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SEROLOGIC REACTIONS OF ORAL GRAM NEGATIVE ANAEROBIC BACILLI.(U)
JAN 77 C E HAWLEY, W A FALKLER DAAB05-72-A-0666
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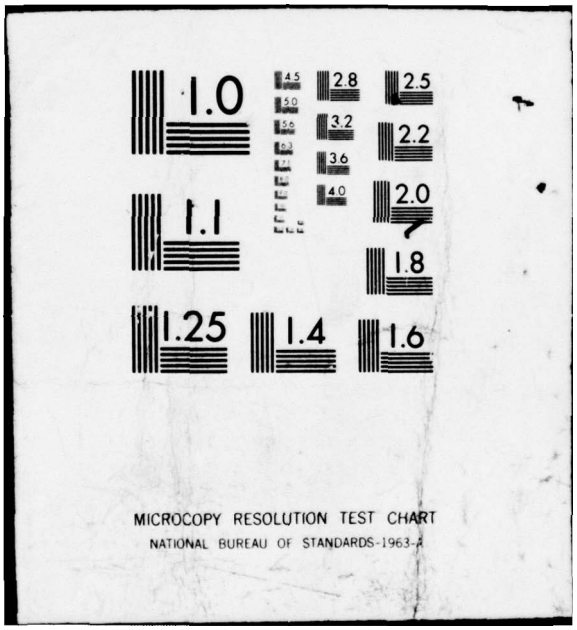
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Serologic reactions were performed using hyperimmune rabbit antisera and antigenic preparations of L. buccalis, F. fusiforme, and F. polymorphum. All tests indicated that there was serologic cross reactivity between the two Fusobacterium species. No cross reactivity could be detected between the Fusobacterium species and L. buccalis. The findings suggest that F. fusiforme and F. polymorphum are similar in their immunogenicity, and that the recent grouping of these organisms as F. nucleatum is justified.

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Serologic Reactions of Oral Gram Negative Anaerobic Bacilli

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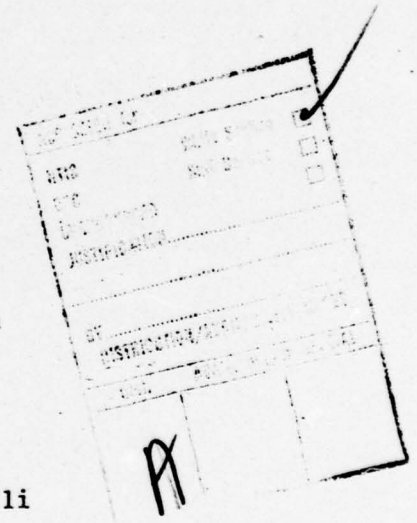

Short Title: Serologic Reactions of Oral Bacilli

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The methods developed in this investigation are basic to current USAIDR mission oriented research on the serologic identification of anaerobic bacteria in oral wound infections.

Synopsis:

Serologic reactions were performed using hyperimmune rabbit antisera and antigenic preparations of *L. buccalis*, *F. fusiforme*, and *F. polymorphum*. All tests indicated that there was serologic cross reactivity between the two *Fusobacterium* species. No cross reactivity could be detected between the *Fusobacterium* species and *L. buccalis*. The findings suggest that *F. fusiforme* and *F. polymorphum* are similar in their immunogenicity, and that the recent grouping of these organisms as *F. nucleatum* is justified.

The non-spore forming, anaerobic, gram negative bacteria of the family *Bacteroidaceae* include members of the genera *Bacteroides, Fusobacterium,* and *Leptotrichia*[1].

The differences that separate *L. buccalis* from the *Fusobacterium* species have been reported by Werner *et al.*[2]. Biochemically, strains of *Fusobacterium* were reported to produce butyric acid, propionic acid and acetic acid in a weak saccharolytic process. In addition, *Fusobacterium* species were shown to produce ammonia, hydrogen sulfide, and a positive indole reaction. In contrast, *L. buccalis* was found to be highly saccharolytic, but it did not produce butyrate, ammonia, hydrogen sulfide, or a positive indole reaction.

Hyperimmune rabbit and human sera have also been used to distinguish the *Bacteroidaceae*. DeAraujo *et al.*[3] analyzed the differences in antigenic potential between members of this group of microorganisms. Using phenol/water extracted lipopolysaccharide (LPS) preparations of *F. polymorphum* and *L. buccalis,* it was determined that LPS was type specific when reacted in an untanned cell hemagglutination test. It was noted that crude antigen preparations from different strains of either *F. polymorphum* or *L. buccalis* showed marked intra-genus cross reactivity. However, neither the LPS nor the crude antigen preparations showed cross reactivity between *F. polymorphum* and *L. buccalis*. On the basis of tests using phenol or trypsin treated crude antigens, it was established that the group reactive antigens of *F. polymorphum* were protein in nature, and that the type specific antigen of both organisms was LPS.

Kristoffersen[4,5] reported that antisera specific to the group reactive *Fusobacterium* antigen, Precipitinogen 2, would not react serologically with crude preparations of *L. buccalis* or *Bacteroides melaninogenicus*. Human sera from different aged patients showed the presence of antibody that reacted with Precipitinogen 2 which could be removed from the sera by absorption with Precipitinogen 2. How-

ever, the absorption of the sera with *L. buccalis*, *Spherophorus necrophorus*, *Veillonella* species had no effect on the reactions seen with the unabsorbed sera. Mergenhagen *et al.*[6] showed that human sera absorbed with *L. buccalis* failed to react in a hemolysis test with sheep red blood cells coated with a homologous crude antigen preparation. Absorption of the same sera with *Fusobacterium* or *Veillonella* whole cells did not affect the reactivity of the sera.

This study was undertaken to investigate the value of serologic reactions in further taxonomic studies of *Fusobacterium* species and *L. buccalis*.

MATERIALS AND METHODS

Organisms used in this investigation are identified as *Leptotrichia buccalis* (ATCC #19696), *Fusobacterium polymorphum* (ATCC #10953), and *Fusobacterium fusiforme* (ATCC #23726)[a]. The latter two organisms have been recently classified as *F. nucleatum* in the 8th edition of Bergey's Manual.[1]

All three members of the family *Bacteroidaceae* were grown in a liquid modified tryptone media, pH 7.2, containing Bacto-tryptone, 10 g; Bacto-yeast extract, 10 g; K_2HPO_4, 1.25 g; $MgSO_4 \cdot 7H_2O$, 1.25 g; glucose, 2 g; and sodium thioglycollate, 5 g; per liter of distilled water. After autoclaving at 121 C and 15 pounds per square inch and cooling to room temperature, the media was inoculated with one of the above oral anaerobes. The freshly inoculated broth cultures were then placed into the anaerobic growth system[b] and incubated for 48 h at 37 C.

After incubation, the cells were harvested by centrifugation at 10,000 x g for 10 min at 4 C. The cells were washed three times in 0.01 M phosphate buffered saline (0.15 M NaCl) pH 7.2 (PBS). The washed cells were then resuspended in iso-

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[a]American Type Culture Collection, Rockville, Md.
[b]Gas-Pak System
Baltimore Biological Laboratories, Cockeysville, Md.

tonic saline and sonicated[c] at 6 amps in a dry ice/ethanol bath (-40 C) using eight, 30 sec bursts. Antigenic preparations of each of the three organisms were derived from the sonicated cellular debris by centrifugation at 10,000 x g for 10 min at 4 C. The supernatants were frozen at -20 C and designated FP 10000, FF 10000 or LB 10000 for the supernatants from *F. polymorphum, F. fusiforme, buccalis* respectively.

The supernatants from the 10,000 x g centrifugation were tested for total protein by the Lowry-Ciocalteau method.[7] Absorbance of the solution was then measured at 540 nm with a colorimeter[d] and compared against a bovine serum albumin standard curve. The bacterial preparations were frozen at -20 C.

New Zealand albino rabbits weighing approximately 3 to 4 kilograms were injected subcutaneously on the preshaved back twice at one weekly interval with 325 µg of the bacterial antigenic preparations suspended in Freund's Complete Adjuvant[e]. The FP, FF, or LB 10000 antigen preparations were used for immunization in all cases. These injections were followed by subcutaneous injections at 650 µg of the antigen alone at three weekly intervals. After a period of eight days, the animals were ear bled. After the blood had been allowed to stand at room temperature for 2 h the sera were separated by centrifugation at 600 x g. The prepared sera were appropriately labelled anti-FP, anti-FF or anti-LB and stored at -20 C.

FP, FF, or LB 10000 preparations from each of the organisms were examined using disc polyacrylamide gel electrophoresis *via* the Canalco system[f]. Each

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[c]Branson Sonifier
Branson Instruments, Inc., Stamford, Conn.
[d]Klett-Summerson Photo Electric Colorimeter
Klett Mfg. Co., New York, N.Y.
[e]Difco Laboratories, Detroit, Mich.
[f]Canalco System, Canalco, Rockville, Md.

preparation, 100 µg, was mixed with the loading gel and the current applied at 2 amps/gel for 4 h at 4 C. The migration of proteins along the 11 cm gels was monitored with tracking dye. The positions of the migrating proteins were established after trichloroacetic acid fixation and staining the gels with 1% Coomasie blue followed by destaining with 0.7% acetic acid.

The FP and FF 10000 preparations were also electrophoresed in 1% A grade agarose[g]. The agarose (1 g) was dissolved by heating a solution containing 95 ml isotonic saline and 5 ml borate buffer (boric acid, 6.18 g; sodium tetraborate, 9.536 g and NaCl 4.384 g in 1,000 ml distilled water). The warm agar (2 ml) was allowed to gel on 26 x 76 mm glass microscope slides. After depositing 25 µl of the antigen at 2,800 µg/ml in the wells, electrophoretic separation was carried out across a potential of 6-7 volts per cm for 2 h in cold veronal buffer, pH 6.8 (Barbital, N.F., 2.76 g; sodium barbital, 17.52 g; and calcium lactate, 0.768 g in 2,000 ml distilled water). The antigens were then reacted for 48 h in the cold with undiluted homologous and heterologous rabbit antisera.[8]

Double diffusion in agar was performed at 4 C in plastic petri dishes[h] which contained 4 ml 1% agarose (1 g A grade agarose in 95 ml isotonic saline and 5 ml borate buffer, pH 8.0). Five mm diameter wells were cut to a depth of 2 mm with center to center distance of 15 mm. Bacterial preparations of varying protein concentrations ranging from 3,400 µg/ml were placed in the radial wells against homologous or heterologous undiluted rabbit antisera in the center wells. Also, different antigen preparations at a standard protein concentration of 1,700 µg/ml were placed in the radial wells and reacted with each of the rabbit antisera in order to study identity, partial identity, and lack of identity between reactants.[8]

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[g]Calbiochem, San Diego, Cal.
[h]Falcon Plastics, Los Angeles, Cal.

A microtiter modification of the hemolysis test using untanned sheep red blood cells (SRBC) was performed with three bacterial preparations (FP, FF, and LB 10000) and the three rabbit antisera.[6] Each bacterial preparation (1,700 µg/ml) was incubated with an equal volume of a 2.5% PBS washed SRBC suspension for 30 min at 37 C. After a washing step with cold PBS and centrifugation at 600 x g for 10 min, the sensitized cells (50 µl) were placed in a microtiter U plate[i] with 50 µl serial dilutions of homologous or heterologous rabbit antisera that had been previously heat treated (56 C for 30 min) and absorbed for 30 min at 37 C with 50% suspensions. To each of the wells was added 25 µl of a 1:10 dilution of guinea pig complement (GPC') in PBS. The GPC' was titrated so that each well contained approximately 20 $C'H_{50}$ units.[9] After the plates were sealed and mixed for one min, they were incubated for 90 min at 37 C and then placed at 4 C for an additional 18 h before reading. A 1+ hemolysis was considered the end point for each serum titration. Controls for the hemolysis test included the reaction of unsensitized SRBC, antisera, and GPC' as well as the reaction of sensitized SRBC with GPC' in the absence of antisera.

A microtiter modification of the tanned cell passive hemagglutination test (HA) was performed.[10] FP, FF, and LB 10000 were allowed to react for 15 min at room temperature with a 2.5% suspension of SRBC that had been previously washed in PBS and treated with a 1:20,000 solution of tannic acid in PBS. The tanning was accomplished by mixing equal volumes of 1:20,000 tannic acid (0.05 g/liter) and the 0.5% SRBC cell suspension for 10 min at 37 C. The cells were then washed once in cold PBS by centrifugation at 600 x g and resuspended in 0.85% saline. Cell sensitization was accomplished by mixing 1 part tanned cell suspension, 4 parts buffered saline (100 ml 0.85% saline, 32.2 ml 0.5 M Na_2HPO_4, and 67.7 ml 0.15 M KH_2PO_4 adjusted to pH 6.4), and 1 part antigen (1,700 µg/ml).

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[i]Cooke Engineering Co.
Alexandria, Va.

The mixture was incubated for 15 min at room temperature. The cells were washed once in 0.2% gelatin diluent (2 mg gelatin/liter 0.85% saline) and then resuspended in the same diluent. The antigen coated SRBC (50 µl) were placed with 50 µl of heat treated (30 min at 56 C) and 50% SRBC absorbed (30 min at 37 C) homologous or heterologous rabbit antisera that had been serially diluted in the gelatin diluent. After mixing the plates for 1 min, the plates were incubated at room temperature with the results recorded at 3 h and 12 h. A 1+ hemagglutination was established as the end point. Controls using the gelatin diluent in place of the sera as well as controls using unsensitized SRBC were included.

Complement fixation (CF) testing was performed using the LBCF microtiter test system.[12] Briefly, 25 µl of each of the bacterial preparations (FP, FF, and LB 10000) which had been two fold serially diluted in a veronal buffered diluent (VBD), pH 7.3-7.4, were added to microtiter plates containing 25 µl of serially diluted rabbit homologous or heterologous antisera. The antisera had been previously heat treated (56 C for 30 min) and absorbed with a 50% VBD suspension of SRBC. To the antigen/antibody mixtures in each well were added 50 µl of GPC' that was titrated according to LBCF procedure and diluted so that each well would receive 5 $C'H_{50}$ units.[9] The plates were gently mixed and incubated for 18 h at 4 C at which time 25 µl hemolysin sensitized SRBC in a 1.4% suspension were added to each well. Controls to detect the anticomplementary activity of either the antigen or the antisera and the activity of the GPC' in the absence of antigen and antisera were also included.

RESULTS

The molecular heterogeneity of each crude preparation was apparent after staining the disc electrophoresis gels with 1% Coomasie blue. The results are

shown in Figure 1. Major differences in banding were observed between LB 10000 and similar preparations of FP and FF 10000. Only minor differences were observed in the banding patterns of the electrophoresed FP 10000 and FF 10000.

The immunogenic heterogeneity of the FP 10000 and FF 10000 preparations was observed using immunoelectrophoresis in gel with either anti-FP or anti-FF sera. The findings are presented in Figure 2 which shows the electrophoretic patterns that were produced by the reaction of anti-FF sera with both FP 10000 and FF 10000 preparations at 2,800 µg/ml. The precipitation bands revealed at least 5 common antigen-antibody systems between the two *Fusobacterium* antigen preparations, and there were at least two precipitation bands seen in the reaction of anti-FF with FF 10000 that were not seen with FP 10000. In addition, there was at least one line observed with FP 10000 that was not seen with FF 10000.

Immunodiffusion in agar was performed using the rabbit antisera against homologous or heterologous bacterial preparations (FP 10000, FF 10000 or LB 10000). Figure 3 shows the effect of antigen dilution on the reactivity of antisera in agar. FF 10000 antigen concentrations in the radial walls were 3,400, 2,800, 2,000, 1,400, 700, and 300 µg/ml total protein. Dilution of this preparation as well as a similar serial dilution of FP 10000 showed a progressive reduction in the number and intensity of precipitation lines in agar against anti-FF sera.

Double diffusion in agar was also performed in which undiluted anti-LB sera produced 4 precipitation lines with LB 10000 at a concentration of 1,700 µg/ml protein. The same antisera showed no reactivity in agar with the same concentrations of FP or FF 10000. Figure 4 shows that undiluted rabbit anti-FF sera produced 5 precipitation lines against FF 10000 at 1,700 µg/ml protein. Two of these lines showed reactions of identity with the same concen-

tration of FP 10000. There was no evidence of reactivity in agar of antisera prepared to *F. fusiforme* with LB 10000. Reactions similar to those with anti-FF were observed with anti-FP sera. Two major lines of identity were produced with FP 10000 and FF 10000 at 1,700 µg/ml and there was at least one precipitation line with FF 10000 that was not apparent protein with FP 10000. No reactions were seen between antisera prepared to sonicates of *F. polymorphum* and antigenic preparations of *L. buccalis* at 1,700 µg/ml protein.

The results of the hemolysis tests are shown in Table 1. The reaction of each of the bacterial preparations at a concentration of 1,700 µg/ml protein was compared using homologous and heterologous rabbit antisera. Anti-LB sera reacted to a titer of 1:256 with LB 10000 coated untanned SRBC. However, the same antisera showed no cross reactivity (<1:2) with untanned SRBC that had been sensitized with 1,700 µg/ml protein of FP 10000 or FF 10000. Anti-FP and anti-FF showed little, if any, reactivity (1:4 and 1:2 respectively) with LB 10000 coated SRBC, but these antisera did react with FP 10000 and FF 10000 coated SRBC. Anti-FP showed a titer of 1:256 against FP 10000 coated SRBC and a titer of 1:64 against FF coated SRBC. Anti-FF reacted with 1 fold higher titers (1:512) against FP 10000 sensitized SRBC than did anti-FP. Anti-FF also produced 2 fold higher titers against FF sensitized SRBC than did the anti-FP sera.

Table 1. HEMOLYSIS TEST

Source of Antigen Preparation[a]	Hyperimmune Rabbit Sera			
	Anti-LB	Anti-FP	Anti-FF	Anti-sera Control (PBS)
L. buccalis (LB) ATCC #19616	1:256	1:4	1:2	<1:2
F. polymorphum (FP) ATCC #10953	<1:2	1:256	1:512	<1:2
F. fusiforme (FF) ATCC #23726	<1:2	1:64	1:64	<1:2
Unsensitized SRBC Control	<1:2	<1:2	<1:2	<1:2

[a]1,700 µg/ml protein

The HA results are shown in Table 2. Anti-FP and FF reacted with homologous and heterologous *Fusobacterium* preparations when FP 10000 and FF 10000 were used at a concentration of 1,700 µg/ml protein to sensitize tanned SRBC. Anti-FP reacted to titers of 1:512 and 1:128 with FP 10000 coated and FF 10000 coated SRBC respectively. Similarly, anti-FF sera reacted to titers of 1:512 and 1:1024 with SRBC sensitized with FP and FF 10000 respectively. Anti-FP and anti-FF did not react with LB 10000 coated tanned SRBC in the passive hemagglutination assay. Anti-LB did however react with the LB 10000 coated SRBC to a titer of 1:64.

Table 2. HEMAGGLUTINATION TEST

Source of Antigen Preparation[a]	Hyperimmune Rabbit Sera			
	Anti-LB	Anti-FP	Anti-FF	Antisera Control
L. buccalis ATCC #19616	1:64	<1:4	<1:4	<1:4
F. polymorphum ATCC #10953	ND	1:512	1:512	<1:4
F. fusiforme ATCC #23726	ND	1:128	1:1024	<1:4
Unsensitized SRBC Control	<1:4	<1:4	<1:4	<1:4

[a]1,700 µg/ml protein

The results of the CF tests are shown in Table 3 and indicate that there was no detectable cross reactivity between LB 10000 and the similarly prepared FP 10000 and FF 10000.

Table 3. COMPLEMENT FIXATION TEST

Source of Antigen Preparation	Anti-LB	Anti-FP	Anti-FF
L. buccalis (LB) ATCC #19616 (425 µg/ml)	1:128	<1:4	<1:64
F. polymorphum (FP) ATCC #10953 (700 µg/ml)	<1:16	1:256	1:1024
F. fusiforme (FF) ATCC #23726 (650 µg/ml)	<1:16	1:256	1:4096

Anti-LB sera contained CF antibodies that reacted with the homologous antigen preparation to a titer of 1:128 while anti-LB did not react with FP 10000 or FF 10000 preparations greater than the anti-complementary level of the sera itself (1:16). On the other hand, neither anti-FP nor anti-FF sera showed any CF activity with the LB 10000 preparation within the anticomplementary limit of each sera which was 1:4 for anti-FP and 1:64 for anti-FF. Anti-FP did react with both FP 10000 and FF 10000 to a titer of 1:256. Anti-FF showed the presence of CF antibodies to titers of 1:1024 with FP 10000 and 1:4096 with FF 10000.

DISCUSSION

Using disc polyacrylamide gel electrophoresis, it was determined that there were major electrophoretic differences in the distribution of FP 10000 or FF 10000, and LB 10000 components. This suggests that there may be potential taxonomic value in polyacrylamide electrophoresis for isolation and serologic testing of species specific antigens. Eluates from the slices of the LB 10000 gels have already been evaluated by Falkler and Joseph[12] for reactivity in various serologic tests. Their data showed that some eluates reacted in a CF test and an immunodiffusion test while others reacted in only one of the two tests studied. In addition, those eluates that reacted in the CF test showed no reactivity in the hemolysis test indicating the isolation of a protein antigen.

In the series of serologic reactions reported here, it has been further shown that marked antigenic differences separate *L. buccalis* and the two *Fusobacterium* species examined. All tests indicated that the antisera produced in rabbits to *L. buccalis* would not react with antigenic preparations of *Fusobacterium* species while reacting with homologous preparations. These findings are in support of others who have reported the genus specificity of LPS from *L. buccalis*[3,6] and of precipitinogen 2 from *Fusobacterium* species.[4,5] In addition to supporting the antigenic dissimilarity of *L. buccalis* and the two *Fusobacterium*

species, the immunodiffusion tests further showed that there were at least two shared antigen/antibody systems between antigenic preparations of *F. polymorphum* and *F. fusiforme*. Using immunoelectrophoresis, the antigenic similarity of both FP 10000 and FF 10000 was also revealed by 5 cross reacting antigen/antibody systems. Immunoelectrophoresis is currently being employed in our laboratories to confirm the isolation and immunogenic homogeneity of antigens prepared from other *Bacteroidaceae*. The finding of strong serologic cross reactivity between FP 10000 and FF 10000 by immunodiffusion and immunoelectrophoresis testing indicates that there may be significant justification for grouping *F. fusiforme* and *F. polymorphum* onto one species of *Fusobacterium, F. nucleatum*.

In the other serologic tests reported here, the cross reactivity of FP 10000 and FF 10000 was confirmed as was the serologic non-reactivity of *L. buccalis* when compared with members of the genus *Fusobacterium*. It has been suggested[6,13,14] that the untanned cell hemolysis test is well suited for the detection of antibodies in human sera that react with LPS or polysaccharide antigens. It has also been reported[4,5,15] that tanned cells tend to adsorb protein antigens. This would indicate that the tanned cell hemagglutination test employed here detected antibodies that probably reacted with protein antigens which differed from the polysaccharide antigens detected in the hemolysis test. The results of hemolysis and hemagglutination testing also indicate the potential immunogenicity of protein and polysaccharide antigens obtained from these gram negative bacteria. The presence of antibodies in human sera that react with antigenic preparations of *L. buccalis*[13,15] and *Fusobacterium* species[4,5,14] in different serologic tests suggests a potential role for these multiple antigen/antibody systems in hypersensitivity states involved in the pathogenesis of periodontal diseases.[16]

CONCLUSION

The serologic tests employed in this investigation showed that antigenic preparations of *F. polymorphum* and *F. fusiforme* were similar in their immunogenicity in rabbits, that there were at least 5 common antigen/antibody systems shared by the two *Fusobacterium* species, that antigenic preparations obtained from *L. buccalis* would not react in serologic tests with anti-*Fusobacterium* sera, and that preparations from *F. polymorphum* and *F. fusiforme* would not react with anti-*L. buccalis* sera.

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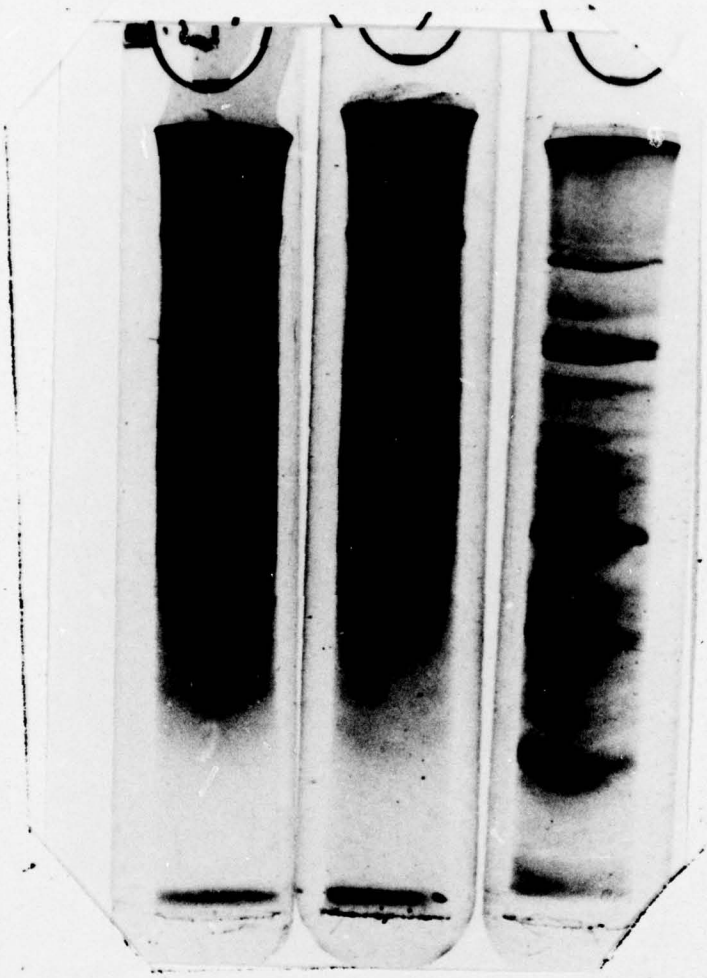

Figure 1.

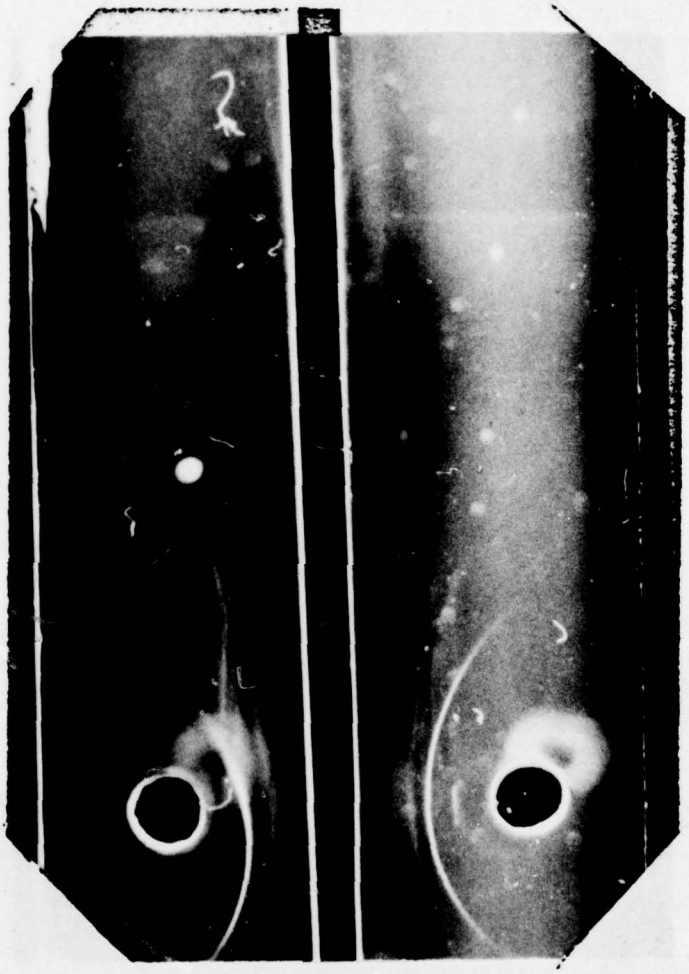

Figure 2.

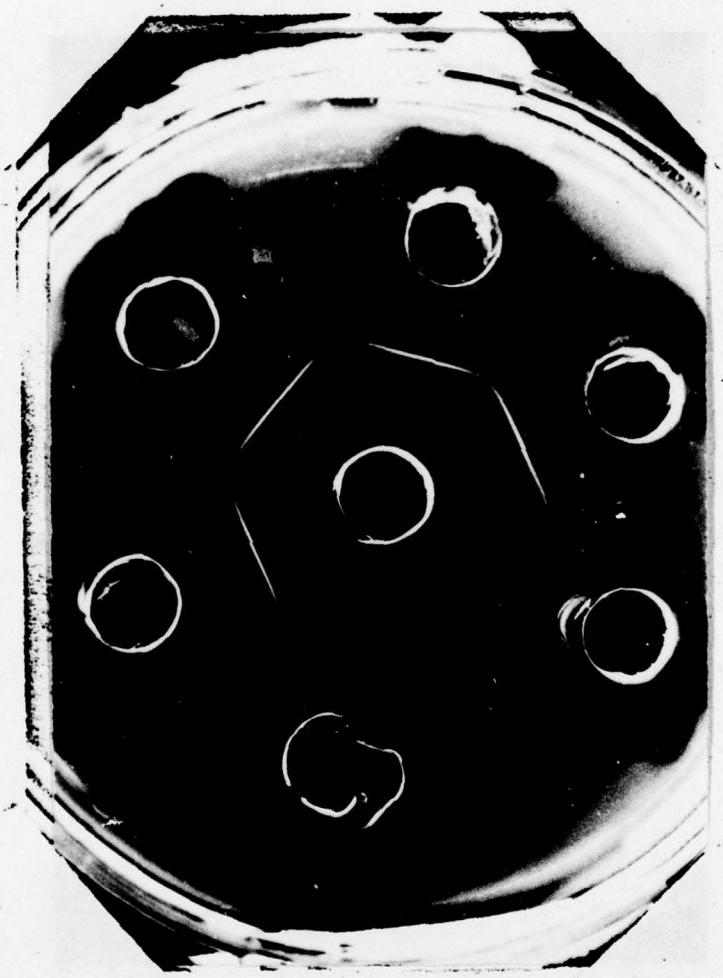

Figure 3.

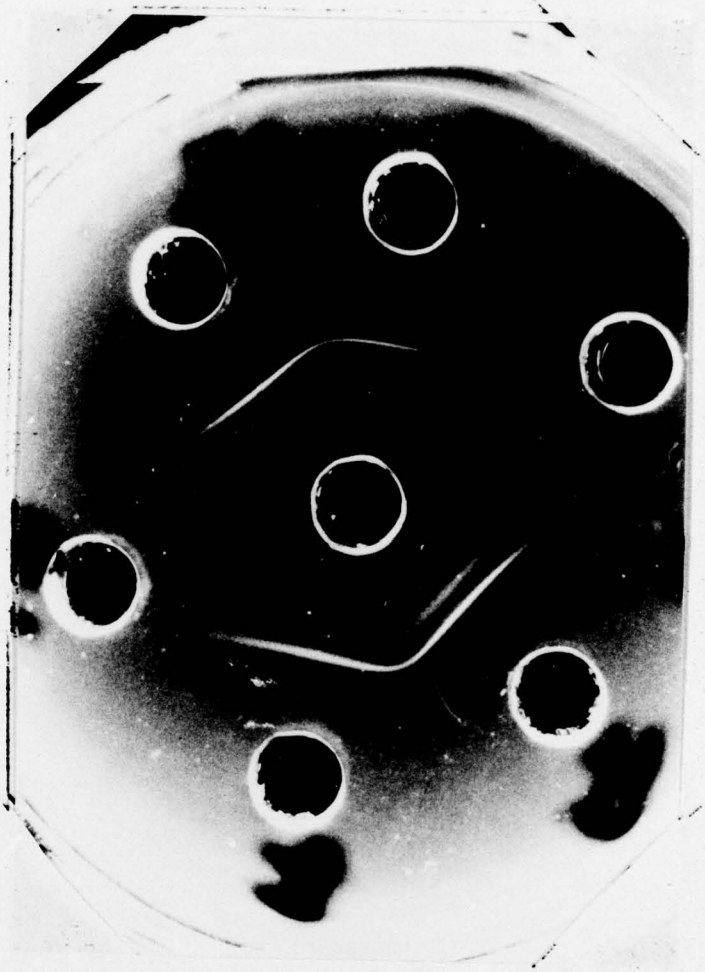

Figure 4.

Figure 1. This figure is a photographic record of disc electrophoresis performed with LB 10000 (LB), FF 10000 (FF), and FP 10000 (FP).

Figure 2. This figure is a photographic record of the immunoelectrophoresis performed with FF 10000 (FF) and FP 10000 (FP) against anti-FF sera. There were at least two precipitation lines formed with FF 10000 that were not formed with FP 10000 (arrows).

Figure 3. This figure shows the effect of diluting FF 10000 in immunodiffusion with anti-FF sera. Reading clockwise from well marked 3400, the concentration of antigen in the radial wells was 3,400, 2,800, 2,000, 1,400, 700 and 300 µg/ml protein.

Figure 4. This figure shows the reactions in immunodiffusion with anti-FF sera in the center well and 1,700 µg/ml LB 10000, FP 10000, and FF 10000 in the six radial wells.