Immunogenicity of Streptococcus equisimilis

Roger Dean Woods
Iowa State University

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Immunogenicity of *Streptococcus equisimilis*

by

Roger Dean Woods

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of
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DOCTOR OF PHILOSOPHY

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INTRODUCTION

Suppurative arthritis in young pigs is generally caused by beta-hemolytic streptococci belonging to Lancefield's serological Group C (426). The principal etiological agents have been reported to be *Streptococcus zooepidemicus* and *Streptococcus equisimilis* (78, 246). Collier (78) originally suggested the importance of *S. equisimilis* in young swine and his findings have been confirmed by others (330, 375, 471). Suppurative arthritis in young swine has also been produced by organisms found in Lancefield Groups D (142) and L (478).

The importance of the disease has been indicated by Collier (78), Helms (200), Doty and Vorhies (114) and Shuman and Wood (426). Approximately 50 per cent (236 of 493) of swine streptococcal isolates from diagnostic laboratory cases were Group C organisms (426). Besides septicemia and suppurative arthritis in young pigs, *S. equisimilis* has been isolated frequently from the reproductive tracts (195, 200) and mammary glands of sows (48, 178, 373) where it has been associated by some veterinary practitioners and diagnosticians with abortion and other forms of reproductive failure (114). However, definite proof that the organism is responsible for these disorders has not been reported (382, 449). No economic
surveys have been reported relative to losses attributed to Group C organisms, particularly \textit{S. equisimilis}.

\textit{S. equisimilis} produces septicemia and suppurative arthritis in swine from birth to six weeks of age (78, 246). The infection is manifested primarily as an acute suppurative arthritis characterized by an evaluated temperature, pain, lameness and swelling of affected joints (381). Mortality is generally low but morbidity may reach 100 per cent in affected litters.

The pathogenesis of the experimental disease induced in swine by \textit{S. equisimilis} was characterized by Roberts et al. (374, 375, 376). However, the pathogenesis of \textit{S. equisimilis} infections in the field are not completely understood. One assumption is that the organism may invade the baby pigs body by way of the umbilicus, foot and skin wounds or palatine tonsils (200). From the initial entry site, the organism is distributed hematogenously to the areas where it is deposited and promotes suppurative arthritis. This mode of distribution suggests that humoral antibody with bactericidal activity could control economic losses caused by \textit{S. equisimilis}.

The proteinaceous, type-specific antigen of \textit{S. equisimilis} appears similar in many respects to the
M-protein of Group A streptococci (246, 322, 378). Because of the physiological similarity of the proteinaceous type-specific antigen, it may be possible to utilize this organism as a model for studying M-protein-like material in an animal model with the ultimate goal of developing a safe and efficacious vaccine for human use.

Killed whole cell bacterins have been recommended for use in the control of beta hemolytic streptococcus infections in swine (195, 200). These reports suggested curative as well as preventive uses for the bacterins. Bacterins prepared from other Group C streptococci have been used to control mastitis in cattle (61, 107, 455) and strangles in horses (22, 134, 512). Vaccinated animals remained relatively asymptomatic following field exposure or experimental challenge (24, 25, 511).

The purpose of this study was to evaluate immunogenicity of S. equisimilis. The primary objective was development of a vaccine capable of eliciting a protective response in swine. Secondary objectives were development of a serological tool to measure the antibody response and further characterizations of the antigens of S. equisimilis.
REVIEW OF THE LITERATURE

The word streptococcus was used in the form streptococclos in 1874 to describe a certain morphological phase of Coccobacteriæ septica, the universal genus into which Billroth placed all microbes (36). Rosenbach (1884) is credited with giving the first clear description of streptococcus as a genus when he described chains of coccoïd organisms in suppurative lesions in man. He named the organism Streptococcus pyogenes (380).

Subsequent to 1884, streptococci were isolated from a wide variety of lesions in man and animals. Heterogeneity of morphologic and biochemical activity was observed among these isolates. Attempts were made to identify and classify the organisms on the basis of cell size and chain length (285), hemolytic action on blood agar (49, 405), fermentative activity in carbohydrates (8, 332, 473) and a combination of fermentative and hemolytic activity (30, 213). First attempts at serologic classification of streptococci were based on use of agglutination and precipitation (183, 264, 273).

The organisms are now classified into major groups on the basis of specific polysaccharide haptens. Further subdivisions are often made on the basis of other antigens that are polysaccharide, protein or a combination of both.
Certain physiological characteristics are also used for subdivision of the major groups.

The original demonstration of serologically active, polysaccharide haptens in streptococci is attributed to Hitchcock (211) and Lancefield (264, 265, 266). Lancefield is credited with most of the work because she identified the group "C" substance, a specific polysaccharide for each group. The serological grouping system bears her name. On the basis of specificity of the "C" substance, Lancefield originally classified 106 strains of hemolytic streptococci into groups A, B, C, D and E (273). Over the years, serological groups F, G, H, K, L, M, N, O, P, Q, R, S and T have been characterized (96). Two additional groups, U and V, have been proposed (220, 472). The "C" substance is usually an integral part of the cell wall firmly bound to the mucopeptide layer by covalent bonds (384). The group specific determinant of groups D, N and possibly Q differ from those of other Lancefield groups in that they are glycerol-teichoic acids.

Individual Group and Type-Specific Antigens

**Group A**

Over twenty different antigenic materials have been detected in concentrated, supernatant fluids of cultures of Group A streptococci by immunoelectrophoretic analysis
Some extracellular products are enzymes that facilitate invasion of organisms, such as hyaluronidase and streptokinase (140, 476). Other extracellular products include exotoxins such as Streptolysin O (189), Streptolysin S (173) and diphosphopyridine nucleotidase (60). The role of all extracellular products and compounds will be discussed later.

The Group A carbohydrate is an integral part of the cell wall (211, 305, 309), has a molecular weight of approximately 10,000 daltons (149) and is composed of 38 moles of L-rhamnose and 17 moles of N-acetyl-glucosamine (253, 451). Terminal beta-N-acetyl-glucosamine residues are the immunodominant determinants of specificity (303). The polysaccharide is linked to the mucoprotein by bridges of phosphate containing glycerol-rhamnoside (169, 256). In certain strains, known as Group A-variant streptococci, terminal amino sugar residues are absent. Such strains have been used to establish that antigenic specificity is dependent upon the L-rhamnose moiety (202, 203, 310, 311, 365).

The cell wall peptidoglycan of Group A consists of repeating units of N-acetyl-glucosamine (NAG) and N-acetyl-muramic acid (NAM) which are cross-linked by tetrapeptides (L-alanyl-D-glutanyl-L-lysyl-D-alanine) through the carboxyl group of muramic acid (115, 257). Approximately 10 per cent
of the tetrapeptides in the NAG-NAM peptide backbone are not cross-linked and have an additional D-alanine which converts them to antigenic, free-hanging pentapeptides (17). With this type of linkage, the free and non-cross-linked pentapeptide acts as the primary antigen while the cross-linked tetrapeptide acts as a weak antigen. These antigens have been used occasionally in classification of certain Group A strains (84, 299, 378).

The outer component of the Group A streptococcal cell wall is a protein layer that contains three major antigens known as M, T and R proteins (312). At least two minor antigens, "M-associated protein" (MAP) (498) and opacity factor (OF) (180) are also located in the outer layer. The typing antigens T and R have been used in epidemiological studies to trace the spread of an outbreak while MAP and OF have been used primarily as aids in typing (272, 301, 482, 497). At the present time, no biological significance has been attached to T, R, MAP and OF antigens (384).

The earliest method for serologically typing streptococci within Lancefield's groups was on the basis of M-antigen, a substance used in Griffith's original method of serotyping Group A streptococci (183). He established 27 types by means of agglutination. Three of the original 27 types were found later to belong in Group C and one was reclassified in Group G (422). At present, 60 types
have been identified within Group A on the basis of M-antigens (174). Some strains lack or have lost the antigen, some have two different M-antigens (384) and others carry cross-protective determinants (149, 298, 499). These type-specific substances are sensitive to activity of proteolytic enzymes (176). In cultures of streptococcal protoplasts or L-forms, M-protein accumulates in the medium, indicating that, although the end product in the intact cell is deposited in serologically active form on the cell wall and bound there, this is not the major site of its production (72, 186). Biosynthesis of M-protein, as studied by use of non-proliferating streptococci, requires peptides, free amino acids and glucose (259).

M-protein antigen was demonstrated in or on the cell wall of organisms grown in medium containing fluorescein-labeled antibody (181, 462). The surface location of type antigens was indicated by immunoelectron microscopy using ferritin labeled antibody (216). Similar substances have been found on the surfaces of streptococci of other Lancefield groups (298, 512).

Although several methods have been utilized for extraction of M-antigen from cells, Lancefield's original hot acid extraction method is the most commonly used (264). Other methods have included washing with buffered saline
(198), alkaline extraction (151, 152), sonic oscillation (35, 66, 324), enzymatic degradation (27, 88, 117) and lysis by phage (233, 254, 255, 296). Crude M-protein obtained by use of any of the methods has been purified by a variety of protein purification methods including ammonium sulfate precipitation (278), zone electrophoresis in starch (357), precipitation at reduced pH (209), density gradient column electrophoresis (410), ion-exchange or filtration chromatography (223) and combinations of one or more of these methods (147, 150, 331).

M-protein has been shown to possess the following general properties: it is antigenic (268), has a molecular weight of 40,000 daltons (268), belongs to the class of alcohol soluble proteins (268), is destroyed by proteolytic enzymes (276), resists boiling for a short time at pH 2 and pH 10 (152, 264), has an isoelectric pH value of about 5.3 (174), is commonly associated with other protein antigens (312), has a multiple molecular structure (150) and as indicated above is bound to the cell wall (72, 186). Antibodies against two different non-type-specific antigens have been detected serologically in highly purified M-preparations. These antigens have been identified as the MAP and OF antigens which were mentioned previously. Their presence or absence depends upon the type of extraction (27, 88).
Group B

The Group B carbohydrate is located in the cell wall (82, 279) and is composed of 50 per cent L-rhamnose, 9 to 11 per cent D-galactose, and 11 to 12 per cent N-acetylglucosamine (89, 508). L-rhamnose was shown by quantitative precipitin inhibition, to be the immunodominant antigenic determinant (451). Strains of Group B have been found that lack the group antigen, although they did contain type-specific antigens (267). Cross reactions have been observed in precipitin tests between Group B, Group C and Group A variant streptococci. All three have L-rhamnose as an antigenic determinant (89).

Cell walls of Group B organisms also contain other polysaccharides that contain type-specific determinants (267). On the basis of precipitin and mouse protection tests (274), the majority of Group B streptococci were originally placed into 4 types, IA, IB, II and IV. A fifth type, IC, has been added recently (500, 501).

Stableforth (451) reported that by use of precipitin and agglutinin absorption, 16 types of Group B streptococci could be identified. Pattison et al. (348, 349) found two protein antigens, X and Y, in the cell wall. He demonstrated that all 16 types could be placed into three serological types, I, II and III, on the basis of 4 polysaccharide and 2 protein antigens.
The Y antigen was determined to be almost identical to the R antigen that is found in Groups A, C, G and L and it was redesignated R antigen (272). This antigen has been shown to be an active component in cross reactions among all Lancefield groups possessing it (298).

Type-specific antigen IA is a polysaccharide composed primarily of 69 per cent D-galactose and 25 per cent N-acetyl-glucosamine (500). The immunodeterminant group has been shown to be more complex than a monosaccharide, probably N-acetyl-galactosamine in combination with another sugar moiety.

Type IC is also a polysaccharide that contains most of the components found in Type IA polysaccharide in addition to a protein with two determinants that differ in susceptibility to trypsin and pepsin. This protein has been compared to the N-antigen of Group A (500).

Type II strains have been found to produce 2 type-specific antigens extractable with cold trichloroacetic acid, one of which was extractable with hot acid (279). The terminal immunodeterminant group of one antigen was shown to be beta linked D-galactose (157) and sialic acid has been suggested as the other antigenic determinant (267).

Type III antigen was characterized by hydrolysis and chromatography and by nitrogen, phosphorus, amino acid and carbohydrate content. It was shown to be a polysaccharide
composed of 38.9 per cent galactose, 22.8 per cent N-acetylglucosamine, 17.8 per cent glucose, 3.1 per cent uronic acid and less than 0.1 per cent amino acids. Glucuronic acid appears to be one of the immunodominant components of this antigen (387).

**Group C**

Organisms included in Group C have been divided into five main species on the basis of biochemical characteristics: *S. equi*, *S. zooepidemicus*, *S. dysgalactiae*, *S. canis* and *S. equisimilis* (322). Some Group C strains possess a hyaluronic acid capsule and several other extracellular substances similar to those found in Group A (238, 253). The antigenic structure and cellular and colony morphology of *S. equisimilis* are similar to those of *S. pyogenes* (96, 384).

The Group C carbohydrate is located in the cell wall (255) and is composed of 42 per cent L-rhamnose, 5 per cent N-acetylglucosamine and 3.9 per cent N-acetyl-galactosamine. The terminal antigenic determinant was shown by quantitative precipitin inhibition to be N-acetyl-galactosamine (256). Serological reactions between antigens of Group C organisms and Group A antibodies are based on the common L-rhamnose backbone (492).

The composition and location of type-specific antigens in Group C organisms differ among the individual species.
All known type-specific antigens are protein in nature as originally indicated by Lancefield in her work with Griffith's type antigens (258). The protein antigen of S. equisimilis is found at or near the surface of the cell similar to the type-specific protein antigen of Group A (308, 378).

S. equi has 5 serological type-specific protein antigens located in the capsule based on agglutination. However, 8 types have been described based on precipitin-absorption (25, 322). Bazeley (22, 23) has characterized these antigens serologically and biochemically. Only one type of protective antigen has been found. It was located in the capsules of very young cells. No information is available on terminal antigenic determinants.

S. dysgalactiae has at least three type-specific protein antigens that are located in the cell wall (96) and the strain isolated from lambs may be another type (38). No chemical analysis of composition has been reported.

Eight different type-specific antigens were demonstrated in S. zooepidemicus by means of precipitin-absorption tests (322).

The numbers of types within S. equisimilis have varied with the investigator and the typing method used. Simmons and Keogh (434) classified 165 human C (S. equisimilis)
strains into 5 types based on slide agglutination and biochemical activities. The chemical nature of these protein antigens was not determined. Bakshi and Singh (13, 14) divided this species into 4 types on the basis of precipitin-absorption. The antigens were determined to be protein although the determinants were not investigated. Evans (138), using carbohydrate fermentation and agglutination, demonstrated eight serological types among human and animal isolates of S. equisimilis. She stated that these antigens were protein in nature. Khan and Ross (246) classified 62 of 64 swine isolates into 4 serological types utilizing precipitin-absorption. They demonstrated cross-reactions between S. equisimilis type IV and Group L.

**Group D**

The group-specific antigenic determinant has been identified as a glycerol-teichoic acid that may be a constituent of the cytoplasmic membrane or the cytoplasm (495) or it may exist in the periplasmic space. It is not a constituent of the cell wall (225, 379, 423, 439) and it is absent from protoplasts and L-forms (204). Serological specificity appears to be determined by the substituted sugar units kojibiose and kojitriose (53). Barber et al. (16) have disputed the glycerol-teichoic acid concept and claim the determinant antigen is a nucleoprotein which
could be extracted from the whole cells with sodium desoxycholate. The Group D antigen is similar to the Group N antigen and cross reacts with antiserum against it (131, 353, 416).

The type-specific antigens of Group D streptococci are located in the cell wall (128). Sharpe and Shattock examined 353 Group D strains and were able to establish 24 distinct types by agglutination and precipitin tests (418). Types 25 to 39 were added a little later (378). Type I antigen appears to have D-glucose and N-acetyl-glucosamine as the antigenic determinants (39, 40).

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The Group D streptococci are separated into at least five species on the basis of physiological characteristics; S. suis, S. equinus, S. bovis, S. faecium and S. faecalis (96, 373). These species contain varying numbers of type-specific antigens that are polysaccharide in nature and generally contain N-acetyl-glucosamine (124, 128).

S. suis has been demonstrated to contain xylose in the group antigen and only one serotype has been described (129). Type-specificity is associated with a capsular polysaccharide that contains D-glucose, N-acetylglucosamine and N-acetylgalactosamine.

S. bovis contains at least 12 serological types (231, 317, 420) and possibly as many as 32 (96). The type-specific polysaccharide is associated with the capsule in all types,
except 5, in which it is a cell wall component. The capsule contains 22 per cent galacturonic acid, 18 per cent L-rhamnose, D-galactose and D-glucose (231). Type II antigen has been demonstrated to contain 31 per cent N-acetyl-glucosamine. Quantitative precipitin inhibition tests indicated, however, this is not the antigenic determinant. Cross type reactions occur with other streptococci of Group A (232) and Groups E and N (356).

Nineteen different serological types of S. faecium have been described (20). The antigens identified appear to be similar in composition to the S. faecalis polysaccharide, although the exact composition and determinants are not known. The type-specific antigens are located in the cell wall (128).

Eleven serotypes have been established within S. faecalis (126, 496) and the antigens appear to be located in the cell wall (417, 421). Type I antigen contains 22 to 30 per cent L-rhamnose, 14 to 16 per cent D-glucose, 6 per cent N-acetyl-galactosamine and 6 per cent N-acetyl-glucosamine. D-glucose and N-acetyl-glucosamine, alpha linked to L-rhamnose, have been established by quantitative precipitin inhibition to be the antigenic determinants. The other antigens are felt to contain similar polysaccharides. Type III antigen contains at least two antigens; one is a neutral
polysaccharide and the other is a negatively charged polysaccharide (503). The antigenic determinant has not been identified.

Cellular disruption of \textit{S. equinus} is required to demonstrate the group antigen (162, 443). Little information is available on the nature of the type-specific antigen.

\textbf{Group E}

The Group E carbohydrate is located in the cell wall (323, 351, 352, 441) and is composed of 41 per cent L-rhamnose, 20 per cent D-glucose and 2 per cent N-acetyl-glucosamine (437). Terminally located beta-D-glucosyl residues (447) have been shown to be the immunodominant antigenic determinant. This group has been shown to cross react with Groups P and U even though each group has its own specific antigen (104, 426, 442).

The type-specific antigenic components are polysaccharides that are located in the cell wall (323). Five serological types, I to V, as well as several untypable strains have been identified. These antigens contain L-rhamnose and D-glucose in addition to components of the group antigen (447). This suggests that only relatively minor chemical differences exist between the type-specific antigens and the group antigens. Some of the type antigens have also been shown to contain D-galactose; however, the
composition and antigenic determinants are unknown. In recent work, the validity of types I and III was questioned (513).

**Group F**

The Group F carbohydrate is located in the cell wall (82, 343) and is composed of 35 to 40 per cent L-rhamnose, 12 to 15 per cent D-glucose, 2 per cent N-acetyl-glucosamine, N-acetyl-galactosamine and a very small amount of galactose (130, 131). The terminal antigenic determinant appears to be a tetrasaccharide composed of two units of 3-O-B-D-glucopyranosyl-N-acetyl-galactosamine, which is glycosidically linked to an L-rhamnose backbone. This group demonstrates occasional cross-reactions, attributed to a rhamnose moiety, with strains belonging to Groups A, B, C, G, L and T (130). Strains have been identified that contain type-specific antigens but which do not contain the group carbohydrate (199).

Five serological types, I to V, have been demonstrated within this group by use of precipitating and/or agglutinating procedures (41, 319, 320, 343, 502). The type-specific antigens are polysaccharides that are located close to the surface of the cell (506). Some strains have more than one type-specific antigenic determinant.

Type I antigen contains D-glucose, L-rhamnose, D-galactose, mannose and N-acetyl-galactosamine (318, 502).
N-acetyl-galactosamine has been shown by quantitative precipitin inhibition to be the antigenic determinant. This antigen cross reacts with antiserum against Group G strains (506).

Type III antigen has been located in streptococcal Groups C, F and L (505). It is a polysaccharide which contains D-glucose, L-rhamnose, N-acetyl-glucosamine and N-acetyl-galactosamine (502). The disaccharide, N-acetyl-glucosamine and N-acetyl-galactosamine has been demonstrated to be the antigenic determinant (505). This antigen cross reacts with antiserum against strains in Group C (343) and Group L (504).

Type IV antigen has been demonstrated to be a polysaccharide similar in composition to the other types. The antigenic determinant appears to be a beta-glucosidic moiety (506).

Type V antigen is a polysaccharide that has D-glucose as the terminal antigenic determinant. The glucose is adjacent to a galactose or rhamnose moiety (506).

The location of the Group F antigen and the type III antigen was evaluated using ferritin-labeled antiserum and immunoelectron microscopy. Findings demonstrated that type III antigen was on the outermost layer of the streptococcal cell and that it appeared to be capsule like. The
group antigen was located deeper in the streptococcal capsule (216).

**Group G**

The Group G carbohydrate is located in the cell wall (69) and is composed of 39 per cent L-rhamnose, 19 per cent N-acetyl-galactosamine and 22 per cent D-galactose (378). L-rhamnose is the terminal antigenic determinant and as stated previously, it is the same determinant that gives Group B its specificity, although their spatial configuration gives these two groups slightly different specificities (89, 90). The two groups do cross react in the precipitin test. In addition Group G cross reacts with Group A (434).

There are two major types of organisms comprising this group, "minute" and large. The "minute" strains are all antigenically similar to the "minute" type I strains of Group F. Chemical analysis indicated the type-specific antigen is composed of a complex polysaccharide although the terminal active component has not been identified. The large colony type has been separated into three types, I, II and III, by agglutination. The chemical composition of type-specific antigens of the large colony type has not been determined, although, they are thought to be a complex polysaccharide (41, 96).
Group G streptococci often possess a full complement of Group A extracellular products and one strain has been demonstrated to possess an M-protein antigen (298, 300). Because Groups A, C and G share some common antigenic components, fluorescent antibody typing techniques are useless unless absorbed serums are used. The cross reactivity is attributed to the L-rhamnose backbone in the cell walls of all three groups (256).

**Group H**

The Group H carbohydrate is located in the cell wall (440) and is composed of L-rhamnose, D-glucose, D-galactose and fucosamine (377). The terminal antigenic determinant is probably L-rhamnose although, a glycerol-teichoic acid has also been suggested as the determinant (485).

Antigens conferring type-specificity appear to be located in the cell wall (141, 489). The types are designated I, II, III, c and d. Types c and d have filamentous processes that are destroyed by proteolytic enzymes (263). The antigenic determinants have not been characterized. Type I antigen has been shown to be a glycerol-teichoic acid located in the cell membrane. This has been suggested as the group antigen by one investigator (485). Type II antigen contains D-galactose and N-acetyl-galactosamine as the antigenic determinant. Type III antigen has been shown
to cross react with Group E antiserum (378). Some strains have more than one type-specific antigen.

**Group K**

The Group K carbohydrate is located in the cell wall (377, 437) and is composed of L-rhamnose, D-lactose, D-glucose, N-acetyl-glucosamine and N-acetyl-galactosamine. The terminal antigenic determinant is L-rhamnose. This group cross reacts strongly with Group F type III antiserum (249).

The type-specific antigenic determinants are polysaccharide in nature are located in the cell wall. Based on precipitin-absorption tests, serological types I, II and I/II have been described (456). The chemical composition has been shown to be D-galactose, D-glucose, L-rhamnose and O-B-D-galactopyranosyl-(1-6)-D-galactose. The latter compound has been reported to be the antigenic determinant for type II. The third type is apparently a combination of the other types.

Filamentous surface processes that are susceptible to trypsin have been identified in this group (170, 263). Although these antigens have not been fully elucidated, it is thought that they are not related to type-specificity.
Group L

The Group L carbohydrate is located in the cell wall (238) and is composed of N-acetyl-galactosamine, D-galactose, L-rhamnose, N-acetyl-glucosamine, alanine, glutamic acid, lysine and muramic acid (440). The antigenic determinant consists of two components, L-rhamnose and B-N-acetyl-glucosamine. Group L antigen cross reacts with antiserum against Group A (221), Group C and G (281). Khan and Ross (246) reported cross reactions between S. equisimilis serotype IV antiserum and acid-extracted Group L antigen. The group antigen is partially acid labile.

Two type-specific antigens have been suggested. One antigen is trypsin sensitive and the other is trypsin labile. It appears that the trypsin labile antigen is related to the M-protein of Group A streptococci (238).

Group M

The Group M carbohydrate is located in the cell wall (377) and is composed of L-rhamnose, D-glucose, D-galactose, alanine, glutamic acid, lysine, aspartic acid, N-acetyl-glucosamine and muramic acid (377; 440). Three biotypes have been identified by physiological and serological tests (436). Biotype I contains a "group" antigen that is heat stable and insensitive to trypsin. Biotype III contains a "group" antigen that is heat labile and sensitive
to pepsin. Biotype II has a combination of both of these antigens. No type-specific antigens have been described, although Biotype I strains cross-react with antiserum against Group K and this would probably be due to a common component.

**Group N**

The specific antigenic determinant of Group N has been identified as a teichoic acid that is located in the cytoplasmic membrane (127, 442). D-galactose phosphate has been shown by precipitin tests to be the antigenic determinant. The group antigen cross reacts with antiserum against Group D strains because of common antigenic determinants. Cross reactions with Group Q have not been reported, although the latter group also has the same general type of antigenic determinant.

The type-specific antigens, A and B, are located in the cell wall and chemical analysis indicated they consist of a mucopentide backbone with L-rhamnose attached to side chains. Attached to the L-rhamnose are the serologically specific N-acetyl-galactosamine and N-acetyl-glucosamine. Antigen A contains L-rhamnose, muramic acid, N-acetyl-glucosamine, N-acetyl-galactosamine, lysine, alanine, glutamic acid and aspartic acid (96).
Group 0

The Group 0 carbohydrate is located in the cell wall (325, 326) and is composed of D-glucose, D-galactose, N-acetyl-glucosamine and N-acetyl-galactosamine but it does not contain L-rhamnose (377). The antigenic determinant appears to be N-acetyl-b-D-glucosamine. Extraction of the group polysaccharide by Fuller's formamide procedure (160) destroys its specificity (42). Because both Group A and Group L contain a similar antigenic determinants cross reactions occur between them.

Group 0 streptococci have been separated into types I and II (325). Type I antigen, a cell wall protein, may be separated into three fractions, each possessing serological specificity. This protein is considered to be a complex structure of undetermined chemical composition. Type II antigen is a nucleoprotein of undetermined cell location. These antigens are extractable with hot acid and possess properties which may be used to separate them from the streptococcal protein antigens M, T, R, MAP and OF of other Lancefield groups.

Group P

The Group P carbohydrate is located in the cell wall (440) and is composed of alanine, glutamic acid, lysine, N-acetyl-glucosamine, muramic acid, L-rhamnose, D-galactose and D-glucose.
Recent reports have directly related Group P to the newly proposed Group U (104, 424). It was suggested that strains now assigned to Group P simply lacked group antigen and the observed precipitin reactions were due to a type-specific antigen, a situation similar to the α antigens found in Group P. Strains assigned to Group U contain the Group P carbohydrate as well as another type-specific antigen (104).

**Group Q**

The Group Q carbohydrate is located in the cell wall and is composed of L-rhamnose, D-glucose, D-galactose and N-acetyl-galactosamine (185, 442). The antigenic determinant, L-rhamnose, has not been demonstrated in all strains. Most strains also contain an antigen similar to the teichoic acid of Group D and Group N, located between the cell wall and the protoplasmic membrane, which occasionally may be responsible for cross reactions among these groups (337, 338).

Located in the cell wall are two type-specific antigens, Qr and Qw, that were identified by precipitin absorption (337). The antigen designated Qr forms a single band detectable by immunodiffusion which is destroyed by treating the antigen with periodate. The cell wall has been shown to contain several carbohydrates one of which must be the
antigenic determinant. The Qw antigen appears to lack the group carbohydrate but possesses a type-specific antigen of unknown chemical composition.

**Group R**

The Group R carbohydrate is located in the cell wall (377) and is composed of D-glucose, D-galactose, L-rhamnose and N-acetyl-glucosamine. D-galactose in the terminal alpha linkage was shown by precipitin absorption to be the antigenic determinant (437). D-glucose also plays a role in specificity but it is located subterminally and only plays a minor role. Some investigators have suggested that the group antigen is acid labile (355).

Six serologically active forms have been reported (442). Sonic treatment releases antigen forms 4, 5 and 6. The chemical composition and antigenic determinants have not been identified.

**Group S**

The Group S carbohydrate is located in the cell wall (440) and is composed of alanine, glutamic acid, lysine, N-acetyl-glucosamine, D-galactose, L-rhamnose and N-acetyl-galactosamine (377). The antigenic determinant has not been identified but the group cross reacts with Group T.
Group T

The Group T carbohydrate is located in the cell wall and is composed of 23 per cent L-rhamnose, 7 per cent D-glucose, 26 per cent D-galactose, 16 per cent N-acetylglucosamine and 3 per cent N-acetylgalactosamine (437). N-acetylglucosamine and N-acetylgalactosamine have been suggested as forming the antigenic determinant. This group cross reacts with antiserum against Group S.

No information has been reported in relation to type-specific antigens.

Group U

The cell U carbohydrate appears to be located in the cell wall since the antigen is extractable with formamide, although its chemical composition has not been determined (104). The group antigen is not extractable by the conventional hot acid procedure, suggesting that it is acid labile. The Group U antigen cross reacts with antiserum against Group E and Group P (104, 431).

de Moor and Thal (104) have suggested that Group P and Group U streptococci belong to one serological group. The Group P strains, they felt, lacked the group antigen but contained some common type-specific antigenic
determinants. Recent work by Shuman and Nord (424) has confirmed the validity of both Group P and Group U. Their results were obtained by precipitin absorption tests.

**Group V**

The Group V carbohydrate appears to be located in the cell wall since the antigenic component is extractable with hot acid and formamide, although its chemical composition has not been determined (220). The group does not cross react with antigens or antiserum of other Lancefield groups.

No information has been reported in relation to type-specific antigens.

**Non-groupable strains**

Several strains of beta hemolytic streptococci have been isolated from man and animals that either fail to possess a known group antigen or have one that has not been clearly defined. As more and more of these strains are characterized and then grouped, several new Lancefield groups will be added to the present list.

Some of the strains appear to contain antigenic components that cross react with antibodies against type-specific antigens in groups E (323) and F (505). The reasons for this have not been investigated.
Other Antigens of Streptococci

In the development of an understanding of the antibody response to streptococcal infection, initial emphasis was placed on evaluation of antibodies which protected the host from disease. Early reports were often contradictory or inconclusive because little was known about the complex antigenic composition of streptococcal cells. Lancefield (264) and Griffith (183) clarified most of the confusion when they separated streptococci into groups and serotypes.

The serological response to a given streptococcal antigen is quite variable. Within any population, some individuals may not produce any detectable antibodies while others produce a variety of group, type and strain specific antibodies. As previously noted, over 20 different precipitating antigens have been associated with streptococci. In the following discussion, antigens of streptococci other than group and type-specific antigens will be reviewed.

Capsular hyaluronic acid

The hyaluronic acid of the capsule is not antigenic because chemically it is closely related to the hyaluronic acids found in animal tissue (304).
Cell wall

The cell wall of most streptococci contains a variety of components, some of which are proteinaceous in composition and others that are basically carbohydrate in nature. The cell wall appears to be composed of three layers which are either separate layers or intercalated (256). The main components of the cell wall are protein (391), carbohydrate (275) and mucopeptide (385). Covalent bonds have been found between the mucopeptide and carbohydrate layers (384) and their existence between the protein layer and mucopeptide layer has been speculated.

Antigenicity of the protein and carbohydrate antigens has been covered in the first section. The mucopeptide component has been shown to be antigenic in rabbits as evaluated by quantitative precipitin reactions (414). Factors that suggest that this is a specific immunological reaction include; antibody concentration increases following specific immunization of rabbits with mucopeptide (3), reactions are observed only with serum fractions containing IgG immunoglobulins (3), specificity of the reaction was demonstrated by cross-absorption with heterologous antigens and antisem (1) and protein eluted from mucopeptide-antibody complexes was shown to be an IgG globulin by immunodiffusion and immunoelectrophoresis (340). Harakawa and Krause (236) demonstrated that the mucopeptide component has two
antigenic determinants; a peptidic fraction and a hexosamine polymer. Antigenicity of the hexosamine polymer was demonstrated by blocking the precipitin reaction with a hexosamine rich dialyzable fraction of an enzymatic digest of cell walls (237).

Mucoprotein has a pronounced capacity to act as an adjuvant and thus enhance antibody formation when administered with other antigens (214).

Polyglycerophosphate

Glycerol-teichoic acid, which makes up approximately 1 per cent of the dry weight of a cell, has been shown to be located near the cell wall (384). It is the antigenic determinant for Groups D and N. This substance precipitates with some antiserums to Group A streptococci (307). Glycerol-teichoic acid has also been found in other Gram positive bacteria (392).

Protoplast membrane

The protoplast membrane is located beneath the cell wall and encloses the cytoplasm. It is composed of 72 per cent protein, 26 per cent lipid and 2 per cent carbohydrate (156). Immunization of rabbits with Group A cell membranes results in production of specific serum antibody detectable in quantitative precipitin tests. These antibodies have the ability to cause rapid rejection of skin
allografts in guinea pigs suggesting that the antigens may play a role in the induction of altered tissue reactivity in the mammalian host (368). This antigen may also contribute to some cross reactions between Groups C, A and G.

**Intracellular streptococcal components**

The cytoplasm contains a complex of protein, nucleotides, nucleic acids and enzymes. At least eleven distinct components (201) have been distinguished electrophoretically. These intracellular antigenic substances have not been studied as well as the cell wall or extracellular products.

Lancefield (270) demonstrated that the nucleoprotein of streptococci had a high degree of antigenic heterogeneity. Antiserum against nucleoproteins from hemolytic streptococci cross-reacts with nucleoproteins of non-hemolytic streptococci, pneumococci and staphylococci (302).

Beta-glucuronidase is produced by strains of streptococci belonging to Groups A, B, C, G, E and L (457). This enzyme is apparently non-antigenic in its natural state since antibodies against it have not been detected in serum of patients convalescing from streptococcal infections (384).

Lipoproteinase has been shown to be produced in an inverse proportion to the production of M-protein (248, 260). The enzyme resembles Streptolysin S in that it may be eluted
from cells with the aid of serum. Antibodies against this enzyme inhibit its enzymatic activity and they have also been found in human serums.

Sonic disruption releases an intracellular hemolysin that resembles Streptolysin S. The two substances can be distinguished by their differential susceptibility to potassium and sodium ions. The intracellular hemolysin is antigenic and specific antibodies to this enzyme are occasionally found in convalescent humans (411, 412, 413, 419).

Virulence Factors

Virulence, the disease-producing capacity of a microorganism, is attributed to three factors: infectivity, invasiveness and toxigenicity. Infectivity depends on a complex of properties that enable the microorganism to establish an initial seat of infection within the host by evading or overcoming the host's local defense mechanisms. Invasiveness is the ability of the microorganism to leave the initial site of infection and grow in other tissues. Toxigenicity is the ability to produce toxic or poisonous substances that are capable of damaging the host tissue.

Virulence of streptococci has been studied chiefly in relation to Group A strains and the following information refers mainly to this group. Virulence of streptococci
has not been attributed to any single cellular component or product but rather to a combination of physical and biochemical properties. M-protein and the capsular polysaccharide have been the most commonly identified major virulence factors (91, 125, 145, 206, 261, 268, 480). Other cellular components or products that may be associated with virulence include: hyaluronidase, leukotoxic and leukocidal products, ribonuclease and deoxyribonuclease and proteinase. It has been suggested that other factors still not known are essential to the development of virulence of streptococci for different animal species (28, 29).

**M-protein**

M-protein is distributed on the surface of the cell in the form of a capsule-like material (462), not blocked by the hyaluronate envelope of Group A streptococci (145). Furthermore, anti-M-antibody is protective, indicating that the M-antigen is directly involved in streptococcal virulence: indeed, both the hyaluronate capsule and the M-protein are antiphagocytic (145). M-protein prevents intracellular destruction of streptococci unless the cells are coated with type-specific antibody (168). Immunity to Group A streptococcal infections is directly related to the presence of type-specific antibody (26, 153, 154, 294, 458). Virulent M-protein containing strains of *S. pyogenes* have been shown to adhere to human pharyngeal cells *in vitro*, while avirulent M-negative strains only adhered feebly (122b).
Hyaluronic acid

Hyaluronic acid, a mucopolysaccharide, has been suggested as a virulence factor in Group A, Group C and possibly Group G (239). Evidence that hyaluronic acid is associated with virulence has been demonstrated by its enhancing effect on invasiveness and its interference with anti-phagocytosis. Hyaluronic acid was shown to facilitate invasiveness in aerosol infections in mice (91, 222). Mice infected with strains denuded of hyaluronic acid resisted infection while control mice infected with the same strain with an intact hyaluronic acid capsule became infected. This function was felt to be independent of M-protein. The exact mechanisms are unknown, although it has been suggested that hyaluronic acid aids the organism in establishing a foot-hold in the mucosa of the naso-pharynx, possibly because of its chemical similarity to cement substances of the tissue (174).

It has also been shown to participate as an antiphagocytic factor. A correlation between the presence of a large capsule among various streptococcal strains and resistance to engulfment has been demonstrated (125, 145, 206, 282). The use of hyaluronidase under conditions appropriate for its enzymatic action results in a marked increase in susceptibility of treated cells to phagocytosis. Thus, hyaluronic acid appears to be important for establishment of
the organisms in the naso-pharynx, while M-protein determines the course of infection.

Hyaluronic acid of streptococci consists of a copolymer of N-acetyl-D-glucosamine and D-glucuronic acid (243) with a molecular weight of 100,000 to 4,000,000 daltons (282). In cultures, especially young ones, hyaluronic acid is found as a capsule probably complexed with M-protein (491).

**Hyaluronidase**

Hyaluronidase is a substance released during the log phase of growth by most streptococci in Group A, Group C and Group G (140, 358, 388, 389, 390). It is of particular interest because it has been shown to depolymerize hyaluronic acid by splitting glycosidic bonds. This may account at least in part for its instability. However, it is difficult to demonstrate hyaluronidase in cultures of encapsulated strains even after prolonged growth and complete loss of capsules. On the other hand, certain streptococcal strains (Type 4 and 22) produce large amounts of hyaluronidase from the start of growth and do not form visible capsules in vitro (304). Since most patients recovering from streptococcal disease have antibodies to hyaluronidase, it must be elaborated in vivo (139).

Hyaluronidase was originally called the spreading factor because of its striking lytic effect on the ground
substance of connective tissue (87, 116). It has long been thought to play a role in the characteristic tendency of streptococci to spread rapidly through mammalian tissue (166, 208, 239, 488). However, this has not been fully investigated.

**Diphosphopyridine nucleotidase**

Most streptococcal cultures of Group A and several Group C and G, contain a diphosphopyridine nucleotidase that liberates nicotinamide from diphosphopyridine nucleotide (59, 468, 470). Nephritogenic (Type 12) strains are particularly prone to produce this enzyme, but there is no evidence that it plays a role in the pathogenesis of glomerulonephritis (31, 34). Antibodies that inhibit its action are frequently found in serum of patients convalescing from streptococcal disease. The enzyme has also been shown to possess leukotoxic and leukocidal properties (31, 34).

**Mitogen of streptococcal origin**

Mitogen has been shown to cause transformation of lymphocytes to blast cells (60, 84, 467, 468) and it plays a role in the initiation of arthritis in mice when injected concurrently with Streptolysin S (210, 469).

**Streptolysin S**

The role played by Streptolysin S in virulence of streptococci is not completely understood (175), although
it has been demonstrated to possess leukocidal and leukotoxic properties (339). Streptolysin S apparently alters permeability of the cell membrane, probably by interacting with membrane lipids (15, 122a). It has been suggested that Streptolysin S may suppress the cellular defense mechanism of the host at the site of entry into the body (173, 175). In this way Streptolysin S may contribute to the enhancement and persistence of a chronic inflammatory process induced by streptococci and released host cellular enzymes. It has also been suggested that Streptolysin S may do the same in vivo (175). It also causes degeneration of heart muscle and other parenchymatous organs (32, 176, 289, 445).

**Streptolysin O**

The role of Streptolysin O in virulence is unknown and because it is produced by streptococci of Group C and Group G (189, 190), which are usually of low virulence for man, Streptolysin O has been assumed to have little importance as a virulence factor. However, since it possesses leukocidal properties similar to Streptolysin S, it must still be considered as a potential virulence factor (33, 137, 207).
Proteinase

Streptococcal proteinase is capable of destroying another cell factor involved in virulence, the M-protein (123). This enzyme exhibits a relatively broad specificity; it may also affect other extracellular proteins such as the streptolysins and streptokinase. It is released when the pH of the broth medium is between 5.5 and 6.5 (241). Under such conditions, large quantities of proteinase are released into the medium from which it may be isolated in a crystalline form. Like many other proteinases it is activated by sulfhydryl compounds and causes necrotic myocardial lesions in rabbits when injected intravenously (241, 242). The mode of action is not understood, although it is believed that these enzymes may either interact with components of the cell membrane or other factors that affect cell permeability (171, 177, 205).

Streptokinase

In 1933, Tillett and Garner described a substance in culture filtrates of streptococci that promoted lysis of human blood clots (476). It was first termed streptococcal fibrinolysin; it was later shown to catalyze the conversion of plasminogen to plasmin and consequently, it was renamed streptokinase (70, 71). Two different molecular species, A and B, have been isolated from Group A streptococci.
(110). They are immunogenic and induce specific antibody in the course of most diseases caused by Group A streptococci (252, 370). Although their action has been assumed to prevent the formation of effective fibrin barriers at the periphery of streptococcal lesions, thus permitting the organisms to spread with unusual rapidity, there is no conclusive evidence to support this hypothesis. In fact, development of streptococcal lesions is apparently not influenced by antistreptokinase antibody.

**Deoxyribonuclease and Ribonuclease**

Group A streptococci elaborate an enzyme that catalyses the cleavage of the 3' phosphate bond of native DNA, producing 5' terminal fragments of variable lengths, mostly larger than dinucleotides (168, 306, 361). Four immunologically and electrophoretically different types of deoxyribonuclease (A, B, C and D) have been found in streptococcal filtrates (487). These enzymes do not penetrate the plasma membranes of living mammalian cells and they are not cytotoxic (33, 487). They are capable of depolymerizing the highly viscous deoxyribonucleic acid which accumulates in pus resulting from disintegration of polymorphonuclear leukocytes. Enzyme preparations containing both streptokinase and streptococcal deoxyribonuclease were utilized in such diseases as pneumococcal empyema (477).
Deoxyribonuclease production has also been observed in Lancefield Groups B, C, D, E, F, G and L, but it is produced less frequently and usually in small amounts (125).

**Erythrogenic toxin**

Erythrogenic toxin is the product of streptococcal cells that causes the well-known skin rash seen in scarlet fever (108, 109). Strains of Group A streptococci that produce the toxin are lysogenic; however, the amount of toxin produced by different lysogenic strains varies widely (515).

There are at least three immunologically distinct forms of erythrogenic toxin (A, B and C) produced by different streptococcal strains (515). They are thought to be protein. Certain strains of Group C and Group G hemolytic streptococci, as well as staphylococci, produce erythrogenic toxins closely related to those of Group A streptococci (302). The toxins are lethal for rabbits (490). Erythrogenic toxin has been shown to suppress reticulo-endothelial system function (193), antibody formation (144) and phagocytosis (193). These effects could contribute directly to virulence.

**Cell-sensitizing factor of streptococcal origin**

The cell-sensitizing factor has been shown to sensitize a variety of cells to lysis by antiserum and complement
(112, 196, 328, 345) and to induce arthritis in animals (172). Evidence that the factor plays a significant role in tissue injury was reviewed recently (173).

C-polysaccharide

The "C" substance is not considered to play any role in the virulence of streptococci (173). Purified "C" substance, injected intravenously into rats, rabbits and guinea pigs, was eliminated by the kidneys within 30 minutes without any evidence of pathological change (396).

Mucopeptide

Mucopeptide, injected intradermally into rabbits or guinea pigs, caused acute necrotic lesions with massive infiltration of polymorphonuclear leukocytes. It has also been shown to inhibit phagocytosis in vitro (230), to cause necrosis of skin (2), to be pyrogenic (385) and to enhance the resistance of animals to subsequent challenge with streptococci (386).

Mucopeptide and carbohydrate complexes

Complexes of these substances have been shown to be involved in tissue injury (174). However, the specific nature of the action has not been determined.

Streptococcal antigens cross reactive with tissue

There is a large body of evidence for the existence of antigens common to streptococci and mammalian tissues.
Cross reactions have been demonstrated between components of streptococcal cell wall (234) or protoplast membrane (158, 516), with cardiac and skeletal muscle (262), smooth muscle of blood vessels (174), basement membrane of the kidney (280, 290, 291, 292), monosaccharide of heart valves (235), connective tissue (179) and certain transplantation antigens (67, 367, 369). The role of these antigens in tissue damage has been reviewed recently (174). The antigens apparently do not have a specific role as virulence factors, although, they appear to be involved in initiation of certain autoimmune diseases and sequelae such as rheumatic fever and glomerulonephritis (174).

Miscellaneous aspects of streptococcal virulence

Breese et al. (1970) reported that the number of beta hemolytic streptococci found in cultures has great clinical and epidemiological significance. They felt that in the individual patient, clinical disease was usually associated with a large number of streptococci; however, in cervical adenitis, unruptured otis media, acute glomerulonephritis and rheumatic fever they suggested that a few streptococci were significant (46).

Cayeux et al. (62, 63, 64) have presented evidence that there are essentially two genetically different types of bacteria in certain strains of Group A streptococci.
One type, "vm", causes lesions but they are not necessarily fatal. The other type is called virulent or "BM" because they cause a rapid death by septicemia in mice. Virulence is linked to a genetic character that is not carried by all organisms in a given culture but only by a certain proportion of them (346). However, this concept has not been substantiated.

An adhesive, cell-associated polysaccharide is one of the more important determinants of virulence of cariogenic streptococci (170). This polysaccharide is composed of a mixture of dextrans with an alpha 1-6 linkage between the glucose units with alpha 1-3 and 1-4 branches (466). Reduction in the formation of the adhesive dextran results in a decrease of the cariogenic potential of these streptococci.

Of 50 strains of streptococci cultured from blood of patients with bacterial endocarditis, 17 belonged to Group H (125). The virulence of this group was related to the "stickiness" of the bacteria associated with dextran production. The dextran facilitated bacterial adhesion to the heart valve.
Serological Response to Streptococci

Streptococcal antibodies have been detected with complement-fixation (43, 507), active and passive mouse protection (44, 143, 277), bactericidal (269, 297, 460, 461, 479), bentonite flocculation (284), indirect hemagglutination (105, 146, 364, 399, 518), fluorescent antibody (321), latex fixation (136), quantitative precipitation (18, 19, 397), long chaining (120, 121, 187, 459) and immunodiffusion (191) procedures. A variety of antigens, some crude and others highly purified, have been used. The majority of work has been related to Group A strains, although, a little work has also been done with some of the other groups (47).

Group A

During the early period of investigation of streptococcal immunity, procedures were developed for evaluation of bactericidal and/or bacteriostatic antibodies. Rothbard developed the indirect bactericidal test to evaluate the serological response (383). It was clearly demonstrated that acquired resistance to Group A streptococci was dependent upon the presence of circulating antibodies to the specific M-antigen.

Antibodies to M-antigen developed during streptococcal infection in most persons (363, 383, 398). The anti-M-antibodies are type-specific and immunity depends upon
them. Antibiotic treatment substantially decreases their production (93). It has been shown that a significant rise in specific antibody follows streptococcal infections in 92 per cent of individuals not treated with penicillin (106). In contrast to antibodies against extracellular streptococcal products, M-protein antibodies develop rather slowly. Differences in the time of appearance of antibodies to the individual types can be shown to depend on antigenicity of the M-protein of specific types. Anti-M-antibody generally reaches the highest titer two months after the beginning of an infection (433). Lancefield in 1959 demonstrated that anti-M-antibodies were present 10 to 32 years after streptococcal disease in about half of the subjects studied (271).

The lower incidence of streptococcal disease in younger children and a different clinical course of illness suggests a relation between the type of disease and previous exposure. Thus, development of immunity and hypersensitivity may play an important role in streptococcal infections.

**Passive immunity in Group A**

Immunotherapy to various streptococcal infections initially involved the use of antiseraums. Marmorek reported that he had obtained an antistreptococcal serum by the injection of horses with whole cells derived from several species of streptococci (293). With this antiserum, he
successfully treated cases of erysipelas, puerpural fever, phlegmon, streptococcal sore throat and mixed infections. He noted particularly good results in cases of scarlet fever. Others have reported successful use of streptococcal antiserums for treatment of streptococcal diseases (37, 113, 164, 409, 486).

Several Russian publications concerning induction of immunity to scarlet fever appeared in 1906 and 1907. One series of papers demonstrated that scarlet fever streptococci produced a filtrable toxin that stimulated development of an antitoxin (394). In another series, it was demonstrated that a vaccine made from formalized whole cell cultures and broth filtrate protected children from scarlet fever (163, 165).

In the human, antibody to streptococci can be passed from mother to the fetus via the placenta. Quinn and Lowry (362) demonstrated that type-specific M-protein antibodies in cord blood of 7 of 9 infants were the same type and in similar titers to those in the mother's serum. These antibodies disappeared from the serum of the neonates by the fifth post-natal month.

Passive hemagglutination, performed on 107 paired human maternal and cord specimens, demonstrated placental transfer of type-specific streptococcal antibody in 78 percent of these cases. The cord serum also demonstrated
bactericidal activity equal to that of the mother and this correlated with the passive hemagglutination titer. The IgM level was significantly higher in the cord serum from these infants than control cord IgM levels (517).

**Active Immunity**

A number of attempts have been made to induce active immunity in humans against Group A streptococci. In most early attempts, the role of type-specific antigen in protection was not recognized, therefore whole cell vaccines were used. Often these vaccines caused such violent local and systemic reactions in the recipients (366) that the projects were terminated in spite of the fact that type-specific antibody, as detected by bactericidal and passive mouse protection tests, was induced in some individuals (458, 514).

Once M-protein had been accepted as being the most important antigen in relation to virulence and immunity, investigators evaluated procedures for obtaining and characterizing this antigen. Many articles have been published on the antibody response of rabbits to M-protein (148, 197, 233, 247).

In an early attempt to produce a purified M-protein vaccine for human use, Schmidt used a hot acid extract that had been partially purified by acid precipitation, ribonuclease digestion and ammonium sulfate precipitation
(398). This material was used to vaccinate adults and children. The vaccine did not cause any untoward reactions in the recipients. Precipitating and bactericidal antibodies were detected in only 5 of 29 individuals who received the vaccine.

Massell et al. (294, 295) prepared a partially purified, M-protein antigen from a hot acid extract of a type 3 Group A streptococcus. Bactericidal antibody developed in 13 of 14 individuals vaccinated intradermally with the extract. Anamnestic responses were detected in 7 individuals with pre-existing bactericidal antibody. Subsequent to vaccination, 3 instances of rheumatic fever were observed in the vaccinates.

The recall of type-specific antibodies in man by administration of streptococcal cell walls containing M-protein has been demonstrated by bactericidal and long chaining tests (360).

Fox and his associates have obtained promising results using highly purified M-protein from specific types of streptococci. These purified M-proteins were antigenic, protective and non-toxic in individuals who were not previously sensitized. Purified M-protein from as many as 4 different types of Group A streptococci were used in combination as a polyvalent vaccine (148, 344).
During the last 10 years, over 150 adults and 90 children have been vaccinated with vaccines prepared with purified M-protein from several of the more common serotypes (1, 3, 4, 6 and 12) (153, 154, 155, 344). The immunization scheme has consisted of three doses of 50 ug per injection of M-protein precipitated with alum. With this type of vaccine, it was possible to induce a streptococcal antibody response in about 75 per cent of the young children as determined by the indirect bactericidal test and the micro complement-fixation test (154). By selectively screening prospective vaccinees, it was possible to avoid local and systemic reactions to the vaccine. The micro complement-fixation procedure, using highly purified M-antigen, has been suggested as an index of protective antibody in Group A immunity (507).

In a protective study using human volunteers, Fox et al. (154) demonstrated that an alum-precipitated, M-antigen vaccine afforded protection against an upper respiratory type 1 streptococcal infection. Only 1 of 19 vaccinates demonstrated signs of clinical illness, whereas 9 of 19 placebo vaccinates were ill following infective challenge. No correlation was observed between challenge results and serological titers.
Group B

Unlike Group A streptococci, mastitis streptococci do not have a potent array of immunogenic virulence factors (395). The various antigens used in serological typing of Group B streptococci do not play any significant role in virulence. A cell associated component, 'S polysaccharide', that plays an important role in virulence has been described, however, protective antibodies apparently are not formed against it (96, 286, 395).

Although S. agalactiae has relatively low virulence for laboratory animals, Norcross et al. (336) developed a mouse protection test for use in assaying streptococcal antibodies in bovine serum and milk. The test was reported to directly measure immunity provided low levels of streptococci were used to challenge the animals. Antibodies against cellular antigens in goat and bovine serum can also be tested by mouse protection (334, 336, 347), precipitation and complement-fixation (55, 333). Unfortunately, none of these procedures have been shown to measure antibody that is related to protective immunity.

Experimental S. agalactiae vaccines have been produced for over two decades and they have produced variable results when used as cures or prophylactics in bovine mastitis (450). Carpenter (61) found that those cows which received several subcutaneous inoculations of S. agalactiae
experienced a shorter period of clinical disease and a less severe inflammatory reaction than unvaccinated cows. In another study (45), vaccinated cows became free of infection and had lower neutrophil counts than controls following challenge with the organism. In a test under field conditions, there was a minimum level of statistical significance in favor of vaccinated cows.

Norcross et al. (336) have presented information on use of an autogenous bacterin in cattle. This vaccine consisted of formalin inactivated whole cells. Five 1 ml doses were inoculated in the area of the right supramammary lymph node at weekly intervals. Vaccinated cattle resisted challenge with the homologous strain. Results obtained in a passive mouse protection test with antiserum from a cow immunized with a whole cell bacterin containing S. agalactiae type-antigen indicated that the antibodies were type-specific (335).

Johnson and Norcross (224) obtained encouraging results in a dairy herd with S. agalactiae mastitis using a formalized whole-cell vaccine combined with antibiotic therapy of infected quarters.

Kozlowski (250) reported some success using an autogenous, formalin-killed, whole cell vaccine. The vaccine was injected under the skin of the udder of 10 cows with
mastitis. Five of the animals responded with an increase in whey agglutinins. Two animals developed purulent mastitis but recovered.

**Group C**

The serological and protective immune response to Lancefield Group C can be divided into three sections: equine, bovine and swine. The immune response has been evaluated more extensively in the horse and bovine than in the pig.

Group C streptococci are the most frequent primary or secondary causes of pyogenic infections of the horse (24, 25, 51, 111, 118). Bazeley and Battle, working in Australia, made probably the most significant single contribution to understanding of strangles when they identified *S. equi* as the causative agent (25). Through cross immunity studies in mice and clinical evidence from the field, Bazeley demonstrated that only one protective antigen was associated with the capsule of *S. equi* (23). He also demonstrated that circulating antibody played a role in the defense mechanism against equine strangles. Immunity to strangles depends on the animal's coming in contact with living or dead capsule material (22). Protection has been attributed to an antibody that alters the capsule.

In recent work, Woolcock (512), demonstrated an M-like material that is associated with the capsule of *S. equi*. 
Purified preparations of the material elicited protective antibody in vaccinated animals as evaluated by live challenge. Protection was directly related to the presence of anti-M-like antibodies.

*S. dysgalactiae* is responsible for 8 to 26 per cent of the clinical cases of bacterial mastitis in the bovine (119). Stark and Norcross developed a passive hemagglutination technique for detection of *S. dysgalactiae* antibody in serum of rabbits and cattle (453, 454). It was demonstrated that most adult cattle had *S. dysgalactiae* hemagglutination titers of 1:80 or more, presumably acquired from inapparent infections. Formalin-killed, whole cell bacterins induced increased antibody levels in cattle with pre-existing antibodies (511). Results obtained by use of a passive mouse protection test indicated that serum from the vaccinated cows contained protective antibodies. Evidence has been obtained that clinical signs and actual infections have been reduced by use of the *S. dysgalactiae* vaccine (455).

Immunization procedures, based on use of autogenous bacterins, for the control of Group C infections in swine have been reported (195, 200). Hare et al. (195) prepared an autogenous, formalized-whole cell culture vaccine to control beta hemolytic streptococcal infection in swine. The vaccine was administered as a curative at 48 hour
intervals in six doses each of 2 ml for suckling pigs and six doses each of 5 ml for sows. As a preventive, the vaccine was given at intervals of seven days in three doses of 1, 2 and 3 ml to suckling pigs and 5, 10 and 15 ml to adult swine. According to the authors, this vaccine significantly reduced the incidence of disease attributed to beta hemolytic streptococci.

Helms (200) reported a reduction in the incidence of navel infection when sows were vaccinated before farrowing with either an autogenous bacterin or a commercially available mixed bacterin.

The species of beta hemolytic streptococci used by Here et al. and Helms were not reported; however, because it is ubiquitous in swine, it is likely that they were working with S. equisimilis.

**Group D**

Vaccination and challenge studies utilizing Group D streptococci in swine were not found in the literature. Elliot et al. and Agarwal et al. successfully protected pigs against Group D by administration of immune serum from sows or other previously exposed pigs (4, 5, 6, 129, 130).

Some vaccination and challenge studies have been conducted in rats and hamsters with dental caries induced
with S. mutans, a Group D species. Variable protection occurred following virulent challenge (466).

Agarwal et al. (4, 5, 6) reported that swine over 8 weeks of age generally had protective levels of antibody directed specifically against the capsular polysaccharide of Group D streptococci. Results obtained in vivo suggested that swine under 4 weeks of age were very susceptible to broth cultures sprayed into the naso-pharynx. With increasing age the blood of swine became inhibitory to the growth of Group D streptococci. This effect was correlated with a decrease in susceptibility to infection. Swine of any age reared in a pathogen free environment did not develop resistance. Thus, immunity against Group D streptococci in older conventional animals apparently arises from inapparent infections.

**Group E**

Swine exposed naturally or experimentally to Group E streptococci (the cause of streptococcal lymphadenitis in swine) have been reported to develop humoral and cellular immunity directed against the organism. Circulating antibodies against Group E streptococci in swine serum have been evaluated by agglutination (132, 429, 438), precipitation (427, 494), hemagglutination (493), immunodiffusion (430, 431, 432) and bactericidal (56, 57, 58, 94) tests. The leukocyte migration inhibition test (58) and cutaneous
reactions to exoantigens have been used to evaluate hypersensitivity (427, 428). The antigen responsible for induction of protective immunity in swine against streptococcal lymphadenitis has not been defined. The reported success of an orally administered, live-attenuated Group E vaccine suggests that protective immunity to streptococcal lymphadenitis may be cell-mediated or secretory in nature. However, proof of this point has not been presented.

Serotype IV has been the most frequently reported Group E type isolated from field cases of streptococcal lymphadenitis (381, 513), although types II, V and several untypable strains have also been isolated. Because of the relatively high incidence of serotype IV, attempts were initiated to develop a bacterin to control the disease. However, autogenous and/or serotype IV bacterins have proven unsuccessful (162, 438). Collier originally evaluated a formalin-killed whole-cell bacterin and reported that his results were inconclusive (76). Conner et al. (83) evaluated an autogenous bacterin prepared from hemolytic streptococci and staphylococci and a commercial bacterin containing organisms isolated from cattle in bred sows and gilts. It was suggested that use of the bacterin reduced the number of pigs that died before weaning and the occurrence of abscesses in pigs. Shuman and Wood (429) also evaluated formalin killed bacterins for prevention of
abscesses. Utilizing either whole cells and adjuvant or cells resuspended in a concentrated culture filtrate (428) combined with adjuvant, they failed to demonstrate protection in 38 of 41 vaccinated animals, although a rise in agglutinating antibodies was demonstrated in the vaccinates.

Collier (79) reported that swine actively infected with Group E streptococci were immune to subsequent challenge. In a study by Engelbrecht and Dolan (135), swine experimentally infected with Group E streptococci resisted subsequent challenge with the same organism. They also reported development and efficacy testing of a modified, live culture, Group E vaccine. When administered orally, the vaccine produced no evidence of increased virulence and protected 88 per cent of swine vaccinated at 10 to 15 weeks of age (134). In field trials, vaccinates and controls remained together at all times and the controls remained susceptible to challenge indicating that the avirulent strain was not transmitted from vaccinates to controls. The strain did not revert to virulence in 5 serial passages in swine. Efficacy of this vaccine has been reported by other investigators (75, 217).

Thus streptococcal lymphadenitis caused by Group E streptococci of serotype IV may be controlled by oral administration of an avirulent live culture (75, 133, 134, 217).
Clinicopathological Entities

The genus *Streptococcus* contains organisms that are among the most important bacterial pathogens of man and animals. They have the capacity to invade any tissue or organ and the range of resulting clinical syndromes is a reflection of the different sites of invasion and consequent host-parasite relationships. Representative strains from every known or proposed Lancefield serological group and many not yet grouped have been isolated from swine (426). Some of these organisms have been associated with diseased tissue while others are from apparently normal individuals.

Streptococci have been isolated from a wide variety of tissues in swine and from swine of all ages. The most frequent manifestations of streptococcal infection are arthritis (78, 142, 184, 316, 330, 415, 464, 478), endocarditis (21, 95, 167, 212, 215, 228, 240, 244, 481), meningoencephalitis (86, 219, 314, 315, 327, 371, 408) and cervical abscesses (9, 10, 73, 74, 77, 97, 98, 99, 226, 329, 444, 452, 513). Miscellaneous diseases that have also been occasionally associated with streptococcal infection include; abortion (84, 85, 188, 251, 393), infertility (114, 200), acute death (7, 52, 195, 484), enteritis (100, 251, 371), metritis (228, 475), mastitis (12, 65, 68,
Arthritis

The most frequent causes of suppurative arthritis in swine from birth to 6 weeks of age are members of the genus *Streptococcus*, principally *S. equisimilis* (381). The literature on streptococcal arthritis in swine consists primarily of case reports and surveys. In an early report, Field et al. (142) indicated that streptococcal arthritis was common in young swine and that infection might be confined to a single litter or in successive litters over a period of many months. The role of the environmental sources and other non-swine sources in the spread of the infection is not understood, although the age of the pigs involved strongly suggests that the sow is the most probable source of infection (142, 195, 200).

Collier (78) studied 13 isolates of beta hemolytic streptococci isolated from arthritic lesions of swine and found 10 of them to be *S. equisimilis*. Switzer et al. in unpublished work cited by Switzer in 1964 (464), isolated beta hemolytic streptococci from 16 of 107 arthritic joints of pigs of market weight and from 9 of 48 arthritic joints of pigs market weight and over. Twenty of the twenty-five beta hemolytic streptococci were later reported to be *S. equisimilis* (374). Tittiger and Alexander (478) isolated 50 beta hemolytic streptococci...
from 478 joints of arthritic swine cultured at slaughter; 28 of the isolates were Group C, 21 Group L and 1 Group E. Nielsen et al. (330) in Denmark studied polyarthritis in 126 litters and found 117 of 722 necropsied swine had lesions of arthritis. Hemolytic streptococci accounted for 67 per cent of the bacteria isolated. The majority of the isolates belonged to Lancefield groups C and L. Roberts et al. (374) isolated *S. equisimilis* from swine with naturally occurring arthritis and reproduced the disease in young pigs by intravenous inoculations of fresh broth cultures of the isolate. They were unable to produce the condition by any other route of inoculation (375, 376). Khan (245) also reproduced the condition by intravenous inoculation. Shuman and Wood (430) produced chronic suppurative arthritis in 5 of 6 hysterectomy-derived pigs by subcutaneous and intravenous injection of *S. zooepidemicus*. In this same study, a strain of *S. equisimilis* of equine origin failed to produce arthritis in 6 pigs following intravenous injection. However, *S. equisimilis* was isolated from tonsils of over 50 per cent of the swine. Shuman and Wood speculated that the inoculated pigs may have been exposed previously and thus, they could have developed protective immunity.

Arthritis has also been experimentally produced in swine with strains from groups A (432, 509), D (129, 130),
E (406, 407), L (227) as well as several non-grouped strains (425).

**Endocarditis**

A wide variety of streptococcal species have been associated with endocarditis in swine; however, *E. rhusiopathiae* (240) is the principal cause of valvular lesions in swine. Kernkamp (244) reported that in 8 cases of streptococcal endocarditis 5 of the organisms isolated were beta hemolotic streptococci. One of the organisms was closely related to *S. fecalis* and the other was related to the enterococci. Hofferber (212) reported that 5 baby pigs in one litter died 8 days to 3 weeks after birth with streptococcal endocarditis. He concluded that an intrauterine infection had occurred.

In 1966, Batis et al. (21) examined 72 streptococcal strains from swine endocarditis and identified 17 as *S. zooepidemicus*, 18 Group D, 5 *S. uberis*; they were unable to classify 32 others. Tomita et al. (481), in Japan, found endocarditis in 212 of 97,952 pigs inspected at slaughter. Streptococci were isolated from 23 of the 212 cases.

Death of 8 sows in a commercial herd was attributed to streptococcal endocarditis by Jones (227, 228, 229). Seven strains of streptococci, 4 Group L, 2 Group C and 1 unidentified, were isolated.
Kast (240) reported an evaluation of 252 cases of bacterial endocarditis in swine at slaughter. He found that about 4/5 of the causative organisms were *E. rhusio-pathiae*, while streptococci and staphylococci accounted for approximately 10 per cent and all other bacteria only 1 per cent. He isolated strains of streptococci from serological groups A, B, C, D, E, L and N.

Endocarditis has been reproduced in young pigs by intravenous injection of streptococci from Group D (129, 130) and Group L (227, 228, 229). Hont and Banks (215) used increasing doses of beta hemolytic streptococci from Group D to produce endocarditis. Cotchin and Hayward (86) injected two pigs intravenously with a single dose of an alpha-hemolytic streptococcus isolated from a natural case of porcine endocarditis. Two other pigs were given repeated intravenous injections with the same strain. Macroscopic lesions of endocarditis were found in one animal and histological lesions in the other animal of the pair receiving the single dose. Jones (228) reproduced endocarditis in 6 of 10 swine inoculated with a single intravenous injection of a Group L strain originally isolated from a natural case. The majority of inoculated swine also developed arthritis.

**Meningoencephalitis**

Various species of streptococci have been isolated from meningoencephalitis of swine. McNutt and Packer (315)
reported several outbreaks of meningitis in baby pigs from which gamma and beta hemolytic streptococci were isolated. The isolates were very heterogeneous biochemically and no single species appeared to be involved in the disease condition. The authors suggested that the disease developed in pigs with lowered resistance because none of the strains were recognized at that time as being pathogenic streptococci. Field et al. (142) isolated a beta hemolytic streptococcus from a similar condition that later was reported by de Moor (101, 102, 103) to cross react with antiserum prepared with streptococci of his proposed Group S. McErlean (314) reported an outbreak of meningocencephalitis in a litter of pigs 4 weeks old. He isolated a motile, pleomorphic non-hemolytic streptococcus from the brain. The strain did not correspond to any of Lancefield groups A through G. The condition was reproduced in young pigs following injection of culture of the organism. Schulte (408) described an outbreak of diplococcal encephalitis in 15 pigs 3 to 4 weeks of age. Alpha-hemolytic streptococci have also been isolated from baby pigs with the disease (327). This condition is still reported to occur in European swine (54, 330, 359, 435, 465) but no reports of the condition in American swine have been reported since that of Ray in 1945 (371).
Abscesses

Abscesses of the head and neck of market pigs are caused primarily by Group E streptococci (9, 77, 99, 226, 513), although several other organisms have also been associated with the condition (426).

Newson (329) described cervical abscesses in swine and stated that a beta hemolytic streptococcus could be isolated from the exudate. Stafseth and Clinton (452) reported Lancefield typing of the streptococci isolated from swine abscesses. These reports demonstrated that the original suggestion of Lancefield that Group E organisms were associated with dairy products needed to be modified. It was suggested that cervical abscesses were spread by means of mechanical transmission. However, Snoeyenbos et al. (444) were unable to transmit the organism by vaccination and they suggested that mechanical transmission was not a major factor in the disease.

Collier found the disease to be enzootic on some farms where it affected pigs of all ages. However, the abscesses were not usually detected until the swine were slaughtered (77).

Deibil et al. (97, 98, 99) evaluated 37 isolates of Group E streptococci and found them to be physiologically homogenous. They found these isolates to be non-pathogenic
for common laboratory animals. However, in subsequent work, Cutlip and Shuman (92) have shown the rabbit to be susceptible to a group E Type IV strain.

Cervical abscesses can be regularly produced in 9 to 14 week old pigs by giving Group E organisms in the feed (77, 400, 401). Intranasal and intrapharyngeal inoculation of the above organism produced similar lesions but inoculation by various other routes failed to induce abscess formation (288).

Reports by various investigators (11, 288, 402) have indicated that 9 to 14 week old swine are more susceptible to Group E streptococci than younger or older swine.

Collier (76) suggested that the sow was the source of Group E streptococcus infection for young pigs. In subsequent work, Collier and Noel (81) and Schmitz and Olson (403, 404) demonstrated transmission of Group E streptococci from carrier swine to susceptible swine. Collier and Noel (80) were able to isolate the organism from tonsil swabs of the carriers, although Schmitz and Olson were unable to isolate the organism from tonsil swabs of known carriers. Schmitz and Olson (400) demonstrated that Group E streptococci could survive in soil for up to 31 days at 37 C.
MATERIALS AND METHODS

Streptococcal Cultures

Source of strains

Five strains of *S. equisimilis* of swine origin were used. Four of the strains, 78-A, R-8, 78-B and F-49 were originally isolated from arthritic joints of pigs or reproductive tracts of sows (245). The fifth strain was obtained by passing strain 78-A through Caesarean-derived colostrum-deprived (CDCD) 6 week old swine 3 times. Two animals were inoculated intravenously with 1 ml of a 6 hour Todd-Hewitt broth$^1$ (THB) culture and observed for symptoms of arthritis. When lameness and joint swelling were observed, the animals were necropsied, fluid from arthritic joints was collected, streaked on blood agar and incubated for 18 hours at 37 C. Typical *S. equisimilis* colonies were picked to THB, incubated for 6 hours at 37 C and reinoculated into swine. This procedure was repeated 3 times and the isolate from the third pig passage was designated 78-A3X. The identity of each inoculated strain was determined by biochemical and serological comparisons with the parent strain.

$^1$Difco Laboratories, Detroit, Michigan.
A *S. zooepidemicus* strain of unknown host origin was obtained from R. A. Packer. S. equi 6580 and Group L 9932 strains were obtained from the American Type Culture Collection.

Comparisons of biochemical activity of strains of *S. equisimilis* were done in phenol red broth base plus 1.0 per cent of the appropriate sugar. Media were inoculated with one drop per tube of 24 hour THB culture, incubated aerobically at 37 C and observed for acid production at 24 and 48 hours.

Hemolysis was determined at 24 and 48 hours incubation at 37 C on 5 per cent horse blood agar. Lancefield grouping serums, obtained from commercial supply houses, were used with capillary pipettes (0.7 to 1.0 mm) to identify group antigen. Serotype specificity was determined by using hyperimmune rabbit antiserum against four known swine *S. equisimilis* serotypes (246).

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1 R. A. Packer, Iowa State University, Ames, Iowa.
2 American Type Culture Collection, Rockville, Maryland.
3 Difco Laboratories, Detroit, Michigan.
4 Burrough Wellcome Co., Tuckahoe, New York.
Stock cultures

Stock cultures of all streptococcal strains were prepared as soon as the strains were received or isolated. Low passage cultures were inoculated into THB using approximately 1 per cent inoculum, the cultures were incubated 16 to 18 hours at 37 C and examined microscopically for purity. Cultures were combined with equal volumes of 1 per cent skim milk, dispensed in 1 ml ampoules and lyophilized. The stock cultures were appropriately labeled, examined for purity and stored at 4 C.

Working cultures

Working cultures were produced by inoculating 1 vial of stock culture into 25 ml of THB which was then incubated for 18 hours at 37 C. Following microscopic examination for purity, the cultures were lyophilized according to the above procedure, labeled, again examined for purity and stored at 4 C. The biochemical and serological identity of the working cultures were determined as described previously. Working cultures were used for production of streptococcal vaccines, sonic-derived antigens and acid or alkaline extracts. All cultures used were no more than 5 passages from the original source.

1The VirTis Corp., Gardiner, New York.
Virulence

Virulence of stock and working cultures was compared by determination of the LD50 of each culture for mice. Cultures were grown 6 hours in THB and 10 fold dilutions were prepared in 1 per cent neopeptone. 0.25 ml amounts of each dilution were given intraperitoneally to each of 10 mice. Virulence of the organism was enhanced by simultaneous intraperitoneal inoculation of 0.15 ml of 5 per cent gastric mucin type W.¹ The death pattern was monitored for 1 week.

Virulence of S. equisimilis for swine was determined by intravenous inoculation of 3 to 6 week old CDCD pigs with a 6 hour broth culture. In these experiments at least 2 animals were inoculated for each 10 fold dilution and clinical signs were observed for 5 days. Animals were necropsied after 5 days for evaluation of gross lesions and collection of samples for bacteriological examination.

Antigen Preparations

Hot acid extraction

Hot acid extracts were prepared according to a procedure originally developed by Lancefield (265) and modified by Lancefield and Perlmann (278). Streptococcal cells grown

for 16 to 18 hours at 37°C in THB were harvested by centrifugation, at 2,000 g for 30 minutes at 4°C and washed once with 0.1 M potassium phosphate buffered saline, pH 7.0. The sedimented cells were mixed with an equal volume of N/5 HCl, heated in a boiling water bath with occasional shaking for 10 minutes, cooled and recentrifuged. The supernatant was decanted into a screw capped vial and a small drop of 0.01 per cent phenol red was added to give the solution a yellow cast. The fluid was neutralized by adding N/5 NaOH in M/15 phosphate buffer until it became pink. The pH was estimated with hydrion papers¹ and appropriate acid or base added until the solution was in the pH range of 7.0 to 7.8. This solution was centrifuged at 5,000 g for 30 minutes at 4°C to remove any insoluble material and stored at -20°C until used.

Alkaline extraction

The alkaline extraction procedure originally developed by Fox and Wittner was used with modifications (152). Streptococcal cells grown for 16 to 18 hours in THB were harvested by centrifugation and washed in 0.1 M phosphate buffered saline, pH 7.0 (PBS-7). A 25 per cent v/v suspension of cells in PBS-7 was adjusted to pH 10 with 1.0 M NaOH, heated in a boiling water bath with occasional shaking

¹Micro Essential Laboratory, Brooklyn, New York.
for 10 minutes, cooled and recentrifuged. The supernatant fluid was removed and the cells were extracted a second time according to the procedure described previously. The supernatant fluids from both extractions were pooled, neutralized with 0.1 M HCl and recentrifuged at 10,000 g for 30 minutes at 4 C to remove insoluble material. This material was stored at -20 C until used.

**Pressure disruption**

THB was seeded with 1 per cent inoculum of a 16 to 18 hour streptococcal culture, incubated at 37 C for 18 hours and the cells were harvested by centrifugation at 27,000 g with a refrigerated continuous flow centrifuge.\(^1\) Sedimented cells were resuspended, rinsed once with PBS-7 and resuspended in the same buffer at a concentration of \(10^{10}\) CFU/ml. The cells were held at 4 C until used.

Cells were disrupted with a French pressure cell in a power laboratory press.\(^2\) The cell suspension was passed through the pressure cell 3 times at a minimum of 18,000 pounds per square inch. The disrupted cell walls were sedimented by centrifugation as described previously and stored frozen at -20 C.

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\(^1\)Model RC2-B. Ivan Sorval, Inc., Norwalk, Connecticut.

\(^2\)American Instrument Co., Silver Spring, Maryland.
**Sonication**

Streptococcal cells from a 16 to 18 hour THB culture were washed in 0.1 M potassium phosphate buffered saline, pH 7.55, and resuspended in the same buffer so that 1 ml contained approximately $10^{10}$ CFU. The number was determined by actual plate count.

The suspended organisms were placed in a glass beaker and the beaker was placed in a crushed ice-salt bath maintained at a temperature of 0 to 4 C. Sonication was performed with a 12 mm ultrasonic probe\(^1\) inserted approximately 3 to 5 mm into the suspension. Sonication was usually done at 90 to 95 per cent full power for 20 minutes, although other intervals and exposure times were utilized. After sonication, the suspension was centrifuged at 500 g to remove whole cells and then frozen for vaccine use or processed further for other antigen preparations.

**Partially purified antigen**

Partially purified, type-specific antigen was prepared according to the procedure used by Fox and Wittner (150). Cell walls were obtained by passing a slurry of cells in PBS-7 through a French pressure cell at 18,000 lbs/in. or from sonically disrupted cell suspensions. Following

\(^1\)Biosonic II. Bronwill Scientific Co., Rochester, New York.
disruption, the suspension was washed three times in PBS-7 and the walls were sedimented by centrifugation at 5,000 g for 30 minutes at 4 C. The walls were resuspended in 4 volumes of PBS-7, treated with ribonuclease (10 ug/ml) for 4 hours at 37 C, washed twice with PBS-7 at 5,000 g and resuspended at a concentration of 25 per cent in PBS-7. The cell walls were extracted with acid according to the usual procedures, centrifuged, neutralized with N/5 NaOH in M/15 phosphate buffer and pooled.

Fractionation of the pooled, supernatant fluids was done with 33 to 60 per cent ammonium sulfate. Initially, 17 grams of ammonium sulfate was slowly dissolved in each 100 ml of supernatant fluids and the mixture was allowed to stand at room temperature over night. The resulting precipitate was removed by centrifugation at 17,000 g for 30 minutes at 4 C and discarded. The supernatant fluid was adjusted to pH 8.0 with 1 N NaOH and brought to 60 per cent saturation by adding 13.9 grams of ammonium sulfate per 100 ml. This solution was mechanically stirred for 1.5 hours at 4 C and then centrifuged at 17,000 g for 30 minutes at

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1 Worthington Biochemical Corporation, Freehold, New Jersey.

2 J. T. Baker Chemical Co., Phillipsburg, New Jersey.
4 C. The 33 to 60 per cent pellet was solubilized in distilled water and dialyzed against PBS-7 for 18 hours at 4 C. This material was used for antigen analysis and protection tests in mice and swine.

Purified type specific antigen

The partially purified antigen was thawed, protein content determined by Lowry's method (287), and 50 mg (dry weight) of the 33 to 60 per cent pellet, was layered onto a carboxymethyl cellulose column (CMC), equilibrated with 0.03 M sodium acetate. Elution was performed according to procedures developed by Fox (146). Five ml samples were collected with a fraction collector and the effluent was monitored with an ultraviolet analyzer at 280 nm with a 3 mm optical path. The column flow rate was regulated to 0.5 to 1.0 ml/min by means of a peristaltic pump. The pH and UV absorption of each sample was monitored and recorded. Elution was begun at tube 1 with 0.1 M sodium acetate buffer pH 5.5. When the effluent reached pH 5.5, a three chambered gradient elution was initiated: 75 ml of 0.1 M potassium phosphate buffer at pH 6.0, 75 ml at pH 6.5 and

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1BioRad Laboratories, New York, New York.

2Instrumentation Specialities Company, Lincoln, Nebraska.

3LKB Instruments Inc., Rockville, Maryland.
200 ml at pH 7.0. The elution was continued until the effluent reached pH 7.0. After fractionation, each peak was lyophilized, reconstituted in a small amount of PBS-7 and dialyzed against PBS-7 for 18 hours at 4 C. After dialysis, the samples were evaluated by immunodiffusion and those containing type-specific antigen were saved for further evaluation.

Type-specific antigen was separated from crude sonicate by a modification of the method used with the partially purified acid extracts of cell walls. Prior to chromatography on CMC, the crude sonicate was dialyzed for 2 days at 4 C against 4 changes of phosphate buffered saline prepared with sodium instead of the potassium salts.

Serological Evaluation

**Antiserum production**

Streptococcal cells utilized for hyperimmunization of rabbits and swine were harvested from 16 to 18 hour THB cultures. The cells were centrifuged, washed once in PBS-7 and resuspended in PBS-7 at a concentration of $3 \times 10^9$ CPU per ml. Sodium merthiolate\(^1\) was added to a final concentration of 1:10,000. The suspension was incubated for 12

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\(^1\)Eli Lilly and Co., Indianapolis, Indiana.
to 24 hours at 37 C and then examined for viability and purity before storing at 4 C.

New Zealand rabbits, which had been determined to be negative for *S. equisimilis* antibodies by indirect hemagglutination and capillary precipitation procedures, were given a series of 6 intravenous (IV) injections of merthiolate killed whole cells (1, 2, 2, 4, 4 and 10 billion) at 3 day intervals. Ten days after the last injection, all rabbits were test bled and given another IV injection of 10 billion cells. Ten days later they were exsanguinated by cardiac puncture and the serums were harvested and stored at -20 C. Serum was heated at 56 C for 30 minutes prior to use in CF or IHA procedures.

Hyperimmune swine serums were prepared from colostrum-deprived swine which had been immunized according to the above protocol. Epinephrine (0.5 ml) was administered IV with each injection to prevent shock. For exsanguination, the swine were anesthetized with CO2 and the axillary and jugular blood vessels were severed. Serums were harvested and stored at -20 C. Prior to use in a serological test, serums were heated at 56 C for 30 minutes.

\[1\] Elanco Products Company, Indianapolis, Indiana.
Immunoelectrophoresis

Immunoelectrophoresis was done on microscope slides coated with 1 per cent Ionagar #2 in 0.05 M barbital buffer at pH 8.6. Electrophoresis was performed for 2 hours at 5 volts per cm. Commercial goat anti-whole swine serum and goat anti-swine IgG were used to identify immunoglobulin classes of fractionated sera.³

Gel electrophoresis

Polyacrylamide gel electrophoresis was conducted according to the method of Hazin and Bottem (372) with a slight modification. Approximately 140 µ liters of antigen solution, either hot acid, sonic-derived or purified type-specific, with a protein content of approximately 150 µg of protein was applied to each tube. Electrophoresis was carried out in 7.5 per cent gel with a cationic system at room temperature for 3 hours at a constant current of 3.5 milliamps per gel.⁴

Precipitation

Precipitation tests were carried out according to the method of Swift et al. (463).

¹Consolidated Laboratories, Inc., Chicago Heights, Illinois.

²Miles Laboratories, Kankakee, Illinois.


Immunodiffusion

Immunodiffusion was done in petri plates prepared with 0.5 per cent Ionagar #2 in distilled water that contained 1 per cent sodium azide and 0.85 per cent NaCl. Wells were approximately 6.0 mm apart and 6 mm in diameter and were filled with 0.1 ml of an appropriate antigen or antisemum. The plates were incubated at room temperature for 48 hours in a moist chamber.

Gel filtration

Gel filtration using Sephadex G-200 was done following the methods described by Flodin and Killander (144). Serums from placebo and vaccinated swine and rabbits were passed through a Sephadex G-200 column which was prepared in tris-buffer.

To prepare the tris-buffer, 66 ml of 0.5 M HCl was added to 133 mls of 0.5 M tris stock solution at room temperature and made up to one liter with 0.1 M NaCl. The pH of the buffer was 8.0. A Pharmacia K15/90 column was used. Inactivated serums were applied in 0.5 ml volumes, eluted at a rate of 10 to 12 ml/hour and collected in 5 ml samples.

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3Nutritional Biochemical Corp., Cleveland, Ohio.
Optical density was determined at 280 nm with an ultraviolet analyzer. Three fractions from each of the first 3 peaks were pooled and concentrated by placing the solution in a dialysis bag and passing a stream of air over it. The CF and IHA streptococcal antibody content of each pool was determined. The predominant immunoglobulin class of each pool was determined by immunoelectrophoresis.

**Starch block electrophoresis**

A starch block (36 x 10 x 1.4 cm), containing 400 grams of purified potato starch mixed with 100 mls potassium phosphate buffer, pH 6.2 and 0.023 M ionic strength, was prepared. Antigen to be electrophoresed was mixed with 5 grams of starch and placed in a 1 x 6 x 9 cm well cut out of the center of the block. Electrophoresis was carried out in the cold (4°C) for 40 hours at 5 to 10 milliamps and 100 volts. At the end of the run, the starch block was cut into 10 two cm strips, starting with the negative pole and each was extracted with 10 ml of phosphate buffer. The extracts were concentrated 10 fold by pervaporation. Total protein content was

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1 Instrumentation Specialities Co., Lincoln, Nebraska.
2 Union Carbide Corp., Chicago, Illinois.
determined by Lowry's method (28?) and the samples were evaluated for serological activity by immunodiffusion. Samples were also evaluated for their ability to elicit protection in mice.

**Complement-fixation**

Sheep red blood cells (SRBC) were collected 7 to 14 days prior to use in Alsevers solution (161) and held at 4 C. Immediately prior to use, cell suspensions were mixed with 3 volumes of cold 1X veronal buffer (VBD) and the material was centrifuged at 600 g for 5 minutes at 4 C. Supernatant fluids were removed along with the buffy white cell layer and the sedimented red cells were mixed with cold VBD. The SRBC were washed 2 additional times with cold VBD and resuspended in VBD to make a 2.8 per cent volume to volume suspension (1 volume packed cells to 34.7 volumes VBD). The 2.8 per cent SRBC suspension was diluted 1:10 with VBD to obtain a 0.28 per cent suspension. This material was combined with an equal volume of VBD containing rabbit hemolysin¹ (1:500).

Two fold dilutions of inactivated serum were prepared in VBD using microtiter plates² and diluters.³ 0.025 ml

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¹ Difco Laboratories, Detroit, Michigan.
³ Cooke Engineering Co., San Mateo, California.
of an appropriate antigen dilution was added to each of the serum dilutions as well as two wells devoid of antiserum. Serum controls were also maintained free of antigen. Lyophilized guinea pig complement\(^1\) was reconstituted with normal serum from a 6 to 8 week old pig. In this way the procomplementary effect of swine serum was accounted for in the titration of guinea pig complement. 0.05 ml of complement containing 5 hemolytic units, was added to each microtiter well. The plates were shaken for 2 minutes on a vibrator,\(^2\) left at 4 C overnight, removed from the cooler the following day and 0.025 ml of sensitized SRBC was added to each microtiter well. The plates were shaken for 30 seconds, placed at 37 C for 30 minutes, removed and the reciprocal of the highest dilution demonstrating less than 30 per cent hemolysis was recorded (161).

**Hemagglutination**

Sheep red blood cells (SRBC) were collected 3 to 5 days before use in an equal volume of Alsevers solution (161) and held at 4 C. Immediately prior to use, the cells were sedimented at 500 g for 5 minutes at 4 C. Packed cells were resuspended in 15 volumes of PBS-7.55 and washed 3

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\(^1\)Grand Island Biological Co., Grand Island, New York.

times. Following the final wash, the cells were resuspended in PBS-7.55 to a 5 per cent v/v concentration. The cells were warmed to 37 C and an equal volume of prewarmed, freshly prepared tannic acid\textsuperscript{1} (1:10,000) was added. The mixture was shaken in a 37 C water bath for 15 minutes and centrifuged at 400 g for 10 minutes. The cells were washed once and resuspended to 5 per cent in warm PBS-7.55.

A 15 ml amount of tanned cells was added to an equal volume of PBS-7.55 solution containing an optimal concentration of antigen. This mixture was incubated in a 37 C water bath for 45 minutes with occasional shaking. Sensitized cells were centrifuged at 400 g for 5 minutes and washed twice in warm PBS-7.55 containing normal rabbit serum (1:150). Centrifugation for each wash was 10 minutes at 400 g. The sensitized cells were resuspended in warm, PBS-7.55 (containing normal rabbit serum 1:100) to an approximate 0.5 per cent concentration.

Two fold dilutions of sera were prepared in microtiter plates with PBS-7.55 and 1:100 normal rabbit serum. 0.025 ml of the sensitized SRBC solution was added to each well. The plates were sealed with transparent tape, shaken for 2 minutes

\textsuperscript{1}Mallinckrodt Chemical Works, St. Louis, Missouri.
and refrigerated overnight at 4 C. Results were recorded the following morning.

When hemagglutination occurred, the sensitized red blood cells settled out as a shield on the bottom of each well. Lack of agglutination was indicated by formation of a small dense button of cells in the center of the well. The titer of a serum was recorded as the reciprocal of the highest dilution with complete agglutination.

Challenge

One vial of lyophilized culture was reconstituted with sterile distilled water and inoculated onto 5 per cent horse blood agar plates. Following incubation for 18 hours, one typical S. equisimilis colony was picked to THB; the culture was incubated at 37 C for 18 hours and examined microscopically for purity. Four drops of the 18 hour culture were transferred to 10 ml of pre-warmed THB. This culture was incubated for 6 hours at 37 C and examined for purity. Cell numbers were estimated by optical density and viability determined by plate count. Growth under these conditions usually resulted in viability counts between 2 and $8 \times 10^8$ CFU per ml. This material was diluted 1:100 in 1 per cent neopeptone$^1$ before use in swine and mice.

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$^1$Difco Laboratories, Detroit, Michigan.
Mouse Experiments

Inbred 3 week old Swiss-Webster mice\(^1\) were used in these experiments. The animals were housed in plastic boxes with no more than 5 animals per cage and given water and a commercial ration\(^2\) free choice.

Mice were held for 4 days following receipt from the supplier before use. Equal numbers of males and females were allotted to each group. The animals received two 0.25 ml doses of streptococcal antigen administered subcutaneously at 7 day intervals. Ten days after the last inoculation, vaccinated and control mice were challenged.

Mice were challenged with 0.25 ml of a 6 hour culture produced according to the procedure described previously. The challenge dose was administered intraperitoneally followed immediately by 0.15 ml of a 5 per cent gastric mucin solution. Death resulted within 48 to 96 hours in non-vaccinated mice.

Swine Experiments

Active and passive protection tests were done in young colostrum-deprived, surgically-derived or occasionally in

\(^1\)ARS/Sprague Dewlany, Inc., Madison, Wisconsin.

\(^2\)Lab bloc, Allied Mills Inc., Chicago, Illinois.
young naturally-born swine. Sows were of Yorkshire, Hampshire or cross-bred Yorkshire-Hampshire breeding mated to Yorkshire or Hampshire boars. They were procured shortly before farrowing from local farmers or from a herd maintained at the Veterinary Medical Research Institute.¹

Pigs were obtained by Caesarean section when the sows evidenced mammary gland and other changes indicative of impending parturition. The pigs were removed aseptically, passed to a sterile room, wiped with sterile paper towels, and the umbilical cords were ligated, severed 2 to 3 cm from the abdominal wall and dipped in tincture of iodine. The animals were transferred to individual cardboard boxes or pens and maintained in an isolation unit at 92 to 96 F. They were initially fed 2 to 3 ounces of SPF-lac² four times per day with gradual increases in amount of SPF-lac depending on the appetite and health of the pigs. After 7 to 10 days the pigs were grouped according to size and fed about 4 to 8 ounces per feeding for the next two to three weeks. When the pigs were 2 to 3 weeks of age a complete 18 per cent protein pig starter and water were offered free choice. When the pigs

¹Veterinary Medical Research Institute, Iowa State University, Ames, Iowa.
²Borden Company, New York, New York
were eating the starter well, the SPF-lac was gradually decreased in amount so that they were weaned by 4 to 5 weeks of age.

Streptococcal vaccine for use in swine consisted of antigen and adjuvant which was mixed until a water-in-oil emulsion was achieved. The prepared vaccine was held at 4°C until used. It was administered in a series of 3 doses consisting of 2 ml inoculations given intramuscularly at 4 day intervals.

All swine used in this study were CF and IHA negative for detectable levels of antibodies against \textit{S. equisimilis} at the time of initial inoculation.

Swine were challenged with 1 ml of the diluted 6 hour culture, usually administered into the marginal ear vein. Following challenge, swine generally evidenced elevated temperatures within 24 hours and lameness within 24 to 48 hours. Other clinical signs observed in some but not all challenged animals included roughened hair coats, joint swelling and inappetence. Joint involvement was frequently the most obvious symptom.

Four or five days postchallenge, the pigs were electrocuted and exsanguinated by severance of the brachial

\footnote{Incomplete Freund. Difco Laboratories, Detroit, Michigan.}
blood vessels. All limb joints, as well as liver, kidney, spleen and heart were examined for gross lesions. All joints and organs were seared on the exterior and opened by making an incision with a heat-sterilized knife. Samples were routinely collected from all joints and tissues with sterile cotton swabs\(^1\) and inoculated onto 5 per cent horse blood agar plates which were incubated 24 to 48 hours at 37 C. Following isolation of the organism, biochemical and serological identification was done as described previously.

Sites inoculated with vaccine were routinely examined for gross evidence of abscesses and any other untoward reactions.

RESULTS

Cultural and Biochemical Characteristics of Streptococcal Strains

Zones of beta hemolysis on blood agar were comparable for all strains of *S. equisimilis*, although strain 78-A3X had a slightly larger zone (Figure 1). The zones averaged 4 to 6 mm in diameter at 24 hours incubation and did not increase significantly after an additional 24 hours. All 5 strains fermented dextrose, lactose, sucrose and trehalose within 24 hours and glycerol between 24 and 48 hours incubation. Strains F-49 and 78-B were salicin positive, while the other 3 were negative. Mannitol, sorbitol and raffinose were not fermented. *S. zoosporidemicus* fermented lactose, sucrose, salicin and sorbitol but not the other sugars. Strain 9932 fermented dextrose, lactose, sucrose, glycerol and trehalose but not salicin, mannitol, sorbitol or raffinose. Growth in broth cultures generally tended to be relatively smooth and diffuse, although strains 78-A3X, R-8 and 9932 occasionally formed sediment.

All streptococcal strains used in this study, except 9932, were shown to belong to Lancefield Group C. Strain 9932 reacted with Lancefield Group L antiserum. The serotype specificities of the *S. equisimilis* strains are presented in Table 1 along with biochemical and sero-grouping results.
Figure 1. *Streptococcus equisimilis*

a. Strain 78-A grown 24 hours on blood agar.

b. Strain 78-A grown 24 hours on blood agar.
Table 1. Biochemical and serological characteristics of streptococcal cultures.

<table>
<thead>
<tr>
<th>Streptococcus strain</th>
<th>Carbohydrate Fermentation</th>
<th>Serological Lancefield S. equisimilis similis Group type</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. equisimilis 78-A</td>
<td>Ad</td>
<td>A</td>
</tr>
<tr>
<td>S. equisimilis 78-A3X</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>S. equisimilis R-8</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>S. equisimilis 78-B</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>S. equisimilis F-49</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>S. zooepidemicus</td>
<td>neg</td>
<td>A</td>
</tr>
<tr>
<td>Group L 9932</td>
<td>A</td>
<td>A</td>
</tr>
</tbody>
</table>

\(^a\)Dex. = dextrose; Lac. = lactose; Suc. = sucrose; Sal. = salicin; Man. = mannitol; Gly. = glycerol; Sor. = sorbitol; Raf. = raffinose; Treh. = trehalose.

\(^b\)Group determined by capillary precipitin test.

\(^c\)Serotype determined by immunodiffusion.

\(^d\)Acid produced.

\(^e\)No acid produced.

\(^f\)Not determined.
Swine-passaged strain 78-A3X was biochemically, serologically and microscopically indistinguishable from parent strain 78-A. However, strain 78-A3X appeared to produce slightly larger colonies on blood agar with a larger zone of beta hemolysis (Figure 1). Todd-Hewitt broth cultures of 78-A3X frequently had clear supernatants and a grainy sediment, whereas cultures of the parent strain remained fairly diffuse with less sediment.

Swine-passaged strain 78-A3X was more virulent for mice and swine than strain 78-A. Strain 78-A3X had an LD50 of $4 \times 10^4$ CFU for mice (Table 2), whereas strain 78-A had an LD50 of $5 \times 10^5$ CFU (Table 3). In swine, 78-A3X had an arthritic dose 50 of $4 \times 10^5$ CFU (Table 4), whereas strain 78-A had been shown previously (245) to have an arthritic dose 50 of $7.2 \times 10^8$ CFU. No appreciable difference was detected in the serological response of swine to the two strains.

Complement-Fixation and Hemagglutination

Comparison of non-adsorbed antiserums prepared in rabbits against 4 different serotypes of *S. equisimilis* with the CF procedure revealed some evidence of serotype specificity (Table 5); however, relatively high levels of cross reacting antibodies were often detected. Multiple absorption procedures eliminated most of the cross-reactive antibodies
Table 2. Pathogenicity of *S. equisimilis* 78-A3X for mice.

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of CFU Inoculated/Animal</th>
<th>Survivors/Total Challenged</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2 x 10^8</td>
<td>0/25</td>
</tr>
<tr>
<td>2</td>
<td>2 x 10^7</td>
<td>0/25</td>
</tr>
<tr>
<td>3</td>
<td>2 x 10^6</td>
<td>3/25</td>
</tr>
<tr>
<td>4</td>
<td>2 x 10^5</td>
<td>5/25</td>
</tr>
<tr>
<td>5</td>
<td>2 x 10^4</td>
<td>8/25</td>
</tr>
<tr>
<td>6</td>
<td>2 x 10^3</td>
<td>12/25</td>
</tr>
<tr>
<td>7</td>
<td>2 x 10^2</td>
<td>22/25</td>
</tr>
<tr>
<td>8</td>
<td>2 x 10^1</td>
<td>23/25</td>
</tr>
<tr>
<td>9</td>
<td>Placebo^b</td>
<td>25/25</td>
</tr>
</tbody>
</table>

^aMice were challenged subcutaneously with 0.25 ml of suspension containing appropriate number of CFU as determined by actual plate count. 0.15 ml of a 5 per cent gastric mucin solution was inoculated intraperitoneally at the time of challenge. Deaths were recorded for 7 days.

^bTodd-Hewitt broth diluted 1:10 with 1 per cent neopeptone.
Table 3. Pathogenicity of *S. equisimilis* 78-A for mice.

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of CPU Inoculated/Animal</th>
<th>Survivors/Total Challenged</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$1.6 \times 10^8$</td>
<td>0/20</td>
</tr>
<tr>
<td>2</td>
<td>$1.6 \times 10^7$</td>
<td>3/20</td>
</tr>
<tr>
<td>3</td>
<td>$1.6 \times 10^6$</td>
<td>12/20</td>
</tr>
<tr>
<td>4</td>
<td>$1.6 \times 10^5$</td>
<td>18/20</td>
</tr>
<tr>
<td>5</td>
<td>$1.6 \times 10^4$</td>
<td>18/20</td>
</tr>
<tr>
<td>6</td>
<td>$1.6 \times 10^3$</td>
<td>17/20</td>
</tr>
<tr>
<td>7</td>
<td>$1.6 \times 10^2$</td>
<td>18/20</td>
</tr>
<tr>
<td>8</td>
<td>$1.6 \times 10^1$</td>
<td>20/20</td>
</tr>
<tr>
<td>9</td>
<td>Placebo^b</td>
<td>20/20</td>
</tr>
</tbody>
</table>

^cMice were challenged subcutaneously with 0.25 ml of suspension containing appropriate number of CPU as determined by actual plate count. 0.15 ml of a 5 percent gastric mucin solution was inoculated intraperitoneally at the time of challenge. Deaths were recorded for 7 days.

^bTodd-Hewitt broth diluted 1:10 with 1 per cent neopeptone.
Table 4. Pathogenicity of *S. equisimilis* 78-A3X for 3 week old CDCD swine.

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of Pigs</th>
<th>Number of CFU Inoculated/Animal</th>
<th>Lameness Indexa</th>
<th>No. Joints Positive for <em>S. equisimilis</em>b</th>
<th>No. Joints with Lesionsb</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3</td>
<td>2.05 x 10^7d</td>
<td>0.31</td>
<td>12/36</td>
<td>11/36</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>3.65 x 10^6</td>
<td>0.25</td>
<td>27/48</td>
<td>20/48</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>2.05 x 10^6</td>
<td>0.20</td>
<td>7/36</td>
<td>8/36</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>3.65 x 10^5</td>
<td>0.27</td>
<td>19/48</td>
<td>15/48</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>2.05 x 10^5</td>
<td>0.12</td>
<td>8/36</td>
<td>11/36</td>
</tr>
<tr>
<td>6</td>
<td>4</td>
<td>3.65 x 10^4</td>
<td>0.33</td>
<td>28/48</td>
<td>23/48</td>
</tr>
<tr>
<td>7</td>
<td>3</td>
<td>2.05 x 10^4</td>
<td>0.08</td>
<td>9/36</td>
<td>5/36</td>
</tr>
</tbody>
</table>

*aLameness index=Total number of limbs exhibiting lameness during 5 days postchallenge divided by total observations.*

*bTotal number of arthritic joints over total number of joints observed at necropsy 5 days postchallenge.*

*cTotal number of joints containing *S. equisimilis* over total number of joints cultured at necropsy 5 days postchallenge.*

*dNumber of CFU based on actual plate count.*
Table 5. Comparison of four serotypes of *Streptococcus equisimilis* by complement-fixation.

<table>
<thead>
<tr>
<th>Antigen Preparations</th>
<th>Serotype I 78-A</th>
<th>Serotype II R-8</th>
<th>Serotype III 78-B</th>
<th>Serotype IV F-49</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. equisimilis</em> 78-A HCl(^a)</td>
<td>320(^b)</td>
<td>160</td>
<td>160</td>
<td>40</td>
</tr>
<tr>
<td><em>S. equisimilis</em> 78-A SD(^c)</td>
<td>640</td>
<td>80</td>
<td>80</td>
<td>80</td>
</tr>
<tr>
<td><em>S. equisimilis</em> R-8 HCl</td>
<td>40</td>
<td>2560</td>
<td>320</td>
<td>40</td>
</tr>
<tr>
<td><em>S. equisimilis</em> R-8 SD</td>
<td>40</td>
<td>1280</td>
<td>320</td>
<td>40</td>
</tr>
<tr>
<td><em>S. equisimilis</em> 78-B HCl</td>
<td>20</td>
<td>40</td>
<td>2560</td>
<td>320</td>
</tr>
<tr>
<td><em>S. equisimilis</em> 78-B SD</td>
<td>20</td>
<td>160</td>
<td>2560</td>
<td>320</td>
</tr>
<tr>
<td><em>S. equisimilis</em> F-49 HCl</td>
<td>20</td>
<td>160</td>
<td>160</td>
<td>320</td>
</tr>
<tr>
<td><em>S. equisimilis</em> F-49 SD</td>
<td>20</td>
<td>80</td>
<td>20</td>
<td>640</td>
</tr>
<tr>
<td><em>S. zooepidemicus</em> HCl</td>
<td>10</td>
<td>40</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td><em>S. zooepidemicus</em> SD</td>
<td>10</td>
<td>40</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Group L 9932 HCl</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>80</td>
<td>80</td>
</tr>
<tr>
<td>Group L 9932 SD</td>
<td>&lt;10</td>
<td>20</td>
<td>40</td>
<td>80</td>
</tr>
</tbody>
</table>

\(^a\)Conventional hot acid extract.

\(^b\)Reciprocal titer determined by duplicate block titration.

\(^c\)Sonic derived extract centrifuged at 30,000 g.
but required considerable time and streptococcal cells. Representative titers obtained with adsorbed and non-adsorbed hyperimmune swine and rabbit serums, as evaluated by CF and IHA, are presented in Table 6. Purified hot acid extract from serotype 1 reacted by CF with its homologous antiserum but failed to react with other *S. equisimilis* antiserums (Table 7). The purified serotype I antigen was not evaluated with the IHA procedure.

Comparison of various *S. equisimilis* preparations indicated that supernatant from sonically disrupted cells (SD 30,000 g) was the most potent CF antigen and that it resulted in minimal non-specific hemolysis and anticomplementary activity (Table 8). The SD 30,000 g procedure resulted in a CF antigen yield approximately 180 times greater, on a per cell basis, than that obtained by extraction with hot acid. In addition, the optimally diluted SD 30,000 g preparation reacted at a 4 fold higher dilution of a standard swine antiserum than did hot acid extract.

Immunodiffusion (ID) analysis revealed that SD 30,000 g and SD 3,000 g preparations of strain 78-A3X contained at least 3 separate precipitating antigens when evaluated against a homologous, non-adsorbed rabbit antiserum (Figure 2). Hot acid extracts derived from *S. equisimilis* R-8 (serotype II) and *S. zoopneumoniae* shared one precipitating
Table 6. Effect of whole cell absorption on complement-fixing and indirect hemagglutinating antibody titers of hyperimmune serums.

<table>
<thead>
<tr>
<th>Number</th>
<th>Source</th>
<th>Sero-type</th>
<th>Non-Adsorbed</th>
<th>Adsorbed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Homologous</td>
<td>Heterologous</td>
</tr>
<tr>
<td>5</td>
<td>Swine</td>
<td>I</td>
<td>640</td>
<td>160</td>
</tr>
<tr>
<td>8</td>
<td>Swine</td>
<td>I</td>
<td>160</td>
<td>80</td>
</tr>
<tr>
<td>128</td>
<td>Rabbit</td>
<td>I</td>
<td>40,960</td>
<td>1,280</td>
</tr>
<tr>
<td>129</td>
<td>Rabbit</td>
<td>II</td>
<td>5,120</td>
<td>320</td>
</tr>
<tr>
<td>137</td>
<td>Rabbit</td>
<td>III</td>
<td>2,560</td>
<td>160</td>
</tr>
<tr>
<td>153</td>
<td>Rabbit</td>
<td>I</td>
<td>5,120</td>
<td>2,560</td>
</tr>
<tr>
<td>218</td>
<td>Rabbit</td>
<td>I</td>
<td>5,120</td>
<td>1,280</td>
</tr>
</tbody>
</table>

*a* Reciprocal homologous CF and IHA titers determined by duplicate block titrations.

*b* Non-adsorbed antiserums heat inactivated for 30 minutes at 56°C.

*c* Type-specific antiserums prepared by multiple whole cell absorptions. Serums inactivated at 56°C for 30 minutes.

*d* Immunizing antigen: numbers 5, 8, 128 and 153 were *S. equisimilis* 78-A; 218 was *S. equisimilis* 78-A3X; 129 was *S. equisimilis* R-8; and 137 was *S. equisimilis* 78-B.

*e* Test antigens were sonicates of the respective immunizing antigens (Column 1).

*f* Test antigen was sonicate of *S. equisimilis* R-8 (serotype II) when testing animals immunized with *S. equisimilis* 78-A or 78-A3X and sonicate of *S. equisimilis* 78-A (serotype 1) when testing animal immunized against *S. equisimilis* R-8.
<table>
<thead>
<tr>
<th></th>
<th>Non-adsorbed&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Adsorbed&lt;sup&gt;c&lt;/sup&gt;</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Homo-logous</td>
<td>512</td>
<td>128</td>
<td>Homo-logous</td>
</tr>
<tr>
<td>Hetero-logous</td>
<td>512</td>
<td>8</td>
<td>Hetero-logous</td>
</tr>
<tr>
<td></td>
<td>128</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2,048</td>
<td>2,048</td>
<td></td>
</tr>
<tr>
<td></td>
<td>512</td>
<td>256</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1,024</td>
<td>256</td>
<td></td>
</tr>
<tr>
<td></td>
<td>512</td>
<td>128</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2,048</td>
<td>512</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Indirect Hemagglutination

<sup>b</sup> Non-adsorbed

<sup>c</sup> Adsorbed
Table 7. Comparison of crude hot acid extract and purified serotype I antigen from *S. equisimilis* 78-A3X by complement-fixation.

<table>
<thead>
<tr>
<th>Antigen Preparation</th>
<th>Sero-type I</th>
<th>Sero-type II</th>
<th>Sero-type III</th>
<th>Sero-type IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude hot Acid extract</td>
<td>1280&lt;sup&gt;a&lt;/sup&gt;</td>
<td>320</td>
<td>40</td>
<td>160</td>
</tr>
<tr>
<td>Purified sero-type I antigen</td>
<td>640</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
</tbody>
</table>

<sup>a</sup>Reciprocal titer determined by duplicate block titrations.

Antigen with strain 78-A3X that was probably the Group C carbohydrate. Other antigens demonstrated by ID in hot acid and SD preparations of *S. equisimilis* 78-A3X were a non-type-specific antigen and the *S. equisimilis* serotype I antigen. Precipitating antigens cross-reactive with *S. equisimilis* serotype I antiserum were not detected in a hot acid extract of the Group L strain.
Table 8. Potency of complement-fixing antigen in various preparations of *S. equisimilis* 78-A.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Optimal Dilution for Maximal Sensitivity</th>
<th>Reciprocal Serum Titer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Working&lt;sup&gt;a&lt;/sup&gt; Dilution</td>
<td>Actual&lt;sup&gt;b&lt;/sup&gt; Dilution</td>
</tr>
<tr>
<td>Acid extracted whole cells</td>
<td>16</td>
<td>32</td>
</tr>
<tr>
<td>Acid extracted cell walls</td>
<td>8</td>
<td>16</td>
</tr>
<tr>
<td>Alkaline extracted whole cells</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>SD 3,000 g&lt;sup&gt;d&lt;/sup&gt;</td>
<td>8</td>
<td>1240</td>
</tr>
<tr>
<td>SD 30,000 g</td>
<td>32</td>
<td>5760</td>
</tr>
<tr>
<td>SD 100,000 g</td>
<td>32</td>
<td>5760</td>
</tr>
</tbody>
</table>

<sup>a</sup>Reciprocal of dilution of each preparation which gave the highest titer in a block titration with a standard antiserum.

<sup>b</sup>Relative potency of each preparation. Determined by combining the working dilution factor with the dilution resulting during preparation of the extract from packed cells.

<sup>c</sup>Non-adsorbed, homologous swine serum.

<sup>d</sup>Sonically disrupted and centrifuged at 3,000 g, 30,000 g or 100,000 g.
Post immunization serums of 6 swine exhibited a sequential rise in CF antibodies against *S. equisimilis* 78-A when compared to the pre-immunization serums. The geometric mean titer (GMT) of CF antibody was $\leq 10$ on day 0, 40 on day 14 (3 inoculations) and 267 on day 35 (8 inoculations). The GMT of IHA antibodies in these same serums was $\leq 4$ on day 0, 12 on day 14 and 48 on day 35. None of the serums collected from 4 placebo vaccinates contained antibodies detectable with either CF or IHA procedures.

Fractionation of Streptococcal Antigens

**Gel filtration**

A 20 mg sample of *S. equisimilis* 78-A3X hot acid extract was applied to a Sephadex G-200 column and forty 5 ml samples were collected. Immunodiffusion (ID) analysis indicated that samples 14 through 34 contained streptococcal precipitating antigens. The group polysaccharide antigen was demonstrated in all samples that contained precipitating components. By use of type-specific antiserum in an ID test, the type-specific antigen was located in samples 15 through 21. Mouse protective activity was distributed throughout the eluate (Table 9). Similar results were obtained when 25 or 30 mg of hot acid extract or 30 mg of sonic extract were applied to the column, although mouse protective activity was not observed.
Figure 2. Immunodiffusion analysis of hot acid and sonic extracts. Well AS contained non-adsorbed rabbit antiserum against *S. equisimilis* 78-A (serotype 1), well 1 contained hot acid extract of *S. equisimilis* 78-A, well 2 contained a SD 30,000 g extract of *S. equisimilis* 78-A, well 3 contained a SD 3,000 g extract of *S. equisimilis* 78-A, well 4 contained a hot acid extract of *S. equisimilis* R-8 (serotype II), well 5 contained a hot acid extract of *S. zooepidemicus* and well 6 contained a hot acid extract of Group L 9932.
Table 9. Analysis of fractions of *S. equisimilis* hot acid and sonic extracts eluted from Sephadex G-200.

<table>
<thead>
<tr>
<th>Sample Number</th>
<th>Type-Group C Specific Anti-Prot- Anti-</th>
<th>Type-30 mg Group C Specific Anti-Prot-</th>
<th>Type-Sonic Extract (30 mg) Group C Specific Anti-Prot-</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*First sample eluted containing at least 1 precipitating antigen.*

*Immunodiffusion analysis of samples against non-adsorbed antiserum.*

*Immunodiffusion analysis of samples against type-specific antiserum.*

To determine mouse protection, 10 mice were vaccinated with two 0.25 ml doses of each sample subcutaneously on days 0 and 7. The mice were challenged subcutaneously 14 days later with approximately $4 \times 10^6$ CFU of *S. equisimilis*. 0.15 ml of a 5 per cent gastric mucin solution was injected simultaneously. Deaths were recorded for 5 days. Protection expressed as survivors/total challenged $\times 100\%$.

*Presence of at least one band in immunodiffusion.*

*Absence of any precipitating bands in immunodiffusion.*
### Table 9. (continued)

<table>
<thead>
<tr>
<th>Sample Number&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Type Group C Specific Mouse Anti-protection&lt;sup&gt;b&lt;/sup&gt;</th>
<th>20 mg</th>
<th>30 mg</th>
<th>Sonic Extract (30 mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Type Group C Specific Mouse Anti-protection&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>+</td>
<td>40</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>+</td>
<td>+</td>
<td>40</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>+</td>
<td>+</td>
<td>60</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>+</td>
<td>+</td>
<td>60</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Pooled Samples 8 to 16 10%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>+</td>
<td>-</td>
<td>60</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>+</td>
<td>-</td>
<td>NS&lt;sup&gt;c&lt;/sup&gt;</td>
<td>+</td>
</tr>
<tr>
<td>11</td>
<td>+</td>
<td>-</td>
<td>20</td>
<td>+</td>
</tr>
<tr>
<td>12</td>
<td>+</td>
<td>-</td>
<td>60</td>
<td>+</td>
</tr>
<tr>
<td>13</td>
<td>+</td>
<td>-</td>
<td>20</td>
<td>+</td>
</tr>
<tr>
<td>14</td>
<td>+</td>
<td>-</td>
<td>20</td>
<td>+</td>
</tr>
</tbody>
</table>

<sup>a</sup>No sample.

<sup>b</sup>Anti-protecting.

<sup>c</sup>No sample.
Table 9. (continued)

<table>
<thead>
<tr>
<th>Sample Number</th>
<th>20 mg</th>
<th>30 mg</th>
<th>Sonic Extract (30 mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Type-</td>
<td>Type-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Group C Specific Mouse Serum</td>
<td>Group C Specific Mouse Serum</td>
<td>Group C Specific Mouse Serum</td>
</tr>
<tr>
<td></td>
<td>Anti-</td>
<td>Anti-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>serum</td>
<td>serum</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>+</td>
<td>-</td>
<td>20</td>
</tr>
<tr>
<td>16</td>
<td>+</td>
<td>-</td>
<td>60</td>
</tr>
<tr>
<td>17</td>
<td>+</td>
<td>-</td>
<td>60</td>
</tr>
<tr>
<td>18</td>
<td>+</td>
<td>-</td>
<td>20</td>
</tr>
<tr>
<td>19</td>
<td>+</td>
<td>-</td>
<td>80</td>
</tr>
<tr>
<td>20</td>
<td>+</td>
<td>-</td>
<td>20</td>
</tr>
<tr>
<td>21</td>
<td>+</td>
<td>-</td>
<td>20</td>
</tr>
<tr>
<td>22</td>
<td>-</td>
<td>-</td>
<td>NS</td>
</tr>
</tbody>
</table>

Note: NS = Not stated
Starch block electrophoresis (SBE)

Electrophoresis of sonic (Figure 3) and hot acid extracted antigens in starch produced similar patterns. Precipitating streptococcal antigens were present in electrophoresed sonic extract samples 6 through 14 when evaluated against non-adsorbed hyperimmune serum (Figure 4) and samples 7 through 13 when evaluated against type-specific rabbit antiserum (Figure 5). Immunodiffusion analysis of electrophoresed hot acid extract indicated that precipitating streptococcal antigens were present in samples 5 through 14 and the type-specific antigen was located in samples 7 through 13; the separation pattern was identical to that obtained for the sonic extract. Mouse protection appeared to be related to those fractions containing the type-specific antigen (Figure 3).

Carboxymethyl cellulose (CMC)

The elution profile of a hot acid extract resulting from chromatography on CMC is shown in Figure 6. Similar findings with a sonic extract are shown in Figure 7. Samples of peak 1, peak 2 and peak 3 were pooled and the pools pervaporated to 2 mls each. Immunodiffusion analysis of the pools indicated that type-specific antigen was associated with the last two peaks. Peak 1 reacted with commercial Group C antiserum but failed to react with adsorbed, homologous type-specific
antiserum. Pool 2 contained precipitating antigens reactive with both group and type-specific antiserum. A single band developed when pool 3 was used with type-specific antiserum or non-adsorbed homologous antiserum. It failed to react with commercial Group C antiserum or unadsorbed antiserum against other *S. equisimilis* serotypes (Figures 8, 9). However, pool 3 contained 2 bands when electrophoresed in polyacrylamide.

Gel Filtration of Normal and Hyperimmune Serums

Presented in Figures 10 and 11 are elution patterns of normal and hyperimmune swine serums following fractionation in Sephadex G-200. Elution profiles of normal and hyperimmune rabbit serums are presented in Figures 12 and 13. As indicated on the figures, selected samples were pooled and the pools were pervaporated and analyzed. Immunoelectrophoretic analysis indicated that peak 1 contained predominately IgM and that peak 2 contained predominately IgG. Serological evaluation of the pools indicated most of the IHA antibody was present in the IgM fractions, whereas, the IgG fraction contained most of the CF antibody (Table 10).
Figure 3. Relationship of total protein, mouse protective activity and the group and type-specific antigens in samples from starch block electrophoresed *S. equisimilis* 78-A3X sonic extract. Arrow indicates point of application (cathode on left and anode on right). Immunodiffusion analysis used to locate group and type-specific antigens. To determine mouse protection, 20 mice were vaccinated with two 0.25 ml doses of material from each sample. Fourteen days later they were challenged subcutaneously with *S. equisimilis* 78-A3X. Deaths were recorded for 7 days: survivors/total challenged x100%. 
Figure 4. Immunodiffusion analysis of *S. equisimilis* 78-A sonic extract samples obtained by starch block electrophoresis. Sample 1 nearest cathode and sample 18 nearest anode. Center wells (153) contained homologous hyperimmune rabbit antiserum. HCl=hot acid extract from *S. equisimilis* 78-A3X.
<table>
<thead>
<tr>
<th></th>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>8</td>
<td>7</td>
<td>6</td>
<td>5</td>
<td>10</td>
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<table>
<thead>
<tr>
<th>13</th>
<th>12</th>
<th>11</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>153</td>
<td>153</td>
<td>153</td>
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<td>---</td>
<td>---</td>
</tr>
<tr>
<td>15</td>
<td>16</td>
<td>17</td>
<td>18</td>
</tr>
</tbody>
</table>

HCl
Figure 5. Immunodiffusion analysis of *S. equisimilis* 78-A sonic extract samples obtained by starch block electrophoresis. Sample 1 nearest cathode and sample 18 nearest anode. Center wells (153) contained homologous type-specific rabbit antiserum. HCl=hot acid extract from *S. equisimilis* 78-A3X.
Figure 6. Chromatography of hot acid extract of S. equisimilis 78-A3X on carboxymethyl cellulose. Peak 1 consisted of samples 3 through 6, peak 2 consisted of samples 19 through 22 and peak 3 consisted of samples 31 through 34.
Figure 7. Chromatography of sonic extract of *S. equisimilis* 78-A3X on carboxymethyl cellulose. Peak 1 consisted of samples 3 through 6, Peak 2 consisted of samples 19 through 22 and peak 3 consisted of samples 31 through 34.
Figure 8. Stages in the purification of *S. equisimilis* serotype I antigen. Center well AS contained non-adsorbed rabbit antiserum against *S. equisimilis* 78-A3X, well 1 contained a crude hot acid extract of *S. equisimilis* 78-A3X, well 2 contained a hot acid extract of *S. equisimilis* 78-A3X after ammonium sulfate precipitation and well 3 contained purified type-specific antigen from *S. equisimilis* 78-A3X.
Figure 9. Specificity of purified serotype I antigen.
Center well CMC contained purified serotype I antigen, well 1 contained rabbit antiserum against *S. equisimilis* 78-A3X, well 2 contained rabbit antiserum against *S. equisimilis* R-8 (serotype II), well 3 contained rabbit antiserum against *S. equisimilis* 78-3 (serotype III) and well 4 contained rabbit antiserum against *S. equisimilis* F-49 (serotype IV).
Figure 10. Elution profile of Sephadex G-200 fractionated swine serum (7) before exposure to *S. equisimilis*. Peak 1 consisted of samples 6 through 9 and peak 2 consisted of samples 12 through 16.
Figure 11. Elution profile of Sephadex G-200 fractionated swine serum (7) following immunization against S. equisimilis 78-A (12 days postimmunization). Peak 1 consisted of samples 6 through 9 and peak 2 consisted of samples 16 through 18.
Figure 12. Elution profile of Sephadex G-200 fractionated normal rabbit serum (153) before *S. equisimilis* exposure. Peak 1 consisted of samples 3 through 6 and peak 2 consisted of samples 11 through 14.
Figure 13. Elution profile of Sephadex G-200 fractionated rabbit serum (153) immunized against S. equisimilis 73-A (21 days postimmunization). Peak 1 consisted of samples 4 through 7 and Peak 2 consisted of samples 13 through 17.
Table 10. Characteristics of complement-fixing and indirect hemagglutinating antibodies against *S. aquisimilis* 78-A3X in serum fractionated in Sephadex G-200.

<table>
<thead>
<tr>
<th>Serum Source</th>
<th>Peak Number&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Predominant Immunoglobulin&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Complement-Fixation Reciprocal Titer&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Indirect Hemagglutination Reciprocal Titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-Vaccinated</td>
<td></td>
<td>Ig M</td>
<td>&lt;10</td>
<td>&lt;4</td>
</tr>
<tr>
<td>Swine</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vaccinated Swine</td>
<td>1</td>
<td>Ig M</td>
<td>20</td>
<td>128</td>
</tr>
<tr>
<td>Non-Vaccinated</td>
<td></td>
<td>Ig M</td>
<td>&lt;10</td>
<td>&lt;4</td>
</tr>
<tr>
<td>Rabbit</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vaccinated Rabbit</td>
<td>1</td>
<td>Ig M</td>
<td>40</td>
<td>1024</td>
</tr>
<tr>
<td>Non-Vaccinated</td>
<td></td>
<td>Ig G</td>
<td>&lt;10</td>
<td>&lt;4</td>
</tr>
<tr>
<td>Swine</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Samples of peak 1 and samples of peak 2 were pooled, pools pervaporated and analyzed.

<sup>b</sup>Immunoglobulin class as demonstrated by immunoelectrophoresis.

<sup>c</sup>Reciprocal of homologous CF and IHA titers determined by duplicate block titrations.
Table 10. (continued)

<table>
<thead>
<tr>
<th>Serum Source</th>
<th>Peak Number</th>
<th>Predominant Immunoglobulin</th>
<th>Complement-Fixation Reciprocal Titer</th>
<th>Indirect Hemmaglutination Reciprocal Titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vaccinated Swine</td>
<td>2</td>
<td>Ig G</td>
<td>160</td>
<td>16</td>
</tr>
<tr>
<td>Non-Vaccinated Rabbit</td>
<td>2</td>
<td>Ig G</td>
<td>&lt;10</td>
<td>&lt;4</td>
</tr>
<tr>
<td>Vaccinated Rabbit</td>
<td>2</td>
<td>Ig G</td>
<td>640</td>
<td>64</td>
</tr>
</tbody>
</table>
Immunogenicity of \textit{S. equisimilis} Antigens in Mice

Comparisons of mouse protective activity of different \textit{S. equisimilis} antigen preparations are presented in Table 11. The results listed in the table are a composite of several tests. Whole cell bacterins were unable to elicit a high level of protection regardless of the inactivating agent. Protection in whole cell vaccinates ranged up to 60 per cent. Hot acid extract protected 80 per cent of vaccinated mice while hot alkaline extract protected 55 per cent. A single dose of sonic vaccine protected 60 per cent of those vaccinated whereas 2 doses protected 87 per cent. The addition of various inactivating agents to the crude preparation had little effect on mouse protection. SD 30,000 g extract, which included most of the soluble components minus the cell wall, protected 72 per cent of vaccinated mice. Purified serotype I hot acid extract elicited protection in 90 per cent of vaccinated mice. Placebo vaccinates were not protected against the challenge.
Table 11. Immunogenicity of *S. equisimilis* 78-A3X preparations in mice.

<table>
<thead>
<tr>
<th>Vaccine Treatment(^a)</th>
<th>Number of CFU Administered/Animal(^b)</th>
<th>Protection(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heat-52(^0) for 30 min.</td>
<td>1.5 x 10^9</td>
<td>60%</td>
</tr>
<tr>
<td>b-propiolactone (BPL) 0.2%</td>
<td>1.5 x 10^9</td>
<td>33%</td>
</tr>
<tr>
<td>Merthiolate 1:10,000</td>
<td>1.5 x 10^9</td>
<td>60%</td>
</tr>
<tr>
<td>Formalin (HCHO) 0.3%</td>
<td>1.5 x 10^9</td>
<td>60%</td>
</tr>
<tr>
<td>Hot-acid</td>
<td>1 x 10^{11}</td>
<td>80%</td>
</tr>
<tr>
<td>Hot-alkaline</td>
<td>5 x 10^{10}</td>
<td>55%</td>
</tr>
<tr>
<td>Sonicated</td>
<td>1.5 x 10^9</td>
<td>87%</td>
</tr>
<tr>
<td>Sonicated</td>
<td>7.5 x 10^8</td>
<td>60%</td>
</tr>
<tr>
<td>Sonicated-0.2% BPL</td>
<td>1.5 x 10^9</td>
<td>85%</td>
</tr>
<tr>
<td>Sonicated-merthiolate</td>
<td>1.5 x 10^9</td>
<td>85%</td>
</tr>
</tbody>
</table>

\(^a\)Cells obtained from 18 hour THB and resuspended in phosphate buffered saline at a concentration of 3 x 10^9 CFU/ml where appropriate and treated accordingly.

\(^b\)Number of CFU based on actual colony count or estimated from packed cell volume; 1 ml of wet packed cells equals 5.4 x 10^{11} CFU.

\(^c\)To determine protection, two 0.25 ml doses of each preparation were given to 30 mice subcutaneously on days 0 and 7. The animals were challenged subcutaneously 14 days after vaccination with approximately 2 x 10^6 CFU. Deaths were recorded for 7 days post challenge. Protection expressed as no. survivors/total challenged x 100%.
Table 11. (continued)

<table>
<thead>
<tr>
<th>Vaccine Treatment</th>
<th>Number of CFU Administered/Animal</th>
<th>Protection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sonicated-HCHO</td>
<td>$1.5 \times 10^9$</td>
<td>60%</td>
</tr>
<tr>
<td>Sonicated 30,000 g</td>
<td>$1.5 \times 10^9$</td>
<td>72%</td>
</tr>
<tr>
<td>Purified type-specific</td>
<td>Undetermined</td>
<td>90%</td>
</tr>
<tr>
<td>Placebo vaccinates</td>
<td></td>
<td>0%</td>
</tr>
</tbody>
</table>

Immunogenicity of *S. equisimilis* Antigens in Swine

Comparison of whole cells and sonicated cells

Experiment 1  Eight 3 week old Caesarean derived-colostrum deprived (CDCD) pigs were allotted to 4 groups for comparison of whole cell and sonic derived vaccines. Vaccines were given intramuscularly on days 0, 4 and 8 (Table 12). On day 26, the animals were test bled and challenged by intravenous inoculation of *S. equisimilis*.

Elevated temperatures (2 to 4 °F) were recorded 24 to 48 hours postchallenge. Clinical observations of lameness were recorded for 4 days and a lameness index was calculated for each group.

On day 30, all animals were exsanguinated and necropsied. The number of joints with lesions and the number of joints containing *S. equisimilis* are tabulated in Table 12.
along with the lameness indices. Those animals vaccinated with whole cell and sonic vaccines appeared to be protected against the challenge while non-vaccinated controls were susceptible.

Serum samples, collected at the time of challenge, were analyzed for their *S. equisimilis* antibody content. The geometric mean complement-fixation (GM-CF) titer for whole cell vaccinates was 80, for sonic vaccinates it was 240 and for both control groups it was less than 10.

**Experiment 2** Ten 4 week old CDCD pigs were allotted to 4 groups for comparison of whole cell and sonic derived vaccines. Vaccines were given intramuscularly on days 0, 4 and 8 (Table 13). On day 26, all animals were test bled and challenged by intravenous inoculation of *S. equisimilis*.

All challenged animals had elevated temperatures (2 to 4 °F) 24 to 72 hours postchallenge. Clinical observations of lameness were recorded for 4 days and a lameness index was calculated for each group.

On day 30, all animals were exsanguinated and necropsied. The number of joints with lesions and the number of joints with *S. equisimilis* are tabulated in Table 13 along with the lameness indices. Those animals vaccinated with streptococcal vaccines appeared to be protected against challenge while non-vaccinated controls were susceptible.
Serum samples, collected at the time of challenge, were analyzed for their *S. equisimilis* antibody content. The GM-CF titer for whole cell vaccinates was 80, for sonic vaccinates it was 320 and for both control groups it was less than 10.

**Comparison of hot acid and sonic derived vaccines**

**Experiment 3**  
Nine 3 week old CDCD pigs were allotted to 3 groups for comparison of hot acid extract and sonic derived vaccines. Vaccines were given intramuscularly on days 0, 4 and 8 (Table 14). On day 33, all animals were test bled and challenged by intravenous inoculation of *S. equisimilis*.

Elevated temperatures (1.4 to 3.6 °F) were observed 24 hours postchallenge. Clinical observations of lameness were recorded for 5 days and a lameness index was calculated for each group.

On day 38, all animals were exsanguinated and necropsied. The number of joints with lesions and the number of joints containing *S. equisimilis* are tabulated in Table 14 along with lameness indices. Both vaccinated groups demonstrated similar levels of protection following challenge when compared to the controls.

Serum samples, collected at the time of challenge, were analyzed for their *S. equisimilis* antibody content.
The GM-CF titer for hot acid extract vaccinates was 210, for sonic extract vaccinates it was 533 and for the control group it was less than 10.

**Evaluation of sonic derived vaccine in swine**

**Experiment 4**  
Ten 3 week old CDCD pigs were allotted to 2 groups for evaluation of 2 doses of sonic derived vaccine. The vaccine was given intramuscularly on days 0 and 8 (Table 15). On day 22 the animals were tested and challenged by intravenous inoculation of *S. equisimilis*. Elevated temperatures (0.6 to 3 F) were observed 24 to 48 hours postchallenge. Clinical observations of lameness were recorded for 5 days and a lameness index was calculated for each group.

On day 27, all animals were exsanguinated and necropsied. The number of joints with gross lesions and the number of joints containing *S. equisimilis* are tabulated in Table 15 along with the lameness indices. The vaccinates appeared to be protected against a virulent challenge while the non-vaccinates were susceptible.

Serum samples, collected at the time of challenge, were analyzed for their *S. equisimilis* antibody content by complement-fixation. The GM-CF titer for the sonic vaccinates was 192 and for the control group it was less than 10.
### Table 12. Experiment 1. Immunogenicity of \( S. \text{equisimilis} \) 78-A whole cells and sonicated cells in swine.

<table>
<thead>
<tr>
<th>Group</th>
<th>No. Pigs</th>
<th>Treatment</th>
<th>Lameness Index(^a)</th>
<th>No. Joints Positive for ( S. \text{equisimilis} ) (^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>Three 2 ml doses of whole cells + incomplete Freund adjuvant I.M. challenged I.V. with ( 3.2 \times 10^6 ) CPU-SE.</td>
<td>0.09</td>
<td>2/24</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>Three 2 ml doses of sonicate + incomplete Freund adjuvant I.M. challenged I.V. with ( 3.2 \times 10^6 ) CPU-SE.</td>
<td>0.03</td>
<td>0/24</td>
</tr>
</tbody>
</table>

\(^a\)Lameness index = Total number of limbs exhibiting lameness during 4 days postchallenge divided by total observations.

\(^b\)Total number of arthritic joints over total joints observed at necropsy 4 days postchallenge.

\(^c\)Total number of joints containing \( S. \text{equisimilis} \) over total number of joints cultured at necropsy 4 days postchallenge.

\(^d\)Colonies forming units of \( S. \text{equisimilis} \) as evaluated by plate count.
### Table 12. (continued)

<table>
<thead>
<tr>
<th>Group</th>
<th>No. Pigs</th>
<th>Treatment</th>
<th>Lameness Index</th>
<th>No. Joints with Lesions</th>
<th>No. Joints Positive for S. equisimilis</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>2</td>
<td>Three 2 ml doses of placebo + incomplete Freund adjuvant I.M. challenged I.V. with 3,2 x 10⁶ CFU-SE.</td>
<td>0.42</td>
<td>13/24</td>
<td>11/24</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>Not vaccinated Not challenged</td>
<td>0.00</td>
<td>0/24</td>
<td>0/24</td>
</tr>
</tbody>
</table>
Table 13. Experiment 2. Immunogenicity of S. equisimilis 78-A3X whole cells and sonicated cells in swine.

<table>
<thead>
<tr>
<th>Group</th>
<th>No. Pigs</th>
<th>Treatment</th>
<th>Lameness Index&lt;sup&gt;a&lt;/sup&gt;</th>
<th>No. Joints With Lesions&lt;sup&gt;b&lt;/sup&gt;</th>
<th>No. Joints Positive for S. equisimilis&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3</td>
<td>Three 2 ml doses whole cells + incomplete Freund adjuvant I.M., challenged I.V. with 2.8 x 10^6 CFU-SE&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.03</td>
<td>1/36</td>
<td>0/36</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>Three 2 ml doses sonicate + incomplete Freund adjuvant I.M. challenged I.V. with 2.8 x 10^6 CFU-SE&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.00</td>
<td>1/36</td>
<td>0/36</td>
</tr>
</tbody>
</table>

<sup>a</sup>Lameness index=Total number of limbs exhibiting lameness during 5 days postchallenge divided by total observations.

<sup>b</sup>Total number of arthritic joints over total joints observed at necropsy 5 days postchallenge.

<sup>c</sup>Total number of joints containing S. equisimilis over total number of joints cultured at necropsy 5 days postchallenge.

<sup>d</sup>Colony forming units of S. equisimilis as evaluated by plate count.
Table 13. (continued)

<table>
<thead>
<tr>
<th>Group</th>
<th>No. Pigs</th>
<th>Treatment</th>
<th>Lameness Index$^a$</th>
<th>No. Joints With Lesions$^b$</th>
<th>No. Joints Positive for S. equisimilis$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>2</td>
<td>Three 2 ml doses placebo + incomplete Freund adjuvant I.M. challenged I. V. with $2.8 \times 10^6$ CPU-SE.</td>
<td>0.45</td>
<td>10/24</td>
<td>11/24</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>Not vaccinated Not challenged</td>
<td>0.00</td>
<td>0/24</td>
<td>0/24</td>
</tr>
</tbody>
</table>
Table 14. Experiment 3. Immunogenicity of *S. equisimilis* 78-A3X sonicated cells and hot acid extract in swine.

<table>
<thead>
<tr>
<th>Group</th>
<th>No. Pigs</th>
<th>Treatment</th>
<th>Lameness Index (^a)</th>
<th>No. Joints With Lesions (^b)</th>
<th>No. Joints Positive for <em>S. equisimilis</em> (^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3</td>
<td>Three 2 ml doses of sonicate + incomplete Freund adjuvant I.M., challenged I.V. with 3.9 x 10(^6) CFU-SE (^d).</td>
<td>0.08</td>
<td>9/36</td>
<td>4/36</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>Three 2 ml doses of hot-acid extract + incomplete Freund adjuvant I.M., challenged I.V. with 3.9 x 10(^6) CFU-SE.</td>
<td>0.06</td>
<td>4/36</td>
<td>1/36</td>
</tr>
</tbody>
</table>

\(^a\) Lameness index = Total number of limbs exhibiting lameness during 5 days postchallenge divided by total observations.

\(^b\) Total number of arthritic joints over total joints observed at necropsy 5 days postchallenge.

\(^c\) Total number of joints containing *S. equisimilis* over total number of joints cultured at necropsy 5 days postchallenge.

\(^d\) Colony forming units of *S. equisimilis* as evaluated by plate count.
Table 14. (continued)

<table>
<thead>
<tr>
<th>Group</th>
<th>No. Pigs</th>
<th>Treatment</th>
<th>Lameness Index</th>
<th>No. Joints Positive for S. equisimilis</th>
<th>No. Joints With Lesions</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>3</td>
<td>Three 2 ml doses of placebo + incomplete Freund adjuvant I.M., challenged I.V., with 3.9 x 10^6 CPU-SE.</td>
<td>0.48</td>
<td>19/36</td>
<td>11/36</td>
</tr>
</tbody>
</table>
Table 15. Experiment 4. Immunogenicity of *S. equisimilis* 78-A sonicated cells in swine.

<table>
<thead>
<tr>
<th>Group</th>
<th>No. Pigs</th>
<th>Treatment</th>
<th>Lameness Index&lt;sup&gt;a&lt;/sup&gt;</th>
<th>No. Joints With Lesions&lt;sup&gt;b&lt;/sup&gt;</th>
<th>No. Joints Positive for <em>S. equisimilis</em>&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>Two 2 ml doses of sonicate + incomplete Freund's I.M.</td>
<td>0.05</td>
<td>2/60</td>
<td>1/60</td>
</tr>
<tr>
<td></td>
<td></td>
<td>challenged I.V. with 7.2 x 10^8 CFU-SEG.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>Not-vaccinated challenged I.V.</td>
<td>0.25</td>
<td>15/60</td>
<td>4/60</td>
</tr>
<tr>
<td></td>
<td></td>
<td>with 7.2 x 10^8 CFU-SEG.</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Lameness index = Total number of limbs exhibiting lameness during 5 days postchallenge divided by total observations.

<sup>b</sup>Total number of arthritic joints over total joints observed at necropsy 5 days postchallenge.

<sup>c</sup>Total number of joints containing *S. equisimilis* over total number of joints cultured at necropsy 5 days postchallenge.

<sup>d</sup>Colony forming units of *S. equisimilis*. Challenge material from frozen culture.
**Experiment 5**

Sixteen 3 week old naturally farrowed pigs were allotted to 2 groups for evaluation of the sonic vaccine. The vaccine was given intramuscularly on days 0, 4 and 8 (Table 16). On day 26, all animals were test bled and challenged by intravenous inoculation of *S. equisimilis*.

Elevated temperatures (2 to 3 F) were recorded 24 to 48 hours postchallenge. Clinical observations of lameness were recorded for 5 days and a lameness index was calculated for each group.

On day 31, the animals were exsanguinated and necropsied. The number of joints with gross lesions and the number of joints containing *S. equisimilis* are tabulated in Table 16 along with the lameness indices.

Serum samples, collected at the time of challenge were analyzed for their *S. equisimilis* antibody titer by complement-fixation. The GM-CF titer for the sonic vaccinates was 192 and for the controls it was less than 10.

**Experiment 6**

Nine 8 week old and five 3 week old CICD pigs were allotted to 3 groups for evaluation of sonic vaccine in swine of two different ages. The vaccine was given intramuscularly on days 0, 4 and 8 (Table 17). On day 26, all animals were test bled and challenged by intravenous inoculation of *S. equisimilis*. 
Elevated temperatures (2 to 4°F) were observed 24 to 72 hours postchallenge. Clinical observations of lameness were recorded for 4 days and a lameness index was calculated for each group.

On day 30, all animals were exsanguinated and necropsied. The number of joints with gross lesions and the number of joints containing *S. equisimilis* are tabulated in Table 17 along with the lameness indices. No significant differences were observed in the response of the two age groups to vaccination or challenge, except for serum antibody.

Serum samples, collected at the time of challenge, were analyzed for their *S. equisimilis* antibody content by complement-fixation. The GM-CF titer for both ages of sonic vaccinates was 160 and for both control groups it was less than 10.

**Location of protective antigen in whole cells and sonic derived vaccines**

**Experiment 7**  
Fifteen 7 week old CDCD pigs were allotted to 4 groups for comparison of unfractionated and 30,000 g supernatant of sonicated cells. The vaccines were given by intramuscular inoculation on days 0, 4 and 8 (Table 18). On day 26, all animals were test bled and challenged by intravenous inoculation of *S. equisimilis*. 
Elevated temperatures (3 to 5 F) were recorded 24 to 72 hours postchallenge. Clinical observations of lameness were recorded for 5 days and a lameness index was calculated for each group.

On day 31, all animals were exsanguinated and necropsied. The number of joints with gross lesions and the number of joints containing *S. equisimilis* are tabulated in Table 18 along with the lameness indices.

Serum samples, collected at the time of challenge, were analyzed for their *S. equisimilis* antibody content by complement-fixation. The GM-CF titer for the sonic vaccinates was 260, for the supernatant vaccinates it was 80 and for both control groups it was less than 10.

**Experiment 8** Thirteen 5 week old CDCD pigs were allotted to 5 groups for comparison of whole cells, cell walls and 30,000 g supernatant of sonicated cells. The vaccines were given by intramuscular inoculation on days 0, 4 and 8 (Table 19). On day 26, all animals were test bled and challenged by intravenous inoculation of *S. equisimilis*.

Elevated temperatures (2 to 4 F) were recorded 24 to 48 hours postchallenge. Clinical observations of lameness were recorded for 4 days and a lameness index was calculated for each group.
On day 30, all animals were exsanguinated and necropsied. The number of joints with gross lesions and the number of joints containing *S. equisimilis* are tabulated in Table 19 along with the lameness indices.

Serum samples, collected at the time of challenge, were analyzed for their *S. equisimilis* antibody content by complement-fixation. The GM-CF titer for whole cell vaccinates was 30, for cell wall vaccinates it was 320, for supernatant vaccinates it was 640 and for both control groups it was less than 10.
Table 16. Experiment 5. Immunogenicity of *S. equisimilis* 78-A3X sonic vaccine in naturally farrowed swine.

<table>
<thead>
<tr>
<th>Group</th>
<th>No. Pigs</th>
<th>Treatment</th>
<th>Lameness Index&lt;sup&gt;a&lt;/sup&gt;</th>
<th>No. Joints With Lesions&lt;sup&gt;b&lt;/sup&gt;</th>
<th>No. Joints Positive for <em>S. equisimilis</em>&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6</td>
<td>Three 2 ml doses of sonicate + incomplete Freund adjuvant I.M., challenged I.V. with 2.9 x 10&lt;sup&gt;6&lt;/sup&gt; CFU-SE&lt;sup&gt;d&lt;/sup&gt;,</td>
<td>0.03</td>
<td>4/72</td>
<td>1/72</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>Three 2 ml doses of placebo + incomplete Freund adjuvant I.M., challenged I.V. with 2.9 x 10&lt;sup&gt;6&lt;/sup&gt; CFU-SE&lt;sup&gt;d&lt;/sup&gt;,</td>
<td>0.03</td>
<td>19/120</td>
<td>3/120</td>
</tr>
</tbody>
</table>

<sup>a</sup>Lameness index=Total number of limbs exhibiting lameness during 5 days postchallenge divided by total observations.

<sup>b</sup>Total number of arthritic joints over total joints observed at necropsy 5 days postchallenge.

<sup>c</sup>Total number of joints containing *S. equisimilis* over total number of joints cultured at necropsy 5 days postchallenge.

<sup>d</sup>Colony forming units of *S. equisimilis* as evaluated by plate count.
Table 17. Experiment 6. Immunogenicity of *S. equisimilis* 78-A3X sonic antigen in swine of different ages.

<table>
<thead>
<tr>
<th>Group</th>
<th>No. Pigs</th>
<th>Age in Weeks</th>
<th>Treatment</th>
<th>Lameness Index&lt;sup&gt;a&lt;/sup&gt;</th>
<th>No. Joints Positive for <em>S. equisimilis</em>&lt;sup&gt;c&lt;/sup&gt;</th>
<th>No. Joints With Lesions&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3</td>
<td>8</td>
<td>Three 2 ml doses of sonicate + incomplete Freund adjuvant I.M. challenged I.V. with $2.4 \times 10^5$ CPU-SE&lt;sup&gt;d&lt;/sup&gt;.</td>
<td>0.06</td>
<td>1/36</td>
<td>3/36</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>8</td>
<td>Three 2 ml doses of placebo + incomplete Freund adjuvant I.M. challenged I.V. with $2.4 \times 10^5$ CPU-SE.</td>
<td>0.04</td>
<td>1/24</td>
<td>2/24</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>8</td>
<td>Three 2 ml doses of placebo + incomplete Freund adjuvant I.M. challenged I.V. with $2.4 \times 10^5$ CPU-SE.</td>
<td>0.22</td>
<td>11/24</td>
<td>12/36</td>
</tr>
<tr>
<td>2</td>
<td>8</td>
<td>8</td>
<td>Three 2 ml doses of placebo + incomplete Freund adjuvant I.M. challenged I.V. with $2.4 \times 10^5$ CPU-SE.</td>
<td>0.25</td>
<td>11/24</td>
<td>6/24</td>
</tr>
</tbody>
</table>

<sup>a</sup>Lameness index=Total number of limbs exhibiting lameness during 4 days post challenge divided by total observations.

<sup>b</sup>Total number of arthritic joints over total joints observed at necropsy 4 days postchallenge.

<sup>c</sup>Total number of joints containing *S. equisimilis* over total number of joints cultured at necropsy 4 days postchallenge.

<sup>d</sup>Colony forming units of *S. equisimilis* as evaluated by plate count.
Table 17. (continued)

<table>
<thead>
<tr>
<th>Group</th>
<th>No. Pigs</th>
<th>Age in Weeks</th>
<th>Treatment</th>
<th>Lameness Index&lt;sup&gt;a&lt;/sup&gt;</th>
<th>No. Joints With Lesions&lt;sup&gt;b&lt;/sup&gt;</th>
<th>No. Joints Positive for &lt;i&gt;S. equisimilis&lt;/i&gt;&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>3</td>
<td>8</td>
<td>Not vaccinated. Not challenged</td>
<td>0.00</td>
<td>0/36</td>
<td>0/36</td>
</tr>
<tr>
<td>1</td>
<td>3</td>
<td>3</td>
<td></td>
<td>0.00</td>
<td>0/12</td>
<td>0/12</td>
</tr>
</tbody>
</table>
Table 18. Experiment 7. Immunogenicity of S. equisimilis 78-A3X sonicated cells and soluble vaccines in swine.

<table>
<thead>
<tr>
<th>Group</th>
<th>No. Pigs</th>
<th>Treatment</th>
<th>Lameness Index^a</th>
<th>No. Joints With Lesions^b</th>
<th>No. Joints Positive for S. equisimilis^c</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3</td>
<td>Three 2 ml doses of sonicate + incomplete Freund adjuvant I.M. challenged I.V. with $2.4 \times 10^6$ CPU-SE^d</td>
<td>0.19</td>
<td>8/36</td>
<td>0/36</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>Three 2 ml doses of soluble^e + incomplete Freund adjuvant I.M. challenged I.V. with $2.4 \times 10^6$ CPU-SE</td>
<td>0.23</td>
<td>10/60</td>
<td>5/60</td>
</tr>
</tbody>
</table>

^aLameness index=Total number of limbs exhibiting lameness during 4 days post challenge divided by total observations.

^bTotal number of arthritic joints over total joints observed at necropsy 4 days postchallenge.

^cTotal number of joints containing S. equisimilis over total number of joints cultured at necropsy 4 days postchallenge.

^dColony forming units of S. equisimilis as evaluated by plate count.

^eSupernatant from 30,000 g centrifuged sonicated cells.
<table>
<thead>
<tr>
<th>Group</th>
<th>No. Pigs</th>
<th>Treatment</th>
<th>Lameness Index (^a)</th>
<th>No. Joints Positive for (S.\ equisimilis) (^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>5</td>
<td>Three 2 ml doses of placebo + incomplete Freund adjuvant I.M. challenged I.V. with (2.4 \times 10^6) CFU-SE.</td>
<td>0.48</td>
<td>29/60</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>Not vaccinated Not challenged</td>
<td>0.00</td>
<td>0/24</td>
</tr>
</tbody>
</table>

\(^a\) Lameness Index
\(^b\) No. Joints Positive for \(S.\ equisimilis\)
\(^c\)
Table 19. Experiment 8. Immunogenicity of *S. equisimilis* 78-A3X whole cell, cell wall and soluble vaccines in swine.

<table>
<thead>
<tr>
<th>Group</th>
<th>No. Pigs</th>
<th>Treatment</th>
<th>Lameness Index&lt;sup&gt;a&lt;/sup&gt;</th>
<th>No. Joints With Lesions&lt;sup&gt;b&lt;/sup&gt;</th>
<th>No. Joints Positive for <em>S. equisimilis</em>&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3</td>
<td>Three 2 ml doses of whole cell + incomplete Freund adjuvant I.M. challenged I.V. with 2.5 x 10&lt;sup&gt;8&lt;/sup&gt; CFU-SE&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.31</td>
<td>11/36</td>
<td>7/36</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>Three 2 ml doses of cell wall + incomplete Freund adjuvant I.M. challenged I.V. with 2.5 x 10&lt;sup&gt;8&lt;/sup&gt; CFU-SE&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.21</td>
<td>7/36</td>
<td>5/36</td>
</tr>
</tbody>
</table>

<sup>a</sup>Lameness index=Total number of limbs exhibiting lameness during 4 days post challenge divided by total observations.

<sup>b</sup>Total number of arthritic joints over total joints observed at necropsy 4 days postchallenge.

<sup>c</sup>Total number of joints containing *S. equisimilis* over total number of joints cultured at necropsy 4 days postchallenge.

<sup>d</sup>Colony forming units of *S. equisimilis* as evaluated by plate count.
Table 19. (continued)

<table>
<thead>
<tr>
<th>Group</th>
<th>No. Pigs</th>
<th>Treatment</th>
<th>Lameness Index&lt;sup&gt;a&lt;/sup&gt;</th>
<th>No. Joints With Lesions&lt;sup&gt;b&lt;/sup&gt;</th>
<th>No. Joints Positive for &lt;i&gt;S. equisimilis&lt;/i&gt;&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>3</td>
<td>Three 2 ml doses of soluble® + incomplete Freund adjuvant I.M. challenged I.V. with 2.5 x 10&lt;sup&gt;6&lt;/sup&gt; CFU-SE.</td>
<td>0.15</td>
<td>5/36</td>
<td>2/36</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>Three 2 ml doses of placebo + incomplete Freund adjuvant I.M. challenged I.V. with 2.5 x 10&lt;sup&gt;6&lt;/sup&gt; CFU-SE.</td>
<td>0.16</td>
<td>13/24</td>
<td>16/24</td>
</tr>
<tr>
<td>5</td>
<td>2</td>
<td>Not vaccinated Not challenged</td>
<td>0.00</td>
<td>0/24</td>
<td>0/24</td>
</tr>
</tbody>
</table>

<sup>a</sup>Supernatant from 30,000 g centrifuged sonicated cells.
DISCUSSION

Two major advances in streptococcosis have resulted from this study: a serological test has been developed for use in evaluation of the streptococcal antibody response in swine and it has been demonstrated that protection against *S. equisimilis* results in swine following intramuscular injection of vaccine prepared from the organism. During development of the serologic test and the vaccine, additional information concerning the antigenic properties of *S. equisimilis* was accumulated.

The cultural and biochemical characteristics of all *S. equisimilis* strains used in this study were typical for the organism (96). All strains were Lancefield Group C and fermented trehalose and glycerol but not sorbitol. The serotype specificity of both stock and working cultures was confirmed as reported previously (245, 246).

Investigators have demonstrated that antigenicity and virulence of Group A streptococci can be enhanced by intraperitoneal passage of the cocci through mice (28, 29). This treatment generally resulted in an increase in the quantity of M-protein present on the cell and an increase in virulence for mice. Whether or not the increased M antigen content is directly responsible for increased virulence has not been established.
Virulence and antigenicity of *S. equi* have been related to the presence of an M-protein-like substance found in the capsule (512). This substance is directly related to the age of the culture. Young cultures commonly contain a relatively large capsule with an abundance of the substance, while older cultures have lost the majority of this capsule and consequently the majority of the M-like substance.

Utilizing these general concepts, a serotype I strain of *S. equisimilis* was passed 3 times through young pigs. The reisolated strain demonstrated an increase in virulence for both swine and mice (Tables 2 and 3). The LD$_{50}$ for mice and the arthritic dose 50 for swine were much lower for the passaged strain than the parent. The reasons for the enhanced virulence were not determined, although apparently it was not directly related to antigenicity since both strains elicited similar levels of streptococcal antibody in swine and rabbits. Serial passage of the strain may have increased the amount of type-specific antigen and thus virulence, or through animal-passage, the strain simply became better adapted for swine and mice by slight physical and biochemical changes. The capsule of 78-A3X was slightly larger than that of the parent strain.\(^1\) Regardless of

the reasons, a 1:100 dilution of a 6 hour broth culture consistently induced arthritis in young, non-vaccinated CDCD swine and death in non-vaccinated mice.

In experiment 5, naturally farrowed pigs were challenged with $2.9 \times 10^6$ CFU of *S. equisimilis* according to procedures described previously, but they developed less severe arthritis than was commonly observed when CDCD animals were challenged. The reason for this difference was not investigated. However, it is quite possible that the pigs may have acquired low levels of antistreptococcal antibodies from the sows milk or they may have received a natural exposure and developed some protective antibodies. As reported previously (426), *S. equisimilis* has frequently been isolated from the reproductive tract and mammary glands of sows. In order to evaluate streptococcal vaccines in naturally farrowed pigs, it may be necessary to increase the challenge dose.

Immunity to group A streptococcal infection in man has been shown to be at least partially type-specific (268). The *in vitro* bactericidal test has been one of the most reliable methods for serological assessment of this immunity (383). Bactericidal results obtained in preliminary work with *S. equisimilis* and swine serum were difficult
to evaluate and inconsistent; thus, other test methods were devised to follow the serological response in swine. Bone et al. (43) used crude acid extracts to detect CF antibody in serum from glomerulonephritic patients that also demonstrated type-specific bacteriostatic activity. Recently, Wittner and Fox (507) developed a micro-CF test for detection of type-specific antibody in serum of patients with Group A infection and presented evidence that the test could be substituted for the bactericidal test as a measure of protective immunity. Using a highly purified hot acid extract, they demonstrated a close correlation between CF antibody and bactericidal activity.

A complement-fixation procedure was developed for use in the study of S. equisimilis. The test was utilized in comparisons of the serological response to various S. equi-similis antigens in swine and rabbits (Table 6). The application of this procedure for detection of antibodies in serum from naturally or experimentally infected swine or rabbits has not been completely investigated. The results obtained using crude and type-specific antigen were encouraging, although no direct correlation was established between the level of humoral antibody and protection.

In the initial work, crude hot acid and sonicated whole cell extracts were used in CF and IHA procedures to demonstrate a serological response in vaccinated swine and rabbits. Because of an apparent lack of specificity, it was necessary to purify the antigens. Following purification of the preparations, it appeared that the principal antigens in the crude extracts involved in the CF test were identical to the type-specific antigen. Evaluations of the role of purified type-specific antigen in IHA were not done.

Evidence has been presented that sonic disruption of streptococcal cells results in liberation of streptococcal M-protein in a more natural form (35) than is obtained with hot acid extraction. The native protein is apparently hydrolyzed during the acid extraction procedure resulting in alteration of its physicochemical form and antigenicity. Extraction under alkaline conditions results in less alteration than occurs with the hot acid extraction procedure (152). Use of these various extraction procedures has apparently resulted in comparable yields of type-specific antigen from Group A streptococci (35). However, sonication of S. equisimilis resulted in a much higher yield of antigen active in the CF test than did hot acid extraction (Table 8). Possibly the type-specific antigen of S. equisimilis is partially destroyed under acidic conditions.
Purification of type-specific antigen from crude sonicate with CMC required a variation in dialysis before application of the column. Dialysis against phosphate buffered saline containing potassium salts resulted in an antigen which became bound to the CMC. Dialysis against the PBS with sodium salts produced a sonicate antigen that behaved similarly to the hot acid extract, although, there still appeared to be some retention of antigen. The reason for this variation was not investigated.

Analysis by ID revealed no difference between the SD 30,000 g and SD 3,000 g preparations of crude sonicate; both preparations contained the group and type-specific components (Figure 2). Evidence obtained by starch block electrophoresis and molecular sieving indicated that the type-specific component in crude sonicate was not of a single species, but rather a composite with different densities and molecular weights all of which appeared to react similarly with the type specific antiserum. The ID patterns suggest this antigen may have a multiple molecular structure similar to the type-specific antigen found in Group A (150). Using a combination of differential centrifugation, ammonium sulfate precipitation and ion-exchange chromatography, it was possible to obtain from either hot acid extracts or crude sonicate a preparation that contained a
single band detectable by immunodiffusion (Figure 9). Electrophoresis of this preparation in acrylamide gel revealed two bands.

As indicated in Table 5, some evidence of serotype specificity was noted in hyperimmune rabbit serums by CF with crude sonicate or hot acid extracts as antigens. There were numerous cross reactions between the various serotypes when non-absorbed antiserums were used. These cross reactions may be attributed to the variety of antigenic components present on or in the whole cells used during the rabbit immunization procedures. Specificity of the type-specific antigen was evaluated using rabbit antiserum against all four known swine serotypes. As indicated in Figure 9, the only visible band was observed between type-specific antigen and its homologous antiserum whereas crude preparations contained at least 3 separate antigens (Figure 8). Serological specificity of the type-specific antigen was evaluated by use of the CF test. Table 7 presents a comparison of crude hot acid extract and purified antigen evaluated against the same four serotypes. Using crude antigen, a 4 fold difference was detected between the two serotypes (Table 5), whereas type-specific antigen demonstrated a strong homologous reaction but failed to detect any complement-fixing antibodies in
heterologous antiserum (Table 7). Thus, from the original crude hot acid or sonic extracts, an antigen was separated which reacted with its homologous serotype in complement-fixation and ID procedures; however, the antigen did not react with either type-specific or non-adsorbed serum from heterologous serotypes of *S. equisimilis* suggesting that it was type-specific. It appears that the CF test is primarily detecting type-specific antibody with homologous antiserum.

Fractionation of serum from vaccinated swine and rabbits by chromatography on Sephadex G-200 revealed that the serums contained the usual protein components. Serological evaluation of pooled samples of peak 1 (IgM) and peak 2 (IgG) demonstrated that antibodies against *S. equisimilis* were present in both fractions (Table 10); a situation analogous to findings reported for immune Group A serum (517). Most of the IHA activity of swine serum was present in the IgM fraction, whereas the IgG fraction contained most of the CF activity. These findings are consistent with previous reports concerning the activity of these two immunoglobulin classes. No attempts were made to determine the role of antibodies in the two immunoglobulin classes in protection.

The type-specific component was not separated from the group polysaccharide by starch block electrophoresis
Protection tests in mice indicated that the protective antigen was concentrated near the point of application (Figure 3) and that it was associated with samples containing type-specific antigen. As listed in Table 9, protection was observed in some vaccinated mice that did not receive type-specific antigen, although this test was not confirmed. However, protection tests using purified type-specific antigen confirmed the protective effect of type-specific antigen in mice (Table 11). This antigen was not evaluated in swine.

*S. equisimilis* has been reported to be a very poor immunity producer, as are most streptococci (114, 492). Adjuvants have been used with many different antigens to improve their immunogenicity and consequently an adjuvant was used in this study. Incomplete Freund mineral oil adjuvant was selected because it produces an optimum effect (159, 510). However, this type of adjuvant could not be utilized in animals used for human food, consequently another adjuvant would be required. There are several commercially available adjuvants that could be evaluated. Possibly, the streptococcal antigen could be incorporated into one of the presently licensed swine mixed bacterins.
The use of whole cell vaccines for control of streptococcal infection in man and animals has been reported previously (22, 154). In animals, immunogenicity appears to be related directly to the animal used and not to the bacterin preparation. Bazeley reported that vaccination of equine foals with heat-killed, young cultures of *S. equi* induced a protective antibody response similar to that conferred by recovery from the natural disease (22).

In bovine streptococcal mastitis, there is no preparation effective in producing complete immunity against *S. agalactiae*. Lancefield and Freimer (279) discussed two type-specific antigens that they felt were related to protection, although animals vaccinated with these antigens were not protected against an experimental challenge. Considerable progress has been made recently in immunity to streptococcal mastitis by the adoption of a passive mouse protection test for the assay of protective antibodies in bovine milk and serum (336). With this test it is possible to compare in the bovine, the results of vaccination with certain antigenic preparations, as well as the effect of the route of inoculation and the duration of immunity. Norcross (335) reported that inoculation of $1 \times 10^{12}$ formalin inactivated whole cells in the area of the supramammary lymph nodes elicited milk antibodies of the
immunoglobulin classes IgM, IgG and IgA. The IgG antibody has been shown to be protective and probably functions at the site of infection to reduce infection and inflammation. Norcross has vaccinated a cow with a polyvalent bacterin which contained all 5 type-specific antigens, and demonstrated by use of the passive mouse protection test that the animal produced circulating protective antibodies to each type. The duration of the heightened resistance was not established.

In swine, whole cell bacterins have been used in attempts to control streptococcal lymphadenitis (SLS), uterine infections and deaths attributed to beta-hemolytic streptococci (195, 200, 429). None of the bacterins used to control SLS were able to significantly reduce the prevalence of the disease and consequently a living avirulent strain was developed to control SLS. In the case of the other two diseases, the whole cell bacterins were reported to be much more successful. One of the basic reasons for the success appeared to be related to repeated inoculations of the bacterins.

In view of the success of streptococcal whole cell bacterins in the control of equine strangles and beta hemolytic streptococcal infections in swine, attempts to develop a S. equisimilis whole cell bacterin were initiated.
Evidence from mouse protection tests indicated that the protective effect was not related to the inactivating agent (Table 11). Thus, a formalin inactivated culture, which was mixed with incomplete Freund adjuvant, was inoculated intramuscularly into swine in 2 doses in an attempt to modify or prevent an artificially induced *S. equisimilis* infection. Following challenge, no protection was demonstrated in the vaccinates when compared to the response of non-vaccinated controls.

Swine receiving 2 doses of whole cell vaccine commonly failed to develop sufficient antibody to afford protection against challenge. The need for multiple injections of streptococcal cells or their components to produce high titer antibodies in man and animals has been demonstrated (154, 295). Bazeley (22) and Engelbrecht (134) found that 3 doses of *S. equi* vaccine were necessary before complete immunity developed in horses. Similar results have been obtained during vaccination attempts in humans with Group A streptococcal extracts. Hare *et al.* (195) used 6 doses of whole cell bacterin to control deaths attributed to beta hemolytic streptococci.

In the present study, a sequential rise in the antibody titer occurred following multiple streptococcal injections. In experiment 1, the animals were vaccinated with three doses of either whole cell or sonic vaccine.
Animals that received whole cell vaccine developed low levels of CF streptococcal antibody in their serums. Following challenge, the vaccinates had less clinical lameness and fewer joints with lesions and *S. equisimilis* than non-vaccinated controls. However, animals that received sonic vaccine had even higher levels of streptococcal antibody and less clinical lameness, joints lesions and joints with *S. equisimilis* than did the whole cell vaccinates. Although a low level of protection was induced in swine by the whole cell vaccine, greater protection was induced by the sonic vaccine.

Shortly after completion of experiment 1, biochemical and serological evaluations of the swine passaged strain 78-A3X, were completed. This strain was used in a protective challenge experiment conducted according to protocol of experiment 1. Following challenge, the clinical picture and necropsy results were similar to those observed in experiment 1. Again, the sonic vaccinates appeared to have higher levels of serum antibody and were more resistant to challenge.

Results obtained in this study with whole cell vaccines are similar to those obtained in experimental trials in humans with whole cell bacterins. No specific reasons have been given for the failure of whole cell vaccines to protect humans, although, one of the predominant reasons
appears to be that the type-specific antigen is only a minor cellular component of the total antigenic complex and antibodies against it are either blocked or slow in being produced. It is quite possible that an analogous situation may exist with *S. equisimilis* since the type-specific antigen is also related to the cell wall (246). Group A whole cell vaccines frequently result in the appearance of untoward reactions in the human vaccinees. No such reactions were noted in any of the swine vaccinated with 2 or 3 doses of *S. equisimilis* whole cell vaccine.

Antigen extracted from Group A streptococcal cells by the hot acid method is the most commonly used material for experimental human streptococcal vaccine (153). Highly purified hot acid extracted M-protein preparations, precipitated with alum and given in a minimum of 3 subcutaneous inoculations 4 weeks apart to human volunteers, induces type-specific antibody in about 75 per cent of vaccinees (154). A protective test in humans indicated that purified M-protein induced type-specific anti-M-antibodies that were able to afford the vaccinees some clinical protection against virulent challenge.

Experiment 3 was designed to evaluate hot acid extract as a potential vaccine for swine and confirm the protective effect induced by a sonic vaccine. The hot acid
extract was prepared according to methods developed for Group A vaccine (154) except it was not purified by CMC. No untoward reactions were observed in any of the vaccinates before challenge and serological evaluations demonstrated relatively high GM-CF titers. Following challenge, protective antibodies were present in vaccinated swine as compared to the response of non-vaccinates. Sonic vaccinates in this trial appeared to have protective levels similar to the hot acid extract vaccinates. Since 3 doses of sonic vaccine appeared to elicit similar protection, it was decided to pursue this type of vaccine.

Alkaline extracts of *S. equisimilis* were evaluated in the mouse protection test (Table 11) and 45 per cent of the mice vaccinated with 2 doses failed to survive challenge. The material was never used in swine as a vaccine because of its low protective effect in mice and it has never been used in human vaccine trials. Of course, mouse protection tests may or may not be a reliable indicator of potential for use in swine. Nevertheless, the mouse test was used in this work to give some preliminary evidence of protective immunogenicity. If any antigen elicited relatively high levels of protection in mice, the material was used to vaccinate swine in a protective challenge study. Whole cell vaccines were less efficacious in mice as well as in swine.
Sonic vibration was found to be an effective means of releasing a variety of antigenic components from *S. equisimilis*. This method disrupted approximately 100 per cent of the cells as determined by microscopic and cultural examinations. Sonicated fractions proved to be antigenic *in vivo* and *in vitro*. Chemical fractionation of bacteria, such as acid extractions, has a tendency to destroy important antigens while sonication apparently does not produce a similar effect.

During development of the CF test it was demonstrated that sonic released antigen was more potent as a CF antigen than hot acid extract on a per cell basis (Table 7). Besdine and Pine (35) reported sonic vibration of heat killed group A streptococci released high molecular weight M-antigen. They reported that sonicated whole cell extract had 4 components by ID: a high molecular weight M-antigen, the group polysaccharide, T antigen and another unidentified antigen. They concluded that this preparation was more antigenic than the low weight material, although as mentioned previously, this preparation has not been used in human vaccine trials.

In experiment 4 immunogenicity of two doses of *S. equisimilis* 78-A sonicate for swine was evaluated. Following challenge with a frozen culture, the sonic vaccinates demonstrated protection against challenge. Few isolates of *S. equisimilis* were made in either group. It appeared that
2 doses of sonic vaccine were able to elicit protective antibodies in this experiment as evaluated by challenge with culture that had been frozen. This study was not repeated with the swine passed strain or with a fresh challenge. This is an area that needs to be investigated further. Use of the frozen challenge appeared to be less severe that that observed in challenge experiments where swine-passaged or fresh culture were used.

In experiment 5 immunogenicity of sonic vaccine in naturally farrowed animals was evaluated. The vaccine induced CF serum antibody levels comparable to that in CDCD swine. However, it is not possible to draw any firm conclusions based on this challenge study. Both vaccinates and controls developed a less severe arthritis than was commonly observed when CDCD animals were challenged. The arthritic dose 50 of S. equisimilis and the immunogenicity of sonic extract for naturally farrowed swine should be determined. Obviously, a vaccine would be used primarily in naturally farrowed swine in the field.

Results of experiment 6 indicated that 3 and 8 week old swine responded similarly to 78-AJX sonic vaccine. Following challenge, no appreciable differences were observed between the 2 groups. Protection was observed in the sonic vaccinates but not in controls.
In experiments 7 and 8 attempts were initiated to determine the location of the protective antigen in crude preparations. Relatively high GM-CF titers were demonstrated in swine vaccinated with sonic and soluble vaccines while the whole cell vaccinates were low. Greater protection occurred in animals that received soluble or particulate vaccines than those animals that received the whole cell or placebo vaccine. A severe response to challenge occurred in these tests. Clinical observations of lameness were almost identical for vaccinates and controls, although other parameters indicated limited protection occurred in most vaccinates. Based on these 2 experiments and the mouse test, evidence was suggestive, not conclusive, that the protective antigen was associated with the soluble components. Evidence obtained from the mouse protection tests indicated that the type-specific antigen was related to protection.

Experimental findings indicated that sonic derived S. equisimilis vaccine was immunogenic in swine from 3 to 8 weeks of age as evidenced by the induction of high levels of circulating CF antibodies following 3 intramuscular injections of the vaccine. There appears to be a correlation between the presence of these antibodies and protection since most of those animals demonstrating high CF antibody levels failed to develop clinical and necropsy evidence
of arthritis following challenge. However, the vaccine was not 100 per cent effective since vaccinates occasionally had symptoms of arthritis following challenge. The CF test was useful for determining the immune status of groups of swine but correlated poorly with the degree of protection in individual animals.

Passive immunity has been used in studies related to Group A organisms. Serums taken from adult swine or pigs 5 weeks after an experimental inoculation with a Group D strain (5, 6) prevented infection by a homologous strain. Administration of serum taken from swine hyperimmunized against *S. equisimilis* to susceptible baby pigs failed to protect them against a challenge dose of *S. equisimilis*. Challenge studies were not encouraging and this method of investigation was not pursued.¹

Evidence was obtained that colostral antibody against *S. equisimilis* could be induced in sows which would protect baby pigs against a challenge dose of *S. equisimilis*.² Administration of 2 or 3 doses of *S. equisimilis* sonic vaccine, by intramuscular or intramammary routes, resulted


in 2 to 8 fold increases in serum streptococcal antibody titers. Some evidence of protection (reduced clinical lameness, joint lesions and joints containing S. equisimilis) was detected in pigs nursing vaccinated sows. Colostral protection appeared to be better when sows were vaccinated with sonic vaccine combined with incomplete Freund adjuvant.

In comparing whole cells, hot acid and sonic derived extracts as potential vaccines, crude sonic extract in combination with an adjuvant, appears to be the best overall preparation for use in swine. Whole cell bacterins simply were not immunogenic enough to protect large numbers of animals. Hot acid extracts appeared to be relatively immunogenic, but a relatively large volume of cells was required in order to produce any quantity of vaccine and the cost could be prohibitive. The sonic extract thus appears to be the most practical material. The major disadvantage with this type of vaccine is that incomplete cell disruption followed by low speed centrifugation may leave viable organisms in the vaccine and these then could be inoculated into susceptible animals with the possibility of spreading the disease, although this problem would be eliminated by the addition of a chemical preservative to the vaccine before it is used in the field. Crude preparations of streptococcal cells have been shown to contain a wide variety of antigens, some of which are detrimental
in humans. Because Group A and Group C cells have similar properties, it is quite possible that an analogous situation may exist with *S. equisimilis*. Thus, the use of purified antigens would eliminate many of the possible undesirable factors associated with relatively crude preparations.
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