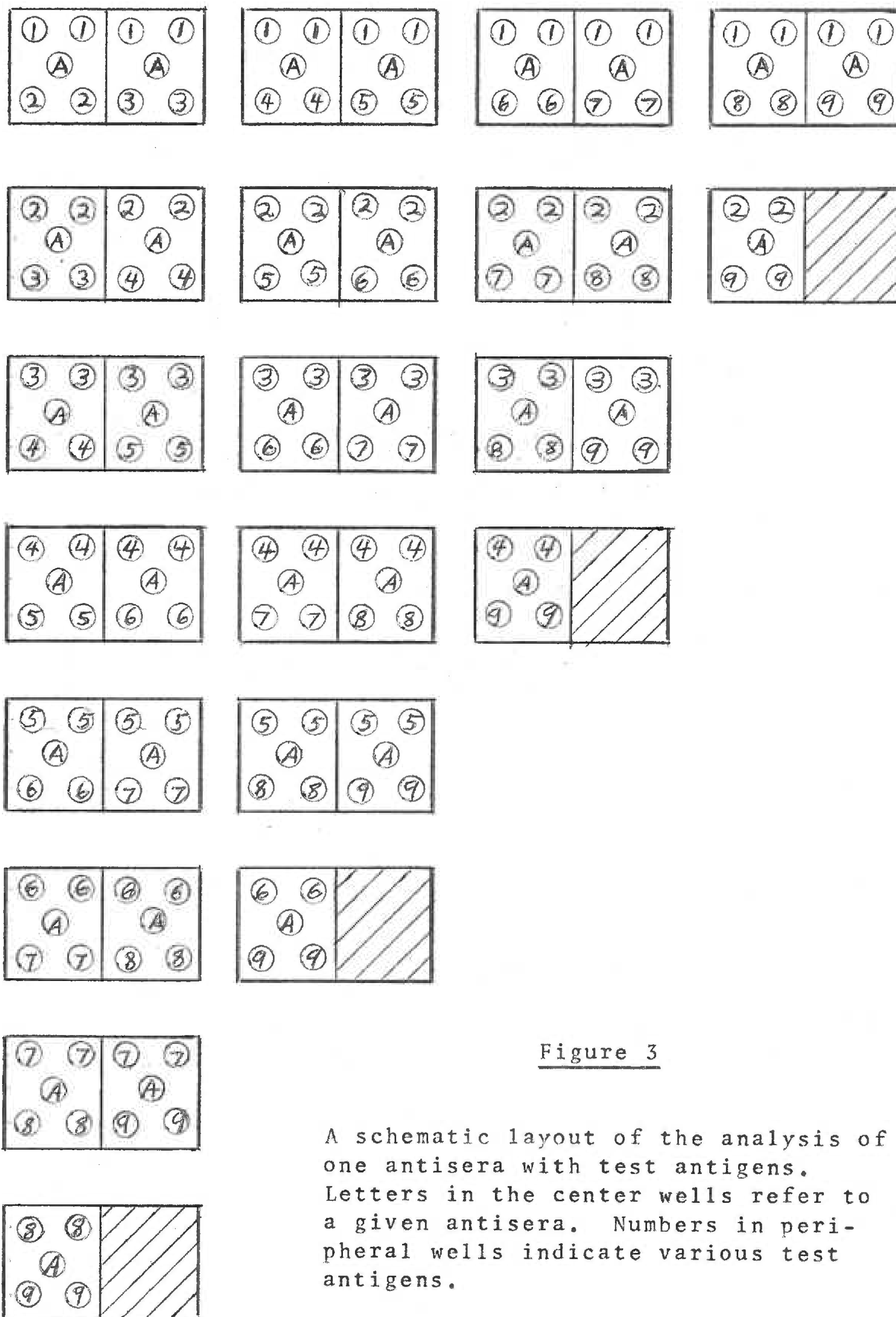



Figure 3

A schematic layout of the analysis of one antisera with test antigens. Letters in the center wells refer to a given antisera. Numbers in peripheral wells indicate various test antigens.

stained with thiazine red as described by Crowley (7) to facilitate visualization of precipitation.

## 3.10 Gross Chemical Analysis of Cells and Components

### 3.10.1 Nitrogen

Nitrogen was determined by Bollin's modification of the Kjeldahl procedure (4). The main feature of this modification is the digestion catalyst. It is composed of 94% potassium sulfate, 5% anhydrous cupric sulfate and 1% ferric sulfate. This mixture is used in addition to a selenized Hengar$^R$ granule*. The procedure was further modified by utilizing a potentiometric rather than a colorimetric means to detect the endpoint of titration. The endpoint of the reaction was taken as pH 4.6.

### 3.10.2 Phosphorus

Total phosphorus was determined by the method of Fiske and Subbarow as described by Umbreit (50).

### 3.10.3 Carbohydrate

Total carbohydrate was estimated by the Anthrone technique as described by Neish (32).

### 3.10.4 Lipid

Total lipid was estimated by the method of Peck (33).

---

* Hengar$^R$ selenized granules, Hengar Co., Philadelphia, Penn.

This technique involves the extraction of so-called loosely bound lipid with ether-alcohol solvents and then with chloroform. More tightly bound material is extracted after acid hydrolysis with the above solvents.

3.10.5 Cellular Production of Reducing Material

Resting cells were prepared by culturing the Fusobacterium following two immediately continuous subcultures on thioglycollate medium. The cells were then harvested and washed three times in cold .067 N phosphate buffer at pH 7.2. Extreme care was taken to protect the cells from exposure to air during washing procedures. This was accomplished by sweeping the vessels containing the organisms with nitrogen gas. The cells were placed in a resting state by suspending them in the buffer at 5° C. overnight under a nitrogen atmosphere. The concentration was adjusted to an optical density of 0.5 as determined on a Bausch and Lomb Spectronic 20 spectrophotometer.

Cells, buffer and/or glucose was added to the protocol as indicated in Figure 4, p.32. Cells and buffer were added and the mixture allowed to equilibrate at 37° water bath. The tubes were charged with an atmosphere of nitrogen prior equilibration. After five minutes at 37°, glucose was added to the indicated tubes and mixed.

Figure 4

| Tubes | Cells | Mg Glucose | Buffer |
|---|---|---|---|
| 1 | 10 ml | 0.1 mg | None |
| 2 | 10 ml | None | 1 ml |
| 3 | None | 0.1 mg | 10 mg |

Protocol used for the determination of reducining substances produced by F. polymorphum cell suspension consists of organisms in sufficient concentration to give an optical density of 0.50. Buffer is .067 molar potassium phosphate at a pH of 7.2. Glucose solution is .01%. One ml of this solution equals 0.1 mg solute.

Incubation proceeded for five minutes after which metabolism was stopped by quenching the suspensions in liquid nitrogen. Particulate matter was removed from the suspension by centrifugation at a relative centrifugal force of 3,020 for 30 minutes. Aliquots of the supernatant were then tested for the presence and amount of reducing substance by the Benedict test.

# IV. FINDINGS

## 4.1 Some Observations on Cultural Characteristics and Morphology of a Strain of Fusobacterium.

### 4.1.1 Cultural Characteristics

Upon isolation from the oral cavity, the fusobacterium were difficult to culture. Plates containing selective medium on which the cells were initially streaked often showed no growth. It was soon discovered that great care had to be taken to minimize the organisms exposure to air after they were streaked on selective medium. The sooner the streaked plates were placed in an atmosphere of 10% carbon dioxide-90% hydrogen, the better was the resulting yield of desired colonies. Generally, if freshly streaked cells were allowed to remain in the air for approximately two minutes or longer after primary isolation from the mouth, no growth would result. Initial subculture of the bacterium in thioglycollate medium enriched with 10% beef serum required two to three days to show signs of growth. After continuous subculture on the same medium, marked growth was obvious after one day. Continued subculture also decreased the need for absolute oxygen lack in the culture medium. After eight to ten continuous subcultures, the fusobacteria were capable of growth after periods of

about four to eight minutes exposure to air when streaked on thioglycollate medium. The need for enrichment of the culture medium with beef serum which was necessary for growth upon initial isolation from the mouth, became nonexistent after several continuous subcultures on thioglycollate medium.

Attempts to grow the fusobacterium on the defined medium proposed by Omata (35) were only partially successful. At best cell growth was very small. Continuous subculture in the medium did not result in improved growth. Marked pleomorphism however was evident. Instead of the usual 10 to 15 micron cell length, the cells were long filamentous forms that exceeded 100 microns. Filamentous forms persisted when the culture was agitated during the culture period. Because the medium was very expensive and the cell growth could not be improved, further work was discontinued. Figure 5, p.37, is a photomicrograph of the bacteria cultured on the defined medium.

Cell masses which were cultured in thioglycollate broth were dark gray in color. This color could not be removed from either the whole bacterial cell or the isolated cell walls. When potato extract agar was utilized as culture medium, cellular color was white.

Growth of Fusobacterium on either thioglycollate or potato extract media produced a foul odor.

Figure 5

*F. polymorphum* cultured on a defined medium at 36 hours of age. The width of these cells is on the order of 0.3 to 0.5 microns. The length is in excess of 100 microns. Magnification is 1,610 diameters.

Figure 6

A photomicrograph of *F. polymorphum* cultured on thioglycollate broth. The cells are 36 hours old. They are on the order of 0.5 - 0.8 microns wide and 10-15 microns long. Magnification is 5,700 diameters.

The organism was not motile. This characteristic was evaluated on cells of 6, 12, 24 and 48 hours by the hanging drop and semi-solid agar techniques.

The organism was Gram negative.

Cells cultured in a semi-solid medium produced isolated cotton ball-like colonies which were spread along the path of the inoculating loop.

Cells cultured in broth formed a tenacious mat on the floor of the vessel which consisted of filamentous forms. If the culture was agitated during incubation, the organisms were evenly distributed throughout the medium and filamentous forms were not evident. Figure 6, p.37, shows the appearance of fusobacterium under oil immersion on a light microscope. Figure 7, p.40, shows the organism under the electron microscope.

### 4.1.2 Morphological Characteristics

#### 4.1.2.1 Colonial

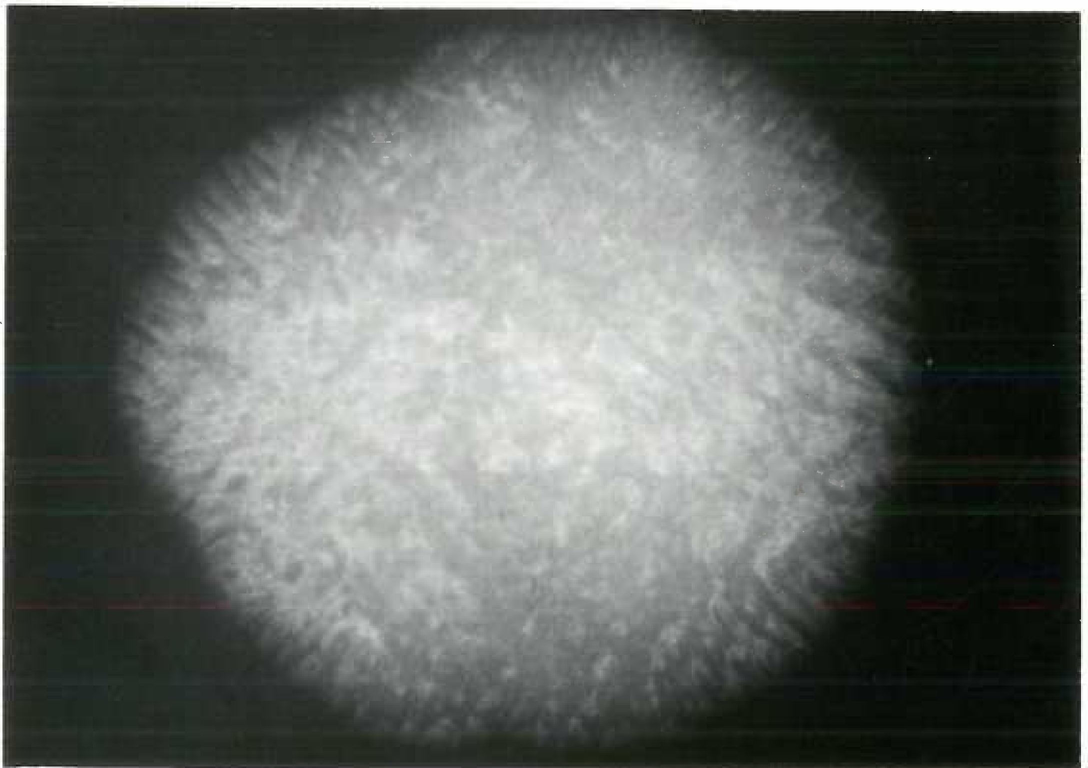
Colonial morphology on blood agar after 24 hours incubation is as follows. Colonies are mucoid and convex in shape and round with a diameter ranging from 0.5 to 3.0 mm. The border is circular and the surface is veined. Color of the colony is translucent greyish-tan. Small white flecks appear to be randomly dispersed through the

Figure 7

An electron photomicrograph of F. *polymorphum* suspended in distilled water. Magnification is 22,000 diameters.

Figure 8

A photograph of a colony of F. *polymorphum* cultured on blood agar at 48 hours of age. The circular nature of the colony is evident. The mucoid colony contains many small beige particle like structures dispersed throughout a translucent matrix.



mucoid matrix. These flecks also become more prominent with age.

Figure 8, p.40, illustrates 72 hour colonies on blood agar.

4.1.2.2 Cellular

The cells appear to have a width of about 0.3 to 0.5 microns and a length approximating the range 5.0 to 15.0 microns when grown on thioglycollate broth. If the organism is cultured on the defined media of Omata (35), cellular length increases to in excess of 100 microns, see Plate I. Definite granules are oriented in the cytoplasm along the long axis of the cell. Generally, two or three are present per cell. In the filamentous forms produced on defined media, the granules are arranged along the length of the cell. As the cell becomes older, the granules tend to lose the Gram positive character and become Gram negative.

The organisms appear to arrange themselves in a more or less random fashion. No alignment of the cell bodies is evident. The ends of the organisms are pointed and the cytoplasm appears homogenous with the exception of the Gram positive granules.

## 4.2 Metabolic Characteristics and Identification of the Organism

The results of tests for various cellular by-products and fermentations are given below.

Figure 9

Cellular by-products expressed as present (positive) or absent (negative).

| Test | Result |
|---|---|
| Indole | - Positive |
| Catalase | - Negative |
| Hydrogen sulfide | - Positive |
| Gas | - Negative |
| Nitrate reduction | - Negative |
| Gelatin liquifaction | - Negative |
| Methylene blue reduction | - Positive |
| Milk coagulation | - Negative |

Fermentation Results

End pH values are expressed as the mean of three determinations

| Medium | | pH |
|---|---|---|
| Basil medium | - | 7.2 |
| Glucose | - | 5.9 |
| Sucrose | - | 6.5 |
| Glycerol | - | 7.1 |
| Fructose | - | 6.0 |
| Lactose | - | 7.0 |
| Maltose | - | 7.1 |
| Inulin | - | 7.1 |
| Mannitol | - | 7.1 |

The data presented by the morphological, cultural, and biochemical features of this organism are coincident with the features of *Fusobacterium polymorphium* as put forth in Bergey's Manual of Determinative Bacteriology (5).

The production of reducing substances during metabolism is shown by data presented in Table 1, p.44.

## 4.3 Cell Wall Isolation

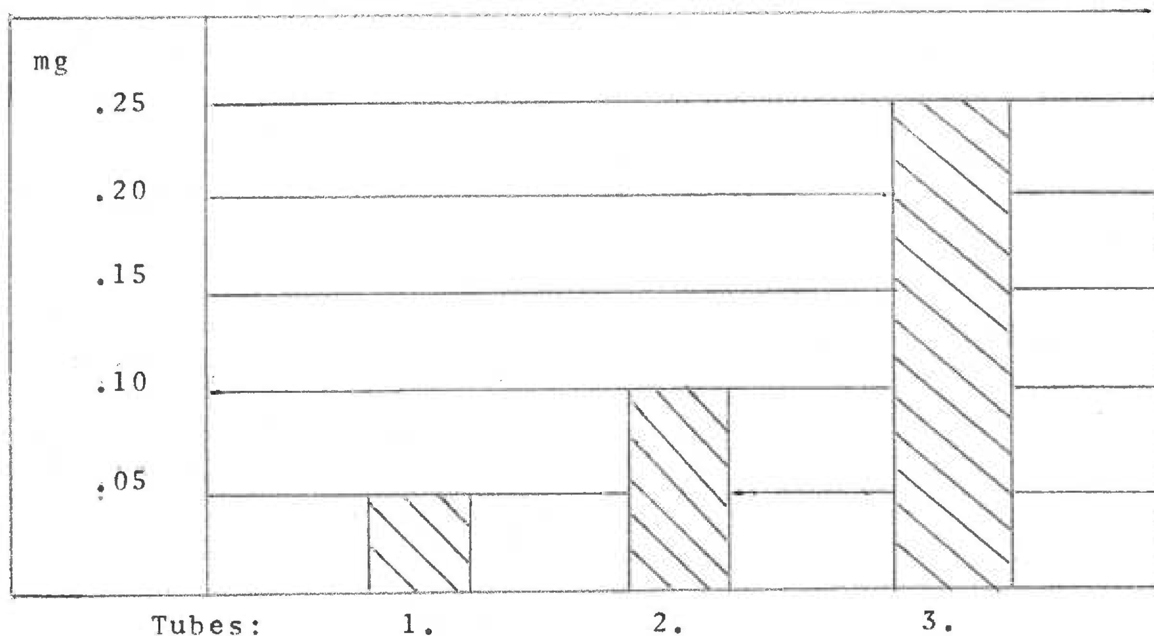
Procedures described by Salton (44, p.4) and others (20) for isolation of cell walls from fusobacterium proved unsuccessful. Removal of contaminating cytoplasm from the walls could not be adequately accomplished by established procedures involving molar potassium chloride, molar sodium chloride or water. Figure 11, p.46, shows wall fragments which are contaminated with cytoplasm. The importance of processing freshly harvested cells immediately, and of maintaining the entire isolation procedure at 5° C. was demonstrated (41). Processing fresh cells at 5° C. with cold one molar potassium chloride and cold distilled water improved the quality of resulting walls. The product, however, was not free from contamination as is evidenced by the electron dense bodies shown in Figure 12, p.46. It was later found that a homogenous product resulted when 10% sodium succinate was added in the final washing. The resulting walls appeared as in Figure 13, p.48. This material was judged free from contamination by cytoplasmic components with electron microscopy (Figure 13) and immunology (Table 2, p.59).

Table I

| Tubes | Reducing Material as Glucose |
|---|---|
| 1. Endogenous | .002 mg |
| 2. Glucose only | .100 mg |
| 3. Cells plus Glucose | .250 mg |

Reducing substances produced by F. polymorphum during glucose metabolism. Reducing material is expressed as glucose. Each value is the mean of two determinations. The range for all means was no greater than ± .002 mg.

Figure 10




A graphic comparison of reducing substances (as mg glucose) produced by endogenous cells and cells supplied with glucose. Tube 2 is a standard for the Benedict Test.

Figure 11

An electron photomicrograph of cell walls which have been washed with distilled water immediately after disruption of the whole cell. The presence of electron dense cytoplasm is evident. Magnification is 12,250 diameters.

Figure 12

An electron photomicrograph of cell walls which have been washed once with 1 molar sodium chloride. Electron dense material is evident. Magnification is 11,352 diameters.

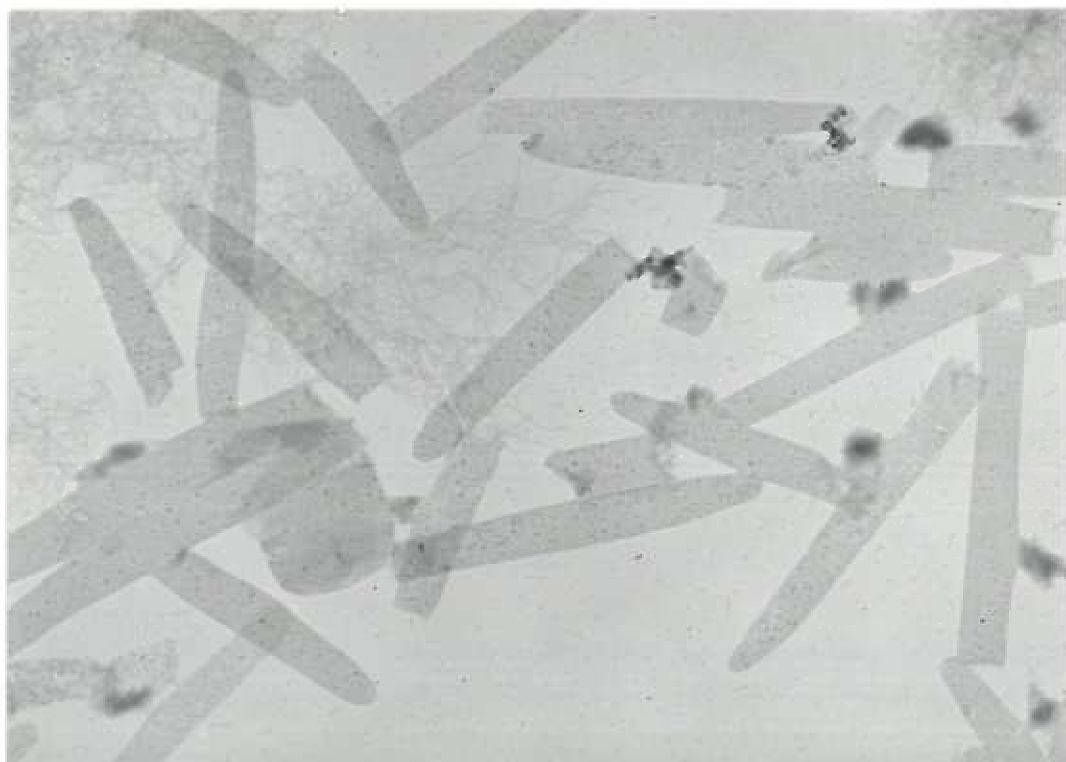
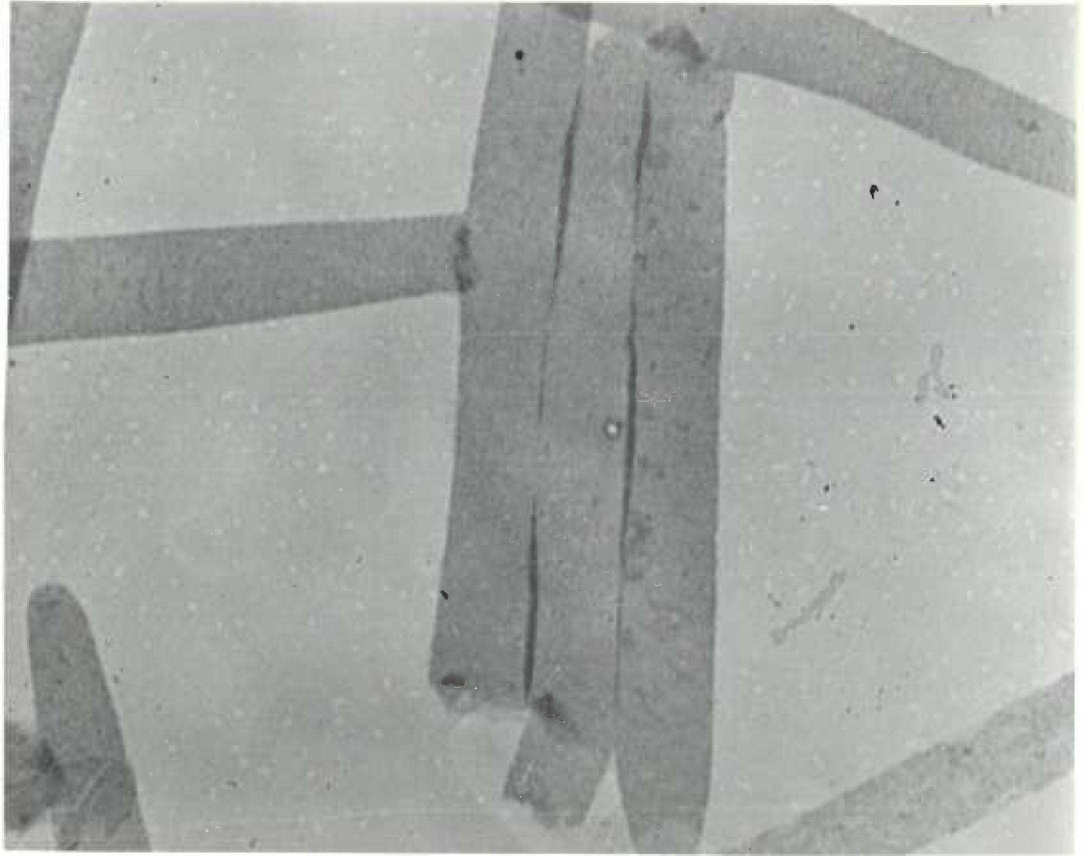


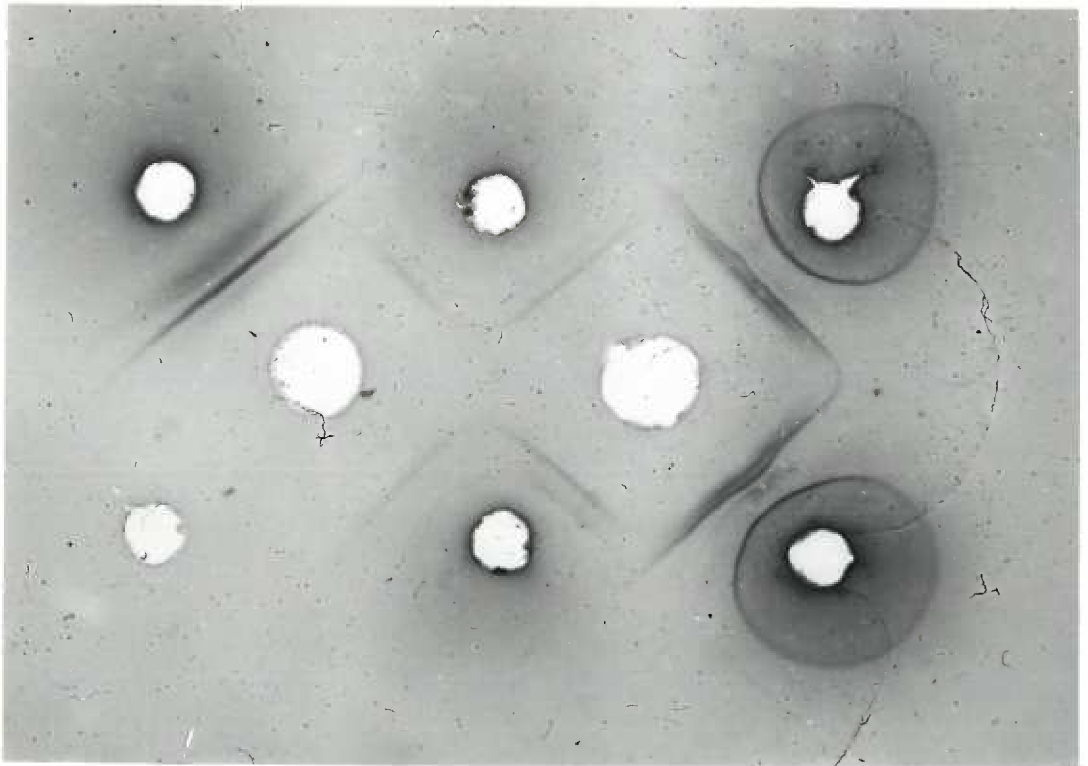
Figure 13

An electron photomicrograph of clean immunologically distinct cell walls. This material was washed with 10% sodium succinate at a temperature of 5° C. Magnification is 12,250 diameters.

Figure 14

A photograph of a gel diffusion plate showing the presence and absence of precipitin lines between center and peripheral wells. The photograph represents magnification of 2 diameters.





4.4 Results of gross chemical analyses on whole cells, cell walls, and test antigens are summarized in Table 3, p.62.

4.5 Table 2, p.59, illustrates the Results of The Gel Diffusion Analyses.

Each positive sign indicates the observance of a visible reaction. A negative sign indicates no visible reaction was observed. Figure 14, p.48, is a photograph of a gel diffusion plate showing positive and negative reactions.

# V. DISCUSSION

## 5.1 Difficulties in Culture of the Organism

The difficulty first encountered in the culture of *F. polymorphum* upon initial isolation and subsequent subculture was probably because of exposure to toxic oxygen levels and to foreign substrates. The isolation of bacteria from their natural habitat and the attempt to culture them on a foreign substrate often results in a decreased generation time (57). Characteristically the anaerobic bacteria are intolerant to the presence of oxygen. The precise reason for oxygen intolerance is not known and the mechanism through which toxicity results is only hypothesized (57, p.83). It appears that the oxidation-reduction potential of the cell environment must be below a specific level before growth can occur. The presence of toxic levels of oxygen in the environment increases the oxidation-reduction to a level not compatible with cellular metabolic machinery. The result is a complete loss of activity.

Any means by which a more negative oxidation-reduction potential can be obtained will result in increased cellular activity. The data presented in Table 1, p.44, show that *F. polymorphum* is capable of creating substantial amounts of reducing products. Such products favor an oxidation-reduction potential more compatible with cellular growth.

Another factor which operates to increase the generation time of freshly isolated bacteria is exposure to a foreign substrate. The need for several continuous generations cultured on the new medium to effect adaptation and maximal growth is well known (57, p.340-341).

In all probability increased oxidation-reduction potential had a retarding effect on growth potential of newly isolated fusobacteria. In this instance it was not known whether the increased oxidation-reduction potential *per se* or the presence of molecular oxygen stopped or retarded cellular activity. Activity is retained if the bacterium receives minimal exposure to oxygen and retained in a reduced atmosphere.

The decreased generation time which was suggested by visual observations of subsequent generations on thioglycollate medium is probably a function of adaptation to new substrates. After cellular adaptation to thioglycollate medium, the production of large amounts of reducing substances could help the cell overcome any marginal oxidation-reduction conditions.

Observations relating to the apparent decreased sensitivity of the organism in these studies to oxygen and the elimination of the need for serum in the culture medium correspond to those of Boe (3, p.58).

Filamentous growth noted in this study in defined medium was evidently the result of a lack of optimal conditions for cellular reproduction and/or growth. It has been stated that predominance of filamentous forms of fusobacterium are in all probability functions of suboptimal growth conditions (3, p.102).

5.2 Morphology of *F. polymorphum*

Morphology of the bacterium varies greatly. Two of the evident factors which influence cell form are age and nutrition. When log phase cells grown on thioglycollate are examined, they are approximately 10 to 15 microns in length and from 0.3 to 0.5 microns in width. Cells which are 72 hours old and are grown on blood agar appear very similar to those log phase cells grown on thioglycollate broth (Figure 7, p.37). Cells which were grown on the defined medium appear as in Figure 6, p.37. No change in the morphology was noted in cells subcultured 12 consecutive times on the defined medium. Such filamentous forms, as was mentioned, have been said to be the result of suboptimal growth conditions (3, p.102). Variation in morphology resulting from a change of environment is in itself not unusual (57). The unusual features here are that the cells increase in length and maintain the same form

over 12 continuous subcultures. It appears that cellular division might be handicapped by factors either present or absent from the medium. Baird-Parker states that fusobacteria divide by constriction of the cell walls so that daughter cells become pinched off--thus forming pointed ends (2, p.463). The presence of long filamentous forms, therefore, suggests a change in the ratio of cell division/protoplasmic growth. It appears as though nutritional factors are adequate for growth of cellular cytoplasm whereas they are inadequate for the cellular division mechanism. It is possible that the relation of Deoxyribonucleic acid (DNA) to cytoplasm is effected by sub-optimal growth conditions so that division can not readily take place (25, p.318).

### 5.3 Fermentation Reactions

Previous studies on the fermentation characteristics of fusobacteria indicate that as a group they are weak acid formers (3, p.114). When F. polymorphum was allowed to metabolize several carbohydrates, only glucose, fructose and sucrose produced significant amounts of acid. Figure 10 p.42, shows the carbohydrates tested and the resulting pH as measured with Radiometer type TTT IC pH meter. Production of acid is usually measured with an indicator dye

such as methyl red which has a pH range of 4.0 to 6.5 pH values above 6.5 cause the indicator to assume a yellow color. Red results when the pH fails to 4.0 or below. Routinely a bacterium is said to ferment a given sugar and produce acid if the indicator is red. If the color is yellow, the bacteria is said not to ferment that sugar. Because *F. polymorphum* is a weak acid former, the methyl red does not show a definite color change when used to indicate acid formation. Boe discusses this problem and claims that normal indicators are inadequate for designation of acid production by these organisms and that a pH meter should be used for reliable results.

Figure 9, p.42 shows the data of the sugars tested only glucose and fructose are readily metabolized. Utilization of fructose by a system which works well on glucose is not surprising in view of the fact that glucose is probably converted to fructose-6-phosphate in the early stages of its metabolism. The utilization of sucrose is conceivable. Either an acid condition of the environment results in the hydrolysis of the sucrose thus providing glucose and fructose (56, p.255), or the cell has lytic enzyme systems capable of attacking sucrose. Indeed the apparent deficient capability of maltose and lactose utilization can be attributed probably to the lack of a lytic

enzyme system for the B 1-4 glucosidic links and the very slow acid hydrolysis rate of lactose and maltose relative to sucrose (56). Another possibility is that the active permeability of the cell is limited to glucose, fructose and sucrose thus allowing only these molecules into the metabolic system.

5.4 Isolation of Antigens

The isolation of cell wall material proved to be by far the most difficult and surprising aspect of the entire study. Criteria for cell wall purity were immunological individuality and freedom from electron dense cytoplasm as judged by electron microscopy (29). The problem was primarily two-fold. First, a suitable method had to be devised by which the cells could be broken apart. The second aspect was to devise a method to rid the cell wall fragments of cytoplasmic contaminants. Sonoration was found to be unsuitable for cell disruption because it fragmented the walls so badly that it was difficult to judge them by electron microscopy. Glass beads were also tried in a shaker apparatus for disruption. The results were fifty to ninety percent breakup of the cells. Attempts to separate intact bacteria from cell wall fragments were unsuccessful. The methods of differential and density gradient centrifugation were unsuccessful. The problem was

spontaneous agglutination of the whole cells and cell parts. This prevented the various sized particles from separating at different centrifugation speeds. Varying ionic strength and density gradient systems did not afford separation. It was eventually decided that a method had to be found which would produce one hundred percent breakup of the organisms and therefore, eliminate the problem of separating whole cells from cell fragments. It was found that the original system used for obtaining cell breakup (200 mesh glass beads and a shaker apparatus) was discarded and one consisting of styrene divinylbenzene copalmer plastic beads and a Servall Omnimixer[R] employed, 100% breakup could be effected. Subsequent to washing freshly ruptured walls with a one molar potassium chloride solution and three aliquots of distilled water, the material was viewed with the electron microscope to judge purity. Figure 11, p.46, shows the walls with adherent cytoplasmic contamination. The electron dense material could not be removed with several wash solutions described in the literature (43).

Considering that Ribonucleic acid (RNA) is a prominent feature of structures immediately adjacent to the cell wall in the intact organism, it was felt that perhaps a good RNA solvent would facilitate cleaning the electron dense

material from crude cell walls. Ten percent sodium succinate was found to readily dissolve dry yeast RNA. When this solution was used to wash the wall material, no electron dense material remained. The conclusion reached by electron microscopy was varified by gel diffusion analysis. Figure 13, p.48, illustrates the clean walls as viewed by electron microscopy. Table 3, p.62, indicates that no visual precipitin reaction occurred when wall antisera reacted with cytoplasm. These immunologic findings confirm the suggestion forwarded by electron microscopy that the cell wall is an independent antigenic agent steriochemically unrelated to the cytoplasm. The results also show that the cytoplasm which was used to produce antisera for testing purposes was not contaminated with cell wall fragments.

Test antigens were isolated from whole cells by techniques which were described in the literature. The isolated material was called protein, polysaccharide, Boivin antigen and water extractable material only because it was isolated by methods commonly used for isolation of those particular chemical entities. The chemical analyses of the test antigens was done for the purpose of characterizing the material.

## 5.5 Antigenic Analysis

The data expressed in Table 2, p.59, is not to be interpreted without qualification. It must be stated that under the conditions of the experiments, the indicated immunologic relations either did or did not present themselves in a visually detectable way. No attempts were made to vary concentrations of test antigen or antisera in an effort to visually detect additional precipitation bands. The presence of a positive sign in the table indicates the minimum number of visually detectable antigen-antibody systems at work between the antisera and the test antigen. In some tests at least one system was operating and in others at least two were evident. The presence of a negative sign in Table 2 indicated that no visible sign of a precipitin antigen-antibody system was evident. On the basis of these criteria, we cannot hope to get the entire picture of the antigenic interrelations between the isolated cell components. We can, however, get some idea of the presence, minimum number present or absence of antigens common to structures.

The presence of wall antigenic material in the base solvent and its absence in an acid solvent is interesting (Table 2). The evidence suggests that base extractable material resides in the cell wall and not the cytoplasm.

Table 2

| | Antisera | | | |
|---|---|---|---|---|
| Test Antigens | Whole Cells | Cell Walls | Cytoplasm | Media |
| Base Extractable Protein | + | + | - | - |
| Acid Extractable Protein | + | - | ++ | - |
| Poly-saccharide | ++ | ++ | - | - |
| Hot Water Extractable Material | ++ | ++ | ++ | - |
| Boivin Antigen | ++ | + | - | - |
| Cytoplasm | - | - | ++ | - |
| Media Control | - | - | - | +++ |

Results of gel diffusion analyses. Each plus sign indicates an immunologic reaction between the antisera and a given test antigen. A negative sign indicates the lack of an immunologic identity.

This denotes a chemical and possibly a structural heterogeneity of these two cell fractions. The acid extractable protein on the other hand is immunologically distinct from the cell wall and resides in association with the cytoplasmic component of the cell. Such evidence also substantiates the heterogeneity between the cell wall and the cell cytoplasm. It is improbable that the difference in antigenic nature of the acid and the base extractable material is an artifact. Denaturation and/or minor rearrangement of the test antigens which might occur under the conditions used are minimal (17). In view of the differing chemical compositions of the acid and base extractable materials, we are justified in ruling out artifact as a cause for the observed antigenic individuality.

The data suggest that antigenic polysaccharide material resides in the cell wall complex. This confirms the work of others (8, 31). The similarity in reactivity between the polysaccharide and the Boivin antigen is not unexpected. The lipo-protein-polysaccharide complex of the Boivin material is largely polysaccharide. Large polysaccharides are capable of stimulating antibody production but classically they are thought to serve as haptenes. It is possible that these determinant groups are removed from the Boivin antigen by the polysaccharide extraction procedure.

The inclusion of water extractable material was for the purpose of a positive control on the gel diffusion tests. Hot water is capable of removing capsular polysaccharide from the cell as well as cytoplasmic components (10). As the data in Table 2, p.59 indicate, material from both the cell wall and the cell cytoplasm were extracted by this treatment.

The control for contamination of cellular antigens by culture medium indicates that the reactions which occurred were not as a result of culture medium contamination. This was evidenced by the results presented in Table 2 which show no reaction between these components.

5.6 Chemical Analyses

Table 3, p.62, presents data on whole cells and cell walls which fall within established ranges of analyzed Gram negative bacteria (12, p.14, 18, 26). Comparison, however, of quantitative data secured by different workers must be viewed with caution unless cultural conditions are rigorously defined. The data do suggest that the cell wall is comprised largely of lipid and polysaccharide and that the majority of these two components resides in the cell wall. The observation that more phosphorus is found in cell walls than in whole cells is not unexpected. The presence of phospholipids in the cell wall and the possible contribution

Table 3

| | Nitrogen | Phos-phorus | Carbo-hydrate | Lipid |
|---|---|---|---|---|
| Whole cells | 13.16±.12 | 1.20±.06 | 22.13±.18 | 10.02±.38 |
| Cell walls | 7.47±.16 | 0.79±.05 | 32.99±.20 | 26.12±.49 |
| Base Ext'r Protein | 4.30±.14 | 0.03±.09 | 4.00±.16 | – |
| Acid Ext'r Protein | 9.63±.09 | 2.01±.02 | 1.55±.09 | – |
| Polysaccharide | 1.46±.09 | 1.59±.14 | 41.25±.14 | – |
| Water Ext'r Mater-ial | 13.02±.09 | 5.38±.06 | 24.97±.12 | – |
| Boivin Antigen | 4.08±.09 | 16.74±.07 | 40.06±.23 | – |

Results of chemical analyses. Values are expressed as percents of the total sample by weight. The confidence interval of the mean was calculated using the statistic "T" with nine degrees of freedom at the 95% level of significance.

of phosphorus from the cytoplasmic membrane (12, p.27) could well account for the difference in phosphorus levels. The difference is further illustrated by the generous phosphorus content of the Boivin antigen which is characteristically unique to the cell walls of Gram negative bacteria (31).

The chemical analyses of test antigens is for the purpose of identity and characterization. The data demonstrate that, each extracted antigen is different in its chemical make up. Since the selection of each test antigen is based on the classical procedures for isolation of protein, carbohydrate and endotoxin (Boivin antigen) etc., the extracts are the result of the effects of precipitation, and of a given procedure when applied to the complex chemical make up of *F. polymorphum*.

# VI. SUMMARY

A Gram negative fusobacterium was isolated from the normal oral cavity. Studies regarding its cultural, metabolic and morphologic characteristics were performed and as a result, the bacterium could be classified as *Fusobacterium polymorphum* according to Bergey's Manual (5). Cell walls and soluble cytoplasm were isolated from the organism. Antisera developed against the isolated cytoplasm, cell walls, whole cells and culture medium was challenged with test antigens which were isolated from the whole cell. Immunological relations between the cell and its parts were studied. Gel diffusion analyses showed that base extractable antigens which reside in the cell wall do not appear in the cytoplasm. Acid extractable antigens reside in the cytoplasm and not in the cell wall. Polysaccharide material is found in the cell wall and not the cell cytoplasm.

Chemical analyses showed that *F. polymorphum* has a nitrogen content of 13.16±.12%, phosphorus 1.20±.06%, carbohydrate 22.13±.18%, and lipid 10.02±38%. The cell walls contain 7.47±.16% nitrogen, 0.79±.05% phosphorus, 32.99±.20% carbohydrate, and 26.12±.49% lipid. A procedure was developed utilizing 10% sodium succinate to isolate pure cell walls of a Gram negative bacterium.

# VII. CONCLUSIONS

The organism which was isolated for this study was *Fusobacterium polymorphum*. The investigation of its cultural and metabolic characteristics revealed that it is an unaerobic, Gram negative, pleomorphic and fastidious organism. Much of its fastidious nature, however, is lost during continuous subculture on complex culture medium. In general, these findings agree with those of previous investigators (3, 27, 29).

The cell wall of the organism appears antigenically distinct from cellular cytoplasm. Nonrelated antigenic factors which reside in different parts of the cell can be extracted by certain techniques. Some factors are native to the cell wall and are not antigenically related to cell cytoplasm. Conversely, cytoplasmic factors exist which are unrelated to the wall. Polysaccharide material and/or Boivin antigens were found to reside only in the cell wall.

Chemical analyses of the nitrogen, phosphorus, polysaccharide and lipid content of *F. polymorphum* indicate that it is similar in composition to other Gram negative bacteria.

# VIII. BIBLIOGRAPHY


1. Babes, H., Veber Bakterielle Hamorrhagische Infectionen Des Menschen. Wein. Med. Wchnschr. 42:1321-1327, Feb., 1872.

2. Baird-Parker, A.C., The Classification of Fusobacteria from the Human Mouth, J. Gen. Microbiol. 22:458-468, Apr., 1960.

3. Boe, J. Fusobacterium: Studies on its Bacteriology, Serology, and Pathogenicity, Norske Viden-Skaps Akad. Oslo. I. 9:1-187, Jan., 1941.

4. Bollen, W.B. Department of Microbiology, Oregon State University, Corvallis, Oregon. Personal Communication.

5. Breed, R.S. et al. Bergey's Manual of Determinative Bacteriology, ed. 7, Baltimore, Williams and Wilkins Co., 1957, p.436.

6. Bruce, H.W. A Case of Vincents Angina in Which the Trachea and Larynx were Involved. Lancet 2:1021-1022 Oct. 1907.

7. Crowle, A.J. Immunodiffusion, New York, Academic Press, 1961, p.265.

8. Davies, D.A.L. Advances in Carbohydrate Chemistry. New York, Academic Press, 1960, Vol. 15 p.271.

9. De Araujo, W.C., et al. Immunochemical Analysis of Human Oral Strains of Fusobacterium and Leptotrichia, J. Bact. 86:837-844, Oct., 1963.

10. Dudman, W.F. and Wikinson, J.F. The Composition of Extracellular Polysaccharides of Aerobacter-Klebsiella Strains Biochem. J. 62:289-295, May, 1956.

11. Ermatinger, L.H. Microorganisms of Lung Abscesses and Bronchiectasis. J. Infect. Dis. 43:390-398, Nov., 1928.

12. Gunsalus, I.C. and Stanier, R.Y. The Bacteria, New York, Academic Press, 1960, Vol. 1, p.13.

13. Hampp, E.G. and Mergenhagen, S.E. Endotoxic Properties by Local Shwartzman Reaction. J. Dent. Res. 39:267-272, Apr., 1960.

14. Heidelberger, M. and Kendall, F. Protein Fractions of a Scarlatnal Strain of Streptococcus Hemolyticus. J. Exptl. Med. 54:515-531, May, 1931.

15. Hine, M.K. and Berry, G.P. Studies of the Genus Fusiformis. J. Bact. 34:517-527, Nov., 1937.

16. Hoffman, H. Bacteriology of Fusobacteria: A Review. O. Surg., O. Med., O. Path. 5:1077-1095, July, 1952.

17. Hourowitz, F. Chemistry and Biology of Proteins. New York, Academic Press, 1950, p.3.

18. Jensen, S.B. and Mergenhagen, S.E. Influence of Endotoxin on Resistance of Mice to Intraperitoneal Infection with Human Oral Bacteria. Arch. Oral Biol. 9:229-239, May, 1964.

19. Jensen, S.B. and Mergenhagen, S.E. Influence of Endotoxin on the Dermal Response of Rabbits to Human Oral Bacteria. Arch. Oral Biol. 9:241-251, May, 1964.

20. Kabat, E.A. and Meyer, M.M. Experimental Immunochemistry Springfield, Ill., C.C. Thomas Co. Inc., 1948, p.740.

21. Kessler, G. and Nickerson, W.J. Glucomannan-Protein Complexes From Cell Walls of Yeasts. J. Biol. Chem. 234:2281-2285, Sept., 1959.

22. Kline, B.S. Experimental Gangrene, J. Infect. Dis. 32:481-483, June, 1923.

23. Knorr, M. Uber Die Fusospirillare Symboise. Die Gattung Fusobacterium und Spirillum Sputigenum, Besonders der Plaut-vincentschen Angina. Centralbl. F. Bakteriol. 87:536-540, June, 1922.

24. Krumwiede, C. and Pratt, J. Fusiform Bacilli. Isolation and Cultivation. J. Infect. Dis. 12:199-207, Feb., 1913.

25. Lamana, C. and Mallette, M. Basic Bacteriology: Its Biological and Chemical Background. Baltimore, The Williams and Wilkins Co., 1959, p.59.

26. Lewkowitz, X. Veber Die Reinkulturen Des Fusiformen Bacillus. Centralbl. F. Bakteriol. 41:153-155, June, 1906.

27. MacDonald, J.B., et al. The Pathogenic Components of an Experimental Fusospirochaetal Infection. J. Infect. Dis. 98:15-20, Jan., 1956.

28. Milner, J.C. et al Structure and Biological Properties of Surface Antigens from Gram Negative Bacteria. Bact. Rev. 27:352-358, June, 1963.

29. Mudd, S.E. and Lackman, D.B. Bacterial Morphology as Shown by Electron Microscopy: I. Structural Differentiation Within the Streptococcal Wall. J. Bact. 41:415-420, March, 1941.

30. Mühlens, P. and Hartmann, M. Zur Kenntnis Des Vaccineerregers. Centralbl. F. Bakteriol. 41:41-42, June, 1906.

31. Munoz, J. J. Immunological and Other Properties of Bordetella Pertussis Antigens. Bact. Rev. 27:325-331, June, 1963.

32. Neish, A.C. Report #46-8-3: Analytical Methods for Bacterial Fermentations. Saskatoon, National Research Council of Canada, 1952, p.33.

33. Nickerson, W. J. Biology of Pathogenic Fungi. Waltham, Mass., Chronica Botanica Co., 1947, p.167.

34. Omata, R.R. and Disraely, M.N. A Selective Medium for Oral Fusobacteria. J. Bact. 72:677-680, Nov., 1956.

35. Omata, R. Studies on the Nutritional Requirements of the Fusobacteria. J. Bact. 77:35-37, Jan., 1959.

36. Omata, R.R. and Braunberg, R.C. Oral Fusobacteria. J. Bact. 80:737-740, Dec., 1960.

37. Ozaki, Y. Zur Kenntnis Der Anaeroben Bakterien Der Mund Hohle. Centralbl. F. Bacteriol. 62:76-79, June, 1912.

38. Pelczar, M.J. et al. Manual of Microbiological Methods New York, McGraw-Hill Book Co., 1957.

39. Plaut, H.C. Werth Des Ausstrich-Praparats Bei Der Diagnose Der Diptheria. Deutsche Med Wehnschr. 21:291-301, July, 1895.

40. Pratt, D. On the Biology of B. Fusiformis. J. Infect. Dis. 41:461-466, Dec., 1927.

41. Ribi, E. Rocky Mountain Laboratory, Hamilton, Montana, Personal Communication.

42. Rosebury, T., et al. A Bacteriologic Survey of Gingival Scrapings From Periodontal Infections by Direct Examination, Guinea Pig Inoculation and Anaerobic Cultivation. J. Dent. Res. 29:718-731, Dec, 1950.

43. Salton, M.R.J. and Horn, R.W. Studies of the Bacterial Cell Wall: II. Methods of Preparation and Some Properties of Cell Walls. Biochim. et. Biophys. Acta 7:177-197, Jan., 1951.

44. Salton, M.R.J. Microbial Cell Walls. New York, John Wiley and Sons, Inc., 1960, p.27.

45. Slanetz, L.W. and Rettger, L. F. A Systematic Study of Fusiform Bacteria. J. Bact. 26:599-621, Dec., 1933.

46. Smith, D.T. Oral Spirochaetes and Related Organisms in Fusospirochaetal Diseases. Baltimore, The Williams and Williams Co., 1932, p.33.

47. Spaulding E.H. and Rettger, L. F. The Fusobacterium Genus I. Biochemical and Serological Classofication. J. Bact. 34:536-548, Nov., 1937.

48. Tunnicliff, R.J. The Identity of Fusiform Bacilli and Spirilla. J. Infect. Dis. 3:148-158, July, 1906.

49. Tynes, B.S. and Utz, J.P. Fusobacterium Septicemia. Amer. J. Med. 29:879-887, Nov, 1960.

50. Umbreit, W.W. et al. Manometric Techniques Minneapolis, Minn., Burgess Publishing Co., 1957, p.273.

51. Varney, P.L. The Serologic Classification of Fusiform Bacilli. J. Bact. 13:275-314, July, 1927.

52. Veillon, A. Recherches sur L'etiologie et la Pathogenie Des Angines Aigues non Diphteritiques. Arch. De Med. Exper. et D'Anat., Path, Par. 6:161-191, May, 1894.

53. Veszpremi, D. Kultur und Tierversuche mit Dem Bacillus Fusiformis und Dem Spirillus. Centralbl. F. Bakteriol. 38:136-137, June, 1905.

54. Vincent, H. Sur L'Etiologie et Sur Les Lesions Anatomopathologigues de la Pourriture D'Hospital. Ann. Inst. Past. 10:488-497, Jan., 1896.

55. Webster, M.E. et al. Studies on the O Antigen of Salmonella typhosa I. Purification of the Antigen. J. Immunol. 74:455-478, June, 1955.

56. West, E.S. and Todd, W.R. Textbook of Biochemistry, Ed. 2. New York, MacMillan Co., 1955, p.255.

57. Wilson, G.S. and Miles, A.A. Topley and Wilson's Principles of Bacteriology and Immunity, Ed.4. Baltimore, The Williams and Wilkins Co., 1955, p.82.

58. Wilson, M.W. and Pringle, B.H. Experimental Studies of the Agar Plate Precipitin Test of Oucterlony. J. Immunol. 73:232-243, Oct., 1954.