



Immunogenic studies of *Clostridium sordellii*  
by Miles Huxtable Bairey

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of  
MASTER OF SCIENCE in Microbiology  
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Abstract:

An investigation of the immunogenic ability of *C. sordellii* was done by vaccinating mice with potential immunizing products and challenging them with lethal doses of *C. sordellii* toxin and spores. The immunizing products used were toxoids, spore bacterins, vegetative cell bacterins and whole culture bacterins, all of which were prepared by first determining how optimal yield), could be produced and then inactivating the preparations with formalin. Commercially prepared aluminum hydroxide precipitated whole culture bacterins were also incorporated into the experiments. Sublethal doses of a toxin preparation were injected into mice and challenged with serial dilutions of lethal toxin.

No animal protection to *C. sordellii* toxin or spores was observed with any of the formalinized products. Protection of mice to low levels of toxin challenge was noted with sublethal toxin injections. Indications of dissociation of the antigen-antibody complex produced by sublethal toxin vaccinations were observed 24 to 48 hours post challenge.

It was concluded from this study that the toxin produced by *C. sordellii* was the immunizing antigen but when inactivated with formalin, the toxin molecule no longer produced an immunogenic response. It was also concluded that antibody production stimulated by *C. sordellii*, toxin was of low avidity.

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## ABSTRACT

An investigation of the immunogenic ability of C. sordellii was done by vaccinating mice with potential immunizing products and challenging them with lethal doses of C. sordellii toxin and spores. The immunizing products used were toxoids, spore bacterins, vegetative cell bacterins and whole culture bacterins, all of which were prepared by first determining how optimal yield could be produced and then inactivating the preparations with formalin. Commercially prepared aluminum hydroxide precipitated whole culture bacterins were also incorporated into the experiments. Sublethal doses of a toxin preparation were injected into mice and challenged with serial dilutions of lethal toxin.

No animal protection to C. sordellii toxin or spores was observed with any of the formalinized products. Protection of mice to low levels of toxin challenge was noted with sublethal toxin injections. Indications of dissociation of the antigen-antibody complex produced by sublethal toxin vaccinations were observed 24 to 48 hours post challenge.

It was concluded from this study that the toxin produced by C. sordellii was the immunizing antigen but when inactivated with formalin, the toxin molecule no longer produced an immunogenic response. It was also concluded that antibody production stimulated by C. sordellii toxin was of low avidity.



## INTRODUCTION

Clostridium sordellii was first isolated from a fatal wound infection in man (Sordelli, 1927). It was later isolated in conjunction with other clostridial infections of cattle (Hall, 1927) and Vawter isolated C. sordellii from dead sheep in 1942. Sordelli (1922) named the organism Bacillus oedematis sporogenes, and a similar organism was isolated and named Clostridium oedematoides by Meloney, Humphreys and Carp (1927). The isolated bacteria of Sordelli (1922) and Meloney et al (1927) were found to be identical (Humphreys and Meloney, 1928; Hall, Rymer and Jungherr, 1929) and the name Bacillus sordellii was proposed (Hall and Scott, 1927) to conform with the nomenclature used at that time.

Most work done on C. sordellii relates to morphologic and physiologic considerations in relation to other closely related bacteria, and little has been done on problems relating to antigenicity, immunity, characteristics of toxins, and mode of transmission. Although there are products on the market containing C. sordellii, there are no published data about their efficacy for producing immunity.

This investigation was designed to study the immunizing antigens of C. sordellii and to estimate the possible protection that can be expected from these antigens. Included in this work are the immunizing effects of toxoids, spore bacterins, vegetative cell bacterins, whole culture bacterins and sublethal doses of toxin.

In preparing the present manuscript several studies about taxonomic differentiation and identifications of C. sordellii and C. bifermentans were cited, but this thesis is not a taxonomic study.

## REVIEW OF LITERATURE

In 1922, a French surgeon, A. Sordelli, observed an acute fatal edematous wound infection in a human postoperative patient in South America. From this wound he isolated a gram positive, spore producing anaerobic organism. He found that filtrates of supernatant culture fluids were highly toxic to guinea pigs and rabbits and that fluids could not be neutralized by antisera prepared against organisms known at that time to produce gas gangrene. He gave this isolated organism the name Bacillus oedematis sporogenes.

Meleney, Humphreys, and Carp (1927) described a similar organism isolated from a postoperative wound infection and from catgut suture material. They produced an antitoxin in rabbits by injecting sublethal doses of 0.05 ml of toxic culture filtrate. This antitoxin would protect white mice against a 0.45 ml subcutaneous (S.C.) injection of toxin produced by this organism. Since they did not recognize that their isolate was identical to the organism isolated by Sordelli, they called their isolate Clostridium oedematoides.

During this same period of time, Hall and Scott (1927) studied Sordelli's isolate, performed cultural and pathological studies, renamed the isolate Bacillus sordelli, concluded that the lethal factor was a potent exotoxin and confirmed that Sordelli isolated a new species.

The identity of these 2 isolates then became the subject for additional studies. Humphreys and Meleney (1928) reported that

Clostridium oedematoides and B. sordellii were one and the same species as determined by cross neutralization tests in mice. Their neutralization was done using rabbit antiserum for each culture. Hall, Rymer and Jungherr (1929) confirmed the results of Humphreys and Meleney, but used antiserum prepared by injecting both whole culture material and a sterile filtered toxin S.C. into rabbits. This was the first description of the antigenicity of the toxin. At this time, Hall (1929) was working with some cultures that he had received from Vawter in Nevada. Vawter had collected these isolates in 1919 and 1921 from cattle that demonstrated the signs of bacillary hemoglobinuria. Hall concluded that these isolates were serologically different from Clostridium hemolyticum but were identical to Sordelli's isolate from South America and Humphreys, Meleney, and Carp's isolate from New York.

Subsequently little work was done with this organism until Clark and Hall (1937) in a comparative study of Bacillus bifermentans and Bacillus centrosporogenes, the latter organism being described by Hall in 1922, included B. sordellii in some additional comparison work of closely related proteolytic anaerobes. They concluded after extensive morphological, cultural, physiological and serological work, that they could not find sufficient evidence to separate these 2 species. This was in agreement with the work of McCoy and McClung (1936) who reported that the H and O factors of the antigenic complex of 2 strains of Clostridium bifermentans and one strain of Clostridium centrosporogenes were identical by reciprocal agglutination absorption experiments. The latter workers concluded that

the 2 species could be combined into a single species, C. bifermentans. While Clark and Hall (1937) described B. bifermentans as a distinctly different species from Bacillus sporogenes, Bacillus tyrosinogenes, Bacillus subterminalis, and Bacillus flabelliferum, they could find no distinct cultural or morphological difference between B. bifermentans, and B. sordellii. But because B. sordellii was pathogenic and B. bifermentans was not, they did not feel that they should be consolidated. However, they did observe an antigenic relationship between these 2 organisms in a weak cross-agglutination reaction. Serum from some rabbits that had been immunized against B. bifermentans produced a passive immunity in guinea pigs challenged against either whole cultures or toxic filtrates of B. sordellii. To confirm their findings they actively immunized rabbits against B. bifermentans, B. sporogenes and B. subterminalis and challenged them with B. sordellii culture. They observed that animals vaccinated against B. bifermentans would withstand 5 to 10 challenge doses more than those animals vaccinated with B. sporogenes, B. subterminalis or those that were retained as unvaccinated controls.

While standardizing Clostridium sordellii antitoxin at the National Institutes of Health in 1938, Stewart observed a relationship between C. sordellii and C. bifermentans. She prepared a dried toxin and a dried antitoxin of C. sordellii, and a serum free of antitoxin of C. bifermentans. She observed that antisera produced against C. bifermentans would protect the mice against intravenous (I.V.) challenge with 2 to 5 minimal lethal

doses (M.L.D.) toxin. In further studies Stewart learned that the 2 strains possessed identical morphology, colony formation and biochemical reactions, as well as cross-agglutination and cross-precipitation reactions. She concluded from her studies that the 2 species were identical and should be combined under the name of C. bifermentans.

Lillie (1938) reported on the gross and histological pathology produced by artificially infecting mice and other laboratory animals and comparing them to observations in natural infections in man.

Prevot and Cordier (1941) reported that the toxin of C. sordellii was the third most potent toxin produced by a member of the genus Clostridium. They observed that Clostridium botulinum was 20 times more lethal and Clostridium tetani was 4 times more lethal than C. sordellii. Included in this work was a study on antigens shared by C. sordellii and C. bifermentans. They concluded that there were enough differences to separate the 2 organisms by species.

Field isolations of 6 cultures of C. sordellii were reported by Vawter (1942) in Nevada. Of these six, 4 were isolated from cattle along with other Clostridia, one was from a post-vaccinated calf and one was from a sheep; the latter was reported as being non-pathogenic. After studying the cultural, biochemical and serological properties of these isolated organisms, Vawter (1942) concluded that C. sordellii produced a potent moderately stable, filterable toxin in culture fluids. He also reported that the toxin could be inactivated in 20 min at 70 C and would become "anatoxic" when exposed to 0.25% formalin for 2 days at 37.5 C.

He also stated that: "the formalinized cultures were antigenic". He found that filtrates of culture or culture fluids were toxic to horses and that antitoxin could be produced in either rabbits or horses by injecting formalinized cultures. In the cross-agglutination work that was done with these strains, it was seen that C. sordellii was antigenically "monovalent". Cross-agglutination occurred not only with these animal strains but also with the human strains that had been isolated in South America. It was also observed that sporulating forms could withstand heating at 90 C and freezing at -70 C as well as dehydration for several years.

A report similar to Vawter's (1942) was made by Pesce-De-Fagonde and Marti-Vidal in 1967 when they isolated C. sordellii from cattle in Argentina that died of icterohemoglobinuria. This bacterium was isolated along with C. hemolyticum. Fasciola hepatica infestation of the liver was also observed.

Other isolations of C. sordellii were reported by Vawter and Smith (1963) in cattle of California feedlots. They reported that these infections appeared to be predisposed to by stress factors such as change in temperature, dust and upper respiratory infection.

The work of Vawter (1942) was supported, as was the work of Stewart (1938), by Michael and McClung (1947) when the latter did extensive studies on the flagellar and somatic agglutination of a number of strains of C. bifermentans and of C. sordellii isolated by Hall (1929), Sordelli (1922)

and Meleney et al (1927). They reported that all strains of both species were serologically identical.

Miles and Miles (1947) confirmed the reports of Clark and Hall (1937) and of Stewart (1938) that C. sordellii, C. centrosporogenes, and C. bifermentans were identical and that C. sordellii was but a toxigenic variety. However, after preparing an anti-lecithinase to C. bifermentans they found that this serum would neutralize neither the lecithinase nor the lethal toxin of C. sordellii.

A method for extraction of the C. sordellii toxin from cells was described by Saissac, Renee, and Raynaud (1951). Using this method they reported that the amount of toxin that can be extracted from the cell is of the same magnitude as that found in the broth when grown in a fluid medium. They concluded that this could not be attributed to traces of toxic broth that might be retained by the cells, and that the toxin must be located either inside the cell or on its surface.

Tardieux and Nisman (1952) studied the immunochemical relationship of these 2 organisms. They extracted the polysaccharide fractions from the cells and reacted them with specific antiserum. They found that when subjected to either the "agglutination test" or the precipitation test the polysaccharide fractions would react to high dilutions with homologous antisera. When "cross-agglutination tests" between the 2 species were attempted, there appeared to be no cross-reaction. When gel-diffusion precipitation tests were done against both homologous and heterologous serums, it was found that C. sordellii antiserum would

precipitate the polysaccharide fraction from the cells of both C. sordellii and C. bifermentans whereas C. bifermentans antiserum would precipitate only the homologous extract. They concluded that while there is an immunological relationship between the 2 organisms, they are nevertheless antigenically distinct.

Huet and Aladone first used the urease test in 1952 by testing 200 anaerobic cultures, these represented 176 species of 26 genera. They reported that only 2 species gave an immediate reaction, C. sordellii and Neisseria vulvovaginitis. This produced a differentiating tool which could be readily utilized by workers attempting to classify C. sordellii and C. bifermentans.

Tataki and Huet (1953) used all of the cultures of C. sordellii and C. bifermentans that were in the collection of the Anaerobic Service of the Pasteur Institute in urease studies, and reported that all their cultures of C. sordellii were urease positive. On the other hand, some of the cultures that had been classified as B. bifermentans, because of non-pathogenicity, were also urease positive. Upon submitting these cultures to agglutination reactions they observed that anti-C. sordellii serum would agglutinate not only suspensions of C. sordellii bacteria but also the urease positive strains of C. bifermentans. They also observed that this serum would agglutinate only weakly and intermittently the urease negative strains. However, they also observed that the anti-C. bifermentans serum reacted about the same with all strains indicating that while there were C. sordellii specific antigens, there was also some antigenic similarity.



These authors postulated that since the species had previously been differentiated by toxigenicity only, some reports of cross-serological reactions could have been due to the use of non-pathogenic strains of C. sordellii.

Smith (1955) in a review of the species considered that the serological similarities between the 2 organisms were such that C. sordellii should be considered as a pathogenic variety of C. bifermentans.

Brooks and Sterne (1956) reported isolating an organism that resembled C. sordellii culturally and biochemically from the intestinal contents of a calf dead from enterotoxemia. When filtered intestinal contents were injected into guinea pigs, the gross pathology was also similar to that found with C. sordellii. The toxin isolated could be neutralized with both the American and the World Health Organization standard C. sordellii antitoxin. This was the first reported isolation of this organism in England or the continent of Europe. Whereas the filtered toxin from the intestinal contents of the dead calf was highly toxic for guinea pigs, filtrates of the cultures grown from the isolation when reinjected into calves produced no pathogenicity.

This same year, Meisel (1956) worked with the toxins of C. sordellii and observed that the amount of toxin produced by a culture could be increased by incorporating glucose into the medium. These studies showed a cross-reaction with C. welchii (C. perfringens) antitoxin and it was observed that this antitoxin neutralized the lecithinase of C. sordellii.

It was from this work that Meisel reported that in addition to lecithinase and hemolysin, C. sordellii, contained a necrotic and lethal toxin.

Meisel and Rymkiewicz (1958) hypothesized that endospores of proteolytic clostridia contained not only the spore antigen, but also the H and O antigens of the vegetative cells. To prove this theory, they treated endospores of C. tetani and C. sporogenes with lysozyme, desoxyribonuclease and trypsin. These enzyme treated spores were then autoclaved for 40 min at 120 C, injected into rabbits and an antiserum produced. The antiserum agglutinated not only the spore antigen, but also H and O vegetative antigens. By comparison, antiserum to either H or O vegetative antigens would not agglutinate this suspension. They concluded that the endospore was not an H or O antigen but could stimulate the production of specific antibodies in the rabbit.

Brooks and Epps (1958) collected 62 different strains from various sources. Using the differentiating criteria of pathogenicity and urease production, they found that they had 24 non-pathogenic, urease negative strains which they called C. bifermentans; 13 strains that were pathogenic and urease positive, classified as C. sordellii (P); and 25 strains that were non-pathogenic but were urease positive and considered to be C. sordellii (N-P). When these cultures were inoculated into glucose, fructose, maltose, mannose, glycerol, sorbitol and salicin these workers observed that the C. bifermentans fermented all of them with the exception of one strain not fermenting sorbitol and one not fermenting salicin.

Both C. sordellii (P) and C. sordellii (N-P) fermented glucose, fructose, maltose and glycerol but none of the other sugars. This resulted in a differentiation of the 2 species by sugar fermentations. These strains were also submitted to chromatographic examination and it was observed that C. sordellii produced large amounts of histidine while C. bifermentans strains produced none. It was their conclusion that the 2 species could be separated on toxigenicity, urease production and ability to ferment mannose, salicin, and sorbitol.

In 1960, Norris and Wolf studied several strains and species of the genus Bacillus and observed that antiserum produced against unheated spores would agglutinate unheated spore antigen, autoclaved spore antigen, and vegetative H antigen but not the vegetative O antigen. Antiserum produced against autoclaved spores would agglutinate autoclaved and unheated spore antigen but neither vegetative H nor O antigen. However, antiserum to vegetative H antigen would agglutinate unheated spore antigen but not autoclaved spore antigen. In no instance would antiserum produced against vegetative O antigen agglutinate a spore antigen. Injection of H antigen into rabbits would stimulate the production of both H and O antibodies. It was interesting to note that these workers reported that antiserum produced against spores treated with formalin would not agglutinate either H or O antigen.

Crane and Poulos (1962) reported that C. sordellii was isolated from feedlot cattle in pure culture in California. These authors postulated

that this organism may be a normal inhabitant of the ruminant digestive tract.

Two isolations of C. sordellii from sheep were reported by Smith, Safford, and Hawkins (1962). The first isolation was made from a ram that died 10 days after being sheared and the second was from 2 rams which had edematous heads and necks. It appeared to these authors that the pathogenesis of this organism may have been due to a wound infection in the first instance and to tissue traumatization as a result of fighting in the latter case. Whereas the isolation of C. sordellii from affected tissue was accomplished easily, it could not be isolated from the soil. Soil sample isolations produced only non-pathogenic, urease negative, C. bifermentans.

Walker (1963) studied spore suspensions of C. sordellii and C. bifermentans that were produced by growing the organism for 3 to 4 days at 37 C, washing the culture in distilled water and centrifuging at 3,000 r.p.m. 6 times, resuspending in water and shaking vigorously, recentrifuging, removing the supernatant fluid and diluting to a Brown opacity standard number 8. After microscopic examination for purity, the spores were autoclaved for 20 min at 121 C to prevent germination and production of H and O antigens. Vegetative cell antigens were prepared by harvesting 12 to 16 hr cultures, centrifuging, washing and standardizing them in the same method as the spores were handled. The H antigen was prepared by submitting this vegetative cell preparation to incubation in 0.08% formaldehyde for 2 hrs. at 37 C. The O antigen was

prepared by steaming the suspension for one hr. Walker observed that C. sordellii and C. bifermentans could be differentiated on the basis of their spore agglutinins but not on their precipitins.

Six distinct antigens of C. bifermentans were determined by Ellner and Green (1963); 5 of these were common to all 4 of the strains used. Five antigens of non-pathogenic C. sordellii and 7 antigens of pathogenic C. sordellii were also found. Five of the latter were common to all of the 9 strains used. Agar-gel diffusion and antigen-antibody absorptions were the methods that were used to distinguish these antigens. These experiments clearly distinguished between pathogenic and non-pathogenic strains. These workers also found that there were 4 antigens common to both C. sordellii and C. bifermentans with the former having, in addition, 3 species specific antigens and the latter 2.

Nishida, Tamai and Yamagishi (1964) studied species differentiation between C. sordellii and C. bifermentans in a somewhat different manner. From previous work on C. perfringens, (Yamagishi, Ishida and Nishida, 1964), they postulated that the ability to sporulate was closely associated with toxigenesis. They observed that strains of the non-pathogenic C. bifermentans sporulated to a greater degree than did the pathogenic strains of C. sordellii. They identified their strains by toxigenicity and urease production and submitted them to heat treatments. They reported that 7 heat-resistant strains of pathogenic C. sordellii became non-toxigenic when exposed to 90 C or 100 C for 10 min. Some of these heat-resistant strains were urease positive and resembled

non-pathogenic C. sordellii, while others became urease negative and resembled C. bifermentans. The sporulation of C. bifermentans was high, that of pathogenic C. sordellii was low and that of non-pathogenic C. sordellii was intermediate between the 2. Tamai and Nishida (1964) continued studies on effects of heat treatment. Two new strains of pathogenic C. sordellii were studied and in one strain, toxigenicity was lost after heat treating at 80 C for 30 min. In the original culture, urease activity was lost at this time also but in a substrain the urease activity was not lost until it was heated at 90 C for 30 min. In another strain, a C. bifermentans-like substrain could not be obtained by heat selection. Huang, Tamai and Nishida (1965) continued this work and added the use of agglutination tests to support their evidence. They reported that the heat-resistant substrains of 3 pathogenic C. sordellii strains not only lost their toxigenic properties but also lost their species specific antigens and acquired the antigenic properties of C. bifermentans. They used cross-agglutination tests and agglutination-absorption tests. These authors concluded that their work proved the close relationship of the 2 species. That they had converted a pathogenic C. sordellii organism into a C. bifermentans further proved the 2 species were actually one and the same.

Prevot (1966) in the first American edition of the Manual for the Classification and Determination of the Anaerobic Bacteria, classified C. sordellii as a variety under the subgenus Clostridium sporogenes

and differentiated it from C. bifermentans on pathogenicity, urease production, sugar fermentation and growth products.

The pathology, along with the pathogenesis of C. sordellii was again studied by Wren in 1968 in guinea pigs and cattle. He found that any parenteral route of administration would produce death in either species but oral administration by several methods would not. He did observe that guinea pigs allowed to drink contaminated water would shed viable organisms for as long as 20 days, indicating the possibility of growth of the organism within the digestive tract without intoxicating the animal. Wren's studies bring up the possibility of carrier animals.

## MATERIALS AND METHODS

### Experimental Animals.

White Swiss mice of both sexes weighing 18 to 22 g used in the various studies were obtained from the Veterinary Research Laboratory animal colony at Montana State University and from a commercial breeder (Carworth Inc. Portage, Michigan). Guinea pigs of both sexes weighing approximately 300 g were used for pathogenic determinations of C. sordellii strains. The guinea pigs were obtained from the animal colony at Montana State University.

### Selection of Strains.

Several strains of C. sordellii were acquired from the Veterinary Biologics Division of the Agricultural Research Service at the National Animal Disease Laboratory in Ames, Iowa and from Dr. Louis D.S. Smith at Montana State University, Bozeman, Montana. These strains were transferred from stock cultures to an anaerobic liver broth medium and incubated anaerobically at 37 C. After growth for 19 hrs stained smears of the organisms were observed microscopically for a preliminary determination of purity. Actively growing cultures were streaked on freshly poured bovine blood agar plates and egg yolk agar plates to observe colonial morphology and to establish hemolytic and lecithinase activity. These plates were incubated anaerobically in Brewer jars at 37 C for 4 days as described by Smith (1955).

In order to determine carbohydrate fermentation, various sugars sterilized by filtration were added to tubes of freshly prepared



thioglycollate medium to give a final concentration of 0.5%. These tubes were then boiled for a few minutes to drive off oxygen and to mix the added carbohydrates with the thioglycollate broth. The cooled tubes were inoculated with approximately 0.5 ml of broth culture, using sterile Pasteur pipettes fitted with rubber bulbs. The inoculated carbohydrate tubes were incubated aerobically at 37 C for 3 days and then observed for fermentation by the addition of a loopful of culture to a pH indicator (1% Brom Thymal Blue) placed on a spot plate. These cultures were re-incubated for an additional 4 days, and at that time the indicator solution was added to the culture for a final assay of carbohydrate fermentation.

The strains of C. sordellii were examined for urease production by inoculating a medium composed of a 31% Urea Broth (Difco, 0272-02) solution mixed 1 to 8 with thioglycollate broth (BBL, 411 665). A heavy inoculum acquired by scraping bacterial growth from a blood agar plate with a wire loop was used to inoculate this medium. These tubes were incubated aerobically at 37 C for 4 hrs.

Cultures were selected for toxigenicity by growing in anaerobic liver broth for 21 hrs, centrifuging to remove the meat particles, and treating the supernatant fluid with 100 mg of aqueous penicillin per ml. A volume of 0.2 ml fluid was injected intraperitoneally (I.P.) into white Swiss mice. Whole culture material was mixed one to one with sterilized 5%

calcium chloride solution and 2.5 ml was injected intramuscularly (I.M.) into guinea pigs to determine the pathogenicity of the strains.

Four strains that produced hemolysis on blood agar plates, fermented glucose, fructose, and maltose but not mannose, salicin or sorbitol were selected for further studies for toxin production. All were considered toxigenic because they killed mice within 24 hrs when injected I.P. with penicillin treated supernatant fluid of broth cultures. These 4 strains were pathogenic for guinea pigs, produced urease and with the exception of one strain, produced lecithinase. Throughout the rest of this manuscript these strains will be known as A-125, 831, 1732 and 3903. The latter strain did not produce lecithinase.

#### Culture Media Used.

Growth culture medium. The medium selected for routine culture growth and used as a base for other media was a liver infusion broth to which was added 1% trypticase (BBL), 0.5% yeast extract (Difco), 0.5% dextrose and 0.01% magnesium sulfate (Lozano, 1965). Into 16 ml tubes were placed 8 ml of this medium and a small amount of dried, ground beef muscle and a pea-sized ball of steel wool. This medium was adjusted to a pH of 7.4, and will hereafter be referred to as standard growth medium.

#### Method for determining effect of length of incubation on toxin yield.

The medium used was the standard growth medium. On the first trial, the 4 strains of C. sordellii were incubated in 4 separate Brewer jars anaerobically at 37 C, 2 cultures of each strain per jar. The cultures

were harvested at 6-hr intervals starting at 6 hrs after inoculation. The toxic fluids were prepared and injected into mice. On the second trial, the 6-hr growth period was eliminated and 30 and 36 hr periods were added.

Method of estimating effect of growth temperature on toxin production.

The procedure was to inoculate cultures of each of 2 selected strains into standard growth medium, and place them under anaerobic conditions. The cultures were incubated in individual incubators (Napco 1000 series, Model 320) at either of the following temperatures 30 C, 33 C, 37 C and 42 C. The cultures were removed at 18 hrs and the toxic fluids prepared and injected into mice. A second trial was conducted, eliminating the 2 lowest temperatures and adding one higher temperature, 45 C.

Method for studying effect of peptone concentration on toxin yield.

To vary peptone concentration of the medium, standard growth medium had to be altered to eliminate the other components that would contribute peptones. The peptone variation broth used was made with 0.5% dextrose, 0.5% sodium phosphate and either 2%, 3%, 4% or 5% Bacto-tryptose (Difco) in distilled water. This preparation was autoclaved for 15 min at 121 C, and then the pH adjusted to 7.4. Tubes of each of these peptone concentrations were inoculated with strain 1732 and incubated at 42 C and harvested at 18 hrs. Tubes were also inoculated with strain 3903 at each of these peptone concentrations, incubated anaerobically at 37 C and harvested at 18 hrs.

Method for studying effect of dextrose concentration on toxin yield.

The medium used for this determination was the same as the peptone variation procedure except that the peptone concentration was maintained at 4% and the dextrose concentration was varied. The dextrose concentrations used were 0.5%, 1.0%, 1.5%, 2.0% and 2.5%. Both strains were incubated anaerobically for 18 hrs before the toxic fluids were harvested and injected into mice. Strain 1732 was incubated at 42 C, whereas strain 3903 was incubated at 37 C.

Toxin production medium. Toxin production flasks were prepared by placing 16 g tryptose (4% peptone), 2 g dextrose (0.5%) and 2 g sodium phosphate (0.5%) into 5 liter Erlenmeyer flasks. Four liters of distilled water were added and mixed until all of the dry ingredients were in solution. The preparation was autoclaved for 20 min at 121 C. The pH was adjusted to 7.4 by the addition of 0.1 N sodium hydroxide.

Spore production medium. It had previously been described in the literature that when C. sordellii was grown on a solid medium for extended periods of time the colonies consisted predominantly of spores with few vegetative cells (Smith, 1955). By microscopic examination of Gram's stained smears of colonies, it was determined that after 4 days growth on a 5% bovine blood agar plate there was a predominance of free spores with only occasional vegetative cells.

Method of Preparation and Storage of Toxic Materials.

Crude toxin. This toxin was prepared as follows: decanting toxic fluid from toxin production cultures, centrifuging (International Centrifuge Size 1, Type SB) at 545 X g, and filtering the supernatant fluid through a 0.45  $\mu$  Millipore filter. Crude toxin preparations were discarded if not used immediately.

Semi-purified toxin. Five seed cultures containing 50 ml of standard growth medium were inoculated with strain 1732 and incubated anaerobically in Brewer jars for 20 hrs at 37 C.

Five production flasks were inoculated by transferring the contents of one seed culture tube into each production flask. The transfer was made after flaming the mouth of both the tube and the flask and passing the contents through sterile cheesecloth to remove the meat particles. The production flasks were then incubated at 42 C for 18 hrs.

The 20 liters of 18-hr growth was harvested by centrifuging (Sorvall SS3 Automatic Superspeed Centrifuge) at 1,000 X g for 20 min and decanting and combining the supernatant fluids in 2 sterile pans. Fifty per cent w/v of ammonium sulfate was mixed into the supernatant fluids and stirring was continued until no more flotsam was formed. This procedure was carried out at 4 C. The toxin was recovered by skimming the flotsam that had risen to the top with a stainless steel screen and placed in 250 ml round bottom flasks. The contents in the round bottom flasks were shell frozen and lyophilized (Virtis Research Equipment).

The dried toxin was removed from the round bottom flasks, pooled into one container and placed in a desiccator jar containing sodium carbonate. Dried toxin inoculated into standard growth medium and incubated at 37 C anaerobically produced no growth and was considered to be free of viable organisms. This was considered to be a semi-purified preparation of C. sordellii toxin.

Spore suspension. Twenty-seven 5% bovine blood agar plates were heavily inoculated and incubated at 37 C anaerobically in Brewer jars for 4 days. Harvest of the spores was done by the method of Long and Williams (1958). The method consisted of scraping the colonies from the agar with a wire loop and washing the agar surface with 0.1 N sodium phosphate buffer. This suspension was centrifuged (Sorvall SS3) at 1885 X g for 30 min. The supernatant fluid containing the vegetative cells was decanted and the precipitated button was rewashed with 0.1 N sodium phosphate buffer and recentrifuged. This process was repeated 4 times. The final suspension was aerated by shaking and refrigerating overnight at 4 C to increase lysis of any vegetative cells which might be remaining. The following morning the spores were washed and centrifuged as before 5 more times and suspended in 60 ml of buffer solution. The spore suspension was examined several times microscopically and no vegetative cells were observed.

The suspension was pipetted into twelve 10 ml freeze-drying ampules, 5 ml to each ampule and lyophilized. The ampules were sealed by heat under vacuum and stored at -20 C.

Methods of Determining Toxicogenicity and Pathogenicity.

Toxin preparation. Mouse lethality was used as the criterion for toxicity of C. sordellii toxin preparations. White Swiss mice weighing 18 to 22 g were given I.P. injections of toxins. All tests were held for 24 hrs and only mortality rates were used to determine effects.

Spore suspensions. Mouse lethality was used as the criterion for pathogenicity of spore preparations. Spore suspensions were injected I.M. into the large muscles of the rear legs along with calcium chloride. Spore titrations were terminated after 48 hrs and only live and dead animals were used in the determinations.

Dilution methods. Toxin dilutions were made in 1% Bacto-peptone (Difco 0118-01) water whereas spores were suspended in sterile distilled saline. The lethality titration dilutions were made in 2-fold, 10-fold or 0.0025 mg increments of the toxic preparations. Lethal doses were calculated by the method of Reed and Muench (1938). All dilutions were made by changing pipettes with each dilution and mixing the preparation by carefully drawing the solution into a pipette and exuding it 10 times for each tube.

Method of Preparing Immunizing Materials.

Toxoids. Toxoids were prepared by diluting dried toxin in 1% peptone water to give 3 preparations of 1000 M.L.D.s per 0.5 ml dose, 100 M.L.D.s per 0.5 ml dose and 10 M.L.D.s per 0.5 ml dose respectively. To each of these preparations a sufficient amount of 36.9% formalin was added and thoroughly mixed by horizontal motion to give a final dilution of 0.7% formalin. The formalinized toxin preparations were incubated at 37 C for 4 days to insure complete inactivation of the toxin. The pH of these toxoids was adjusted to 7.4. Sterility tests were performed on toxoids by heavily inoculating 5 tubes of standard growth medium and incubating 3 tubes anaerobically and 2 tubes aerobically for 10 days at 37 C. Daily observations for evidence of lack of growth and terminal microscopic examinations of stained smears were used for these sterility tests. Safety tests to determine product inactivation were performed by 0.2 ml I.P. injections in white Swiss mice and by 2.5 ml S.C. injections in guinea pigs. Fluids used for guinea pig safety inoculations were mixed with 5% calcium chloride. Satisfactory toxoids were stored at 4 C.

Spore Bacterins. Preparation of spore bacterins was similar to that of the toxoids. The calculated amount of spores that would give 3 preparations of 1000 M.L.D.s per 0.5 ml dose, 100 M.L.D.s per 0.5 ml dose and 10 M.L.D.s per 0.5 ml dose respectively was suspended in sterile distilled saline. Formalin was added to give a final concentration



of 0.7% formalin. The formalinized preparation was incubated for 4 days at 37 C, the pH was adjusted to 7.4 and sterility tests were performed by the same procedure used for the toxoids. Safety tests were carried out by the same method as with the toxoids to insure that the spores were inactivated.. Satisfactory bacterins were stored at 4 C.

Vegetative cell bacterins. To produce a washed cell bacterin it was determined at what phase of growth spores began to form in order to harvest the vegetative cells prior to sporulation. It was necessary to determine at what phase of growth vegetative cells became the most virulent for mice.

Twelve test tubes of standard growth medium were inoculated with strain 1732 and incubated anaerobically at 37 C and 2 tubes were removed from incubation at 4-hr intervals for examination.

Washed cells were processed by pouring cultures through sterile cheesecloth. After centrifuging (Sorvall SS3) at 130 X g for 10 min, removing the supernatant fluid carefully and resuspending the pelleted cells in an equal volume of sterile saline, the resultant suspension was recentrifuged. This procedure was carried out 4 times. The final cell pellet was resuspended in enough sterile saline to produce a Macfarlane nephelometer reading of 8 which was the same as the broth culture before washing the cells. A Bausch and Lomb Spectronic 20 spectrophotometer adjusted to 740 m $\mu$  was used to evaluate nephelometer readings.

This suspension of washed cells with a Macfarlane nephelometer reading of 8, was diluted to  $10^{-3}$  and  $10^{-8}$ . The washed cell bacterins were prepared with cultures from ten 50 ml tubes of standard growth medium that were inoculated with strain 1732 and incubated anaerobically at 37 C for 10 hrs. The pH was adjusted to 7.4. These 2 preparations were inactivated with formalin and tested for sterility and safety in the same manner as the toxoids and the spore bacterins. Those found to be satisfactory were stored at 4 C.

Whole culture bacterins. The whole culture bacterin was prepared by inoculating ten 50 ml tubes of standard growth medium and allowing them to incubate for 10 hrs anaerobically at 37 C. The cultures were poured through sterile cheesecloth to remove meat particles. Dilutions were made to  $10^{-3}$  and  $10^{-8}$  and treated with formalin to inactivate them. The pH was adjusted to 7.4 and sterility and safety tests were performed as described previously. Satisfactory bacterins were stored at 4 C.

Method for Heat Inactivation.

The production of heat resistant substrains of C. sordellii strain 1732 was attempted by the method of Nishida, Tamai and Yamagishi (1964). This consisted of inoculating test tubes of standard growth medium with strain 1732 and allowing them to grow anaerobically at 37 C for 20 hrs. The tubes were placed in water baths adjusted to 80 C, 90 C or 100 C. Cultures removed from the water baths were immediately transferred to

freshly boiled standard growth medium and incubated anaerobically at 37 C for 18 hrs. These cultures were injected into mice to determine if they were pathogenic and were inoculated into urea test medium to see if there was urease production (Tamai and Nishida, 1964; Huang, Tamai and Nishida, 1965).

Serological Methods.

Precipitation reactions between toxins, toxoids and specific antiserum were developed in a plate gel-diffusion system according to Ouchterlony (Ouchterlony, 1968, Campbell et al, 1964).

## EXPERIMENTAL RESULTS

### Preparation of Toxoids.

Factors affecting toxin yield. To facilitate the productions of toxin, several variations were made in the procedures used in toxin production. Also selection of a high toxin producing strain of C. sordellii was necessary.

Preliminary trials indicated that strains of C. sordellii, under these conditions would reach their peak of toxin production at 18 hrs and would maintain this level of production for 24 to 36 hrs depending on the strain.

The temperature of incubation was studied with respect to toxin production using only strains 1732 and 3903. These particular strains were chosen because they produced a potent toxin with regularity.

After 18 hrs growth it was learned that 42 C was the optimal toxin production temperature under these conditions for strain 1732, and 37 C for strain 3903.

The third factor considered was the effect of concentration of peptone on toxin production. The results of the peptone variation studies are summarized in Table I. It may be observed that there was little difference in toxin production with strain 1732 when 3% to 5% peptone was used in this medium. Similar observations were made with strain 3903.

Table I. Effects of peptone concentration on toxin yield

% Peptone	Lethality in mice (dead/total injected)					
	Strain 1732			Strain 3903		
	1:100	1:500	1:1000	1:100	1:500	1:1000
2%	2/2	0/2	0/2	2/2	2/2	1/2
3%	2/2	1/2	2/2	2/2	0/2	0/2
4%	2/2	2/2	2/2	2/2	2/2	0/2
5%	2/2	1/2	2/2	2/2	2/2	0/2

In studies of 5 different concentrations of dextrose, the results of testing at 0.5% to 2.5% in 0.5% increments are recorded in Table II. Since it was desirable to work with only one strain, C. sordellii 1732 was selected because it produced the most toxin under varied conditions. The optimal medium for toxin production with strain 1732 was determined to be: 0.5% dextrose, 4% peptone, 0.5% sodium phosphate buffer in distilled water.

Twenty liters of satisfactory toxin was produced in production medium and the toxin was extracted with ammonium sulfate, lyophilized and stored.

Toxin titration. Before a toxoid could be made, it was necessary to determine the toxicity of the lyophilized toxin preparation. Serial

Table II. Effects of dextrose concentration on toxin yield

% Dextrose	Lethality in mice (dead/total injected)									
	Strain 1732					Strain 3903				
	1:100	1:400	1:800	1:1000	1:5000	1:100	1:400	1:800	1:1000	1:5000
0.5%	2/2	2/2	2/2	2/2	1/2	2/2	2/2	2/2	2/2	0/2
1.0%	2/2	2/2	2/2	2/2	0/2	2/2	2/2	0/2	0/2	0/2
1.5%	2/2	2/2	2/2	0/2	0/2	1/2	0/2	0/2	0/2	0/2
2.0%	2/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2
2.5%	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2

2-fold dilutions were made of this solution to give 7 dilutions, containing 0.1 mg to  $1.56 \times 10^{-3}$  mg, which were injected I.P. into 2 mice each as a screening test. It was observed that all injected mice died indicating a higher level of toxicity than had been anticipated. A second titration was conducted with reduced amounts of toxin using 0.01 mg per 0.1 ml dose for the uppermost dilution level. This experiment was repeated using 5 mice in place of 2 mice per dilution. The results of preliminary toxin titrations are summarized in Table III.

Table III. Preliminary toxin titrations

Trial	Mg of toxin							
	0.01	0.0075	0.005	0.0025	0.001	0.00075	0.0005	0.00025
* #2 ***	1/2	2/2	2/2	2/2	1/2	0/2	0/2	0/2
** #3	ND	ND	5/5	5/5	4/5	2/5	2/5	ND
Total	1/2	2/2	7/7	7/7	5/7	2/7	2/7	0/2

\* #2 test is the screening test using 2 mice per dilution.

\*\* #3 test is the screening test using 5 mice per dilution.

\*\*\* Dead mice/total mice injected.

ND Denotes that the test was not done at this dilution.

It can be seen from the results that the M.L.D. of this toxin preparation was 0.0025 mg per mouse dose when given I.P.

Inactivation of toxins. To insure that when the toxoids were prepared there would be adequate toxin available, the next higher dilution above the titrated M.L.D. was taken as the "toxoiding" dose. Toxoids were thus prepared using 0.005 mg per dose. Inactivation of toxin was accomplished as previously described.

#### Preparation of Spore Bacterins.

Spore suspension production. It was determined that the optimal method to produce large numbers of spores was growth for 4 days on solid media.

Spore suspension titrations. Preliminary titrations of lyophilized spores suspended in 2.5% calcium chloride resulted in death of all mice. Several calcium chloride concentrations were injected I.M. into mice in various volumes. No deaths occurred, eliminating the possibility that death was caused by the calcium chloride.

Further studies were then conducted using various concentrations of spores suspended in either of 3 different concentrations of calcium chloride or sterile distilled saline. The effect of volume of the injections was also studied. The results of these experiments are recorded in Table IV and show that the concentration of calcium chloride used with the spore suspension did make a difference in the lethality. With the smaller volume, 1%, there did not appear to be sufficient



Table IV. Effect of dosage and calcium chloride on pathogenicity of spores

Vol. in ml/CaCl <sub>2</sub>	Spore preparation in mg											
	10 <sup>-1</sup>	10 <sup>-2</sup>	10 <sup>-3</sup>	10 <sup>-4</sup>	10 <sup>-5</sup>	10 <sup>-6</sup>	10 <sup>-7</sup>	10 <sup>-8</sup>	10 <sup>-9</sup>	10 <sup>-10</sup>	10 <sup>-11</sup>	10 <sup>-12</sup>
0.1/H <sub>2</sub> O	*0/2	0/2	0/2	1/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2
0.1/1.0	1/2	1/2	0/2	1/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2
0.1/2.5	2/2	2/2	2/2	2/2	2/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2
0.1/5.0	2/2	2/2	2/2	2/2	2/2	1/2	0/2	0/2	0/2	0/2	0/2	0/2
0.3/H <sub>2</sub> O	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2
0.3/1.0	2/2	2/2	2/2	2/2	2/2	1/2	0/2	0/2	0/2	0/2	0/2	0/2
0.3/2.5	2/2	2/2	2/2	2/2	2/2	2/2	1/2	0/2	0/2	0/2	0/2	0/2
0.3/5.0	2/2	2/2	2/2	2/2	2/2	2/2	2/2	1/2	1/2	2/2	0/2	1/2

\*Dead mice/total mice injected.

calcium chloride to produce suitable conditions for spores to exert their lethal effect. At the 0.3 ml volume there was a significant and uniform relationship between the calcium chloride concentrations and lethality. In the absence of calcium chloride, germination of spores may not take place in sufficient amounts to cause death. It was determined that the 2.5% calcium chloride concentration gave a satisfactory level of anaerobiosis and would be used with spore suspensions hereafter.

In the previous experiment it was observed that there was significant differences between a 0.1 ml dose and 0.3 ml dose containing the same amount of spore suspension with the same concentrations of calcium chloride. Because the injections were made I.M. and the size of the test animal was small, it was desirable to use a small dose. To further study the effect of volume, an experiment was done to compare a 0.3 ml dose to a 0.2 ml dose, both containing the same amount of spore suspension and both having a final concentration of 2.5% calcium chloride. The results of this work are given in Table V. It was learned that the 0.2 ml dose was at least as effective as the 0.3 ml dose. This volume was used for subsequent spore injections into mice.

Table V. Effect of dosage volume on pathogenicity of spore suspension

Volume injected in ml	Spore suspension in mg				
	$10^{-5}$	$10^{-6}$	$10^{-7}$	$10^{-8}$	$10^{-9}$
0.2	* 5/5	5/5	1/5	1/5	0/5
0.3	5/5	2/5	0/5	0/5	0/5

\* Dead mice/total mice injected.

With volume and concentration of the dose and the concentration of calcium chloride established, an experiment to determine a M.L.D. was performed. The results of these tests are summarized in Table VI.

There was considerable variation in results, but it was observed that in 3 of the 5 titrations the M.L.D. was  $1.25 \times 10^{-6}$  mg and this dose was selected for the preparation of spore bacterins.

Preparation of Vegetative Cell Bacterins.

Vegetative cell titrations. Vegetative cells of strain 1732 of *C. sordellii* were washed and prepared for pathogenicity titrations as described previously. Ten-fold dilutions were made of the final washed cell suspension and 8 dilutions each were injected into 10 white Swiss

Table VI. C. sordellii spore suspension titrations.

Repetition	Spore suspension in mg										
	* 100	50	25	12.5	6.25	3.125	1.563	0.78	0.39	0.195	0.098
#1	** 5/5	4/5	4/5	4/5	4/5	3/5	1/5	1/5	1/5	1/5	0/5
#2	5/5	5/5	5/5	5/5	3/5	3/5	1/5	1/5	1/5	2/5	1/5
#3	ND	ND	4/5	4/5	3/5	2/5	0/5	0/5	ND	ND	ND
#4	5/5	5/5	5/5	5/5	3/5	3/5	2/5	3/5	ND	ND	ND
#5	5/5	5/5	5/5	5/5	5/5	4/5	4/5	3/5	0/5	0/5	ND
Total	20/20	19/20	23/25	23/25	18/25	15/25	8/25	8/25	2/15	3/15	1/10

\* All numbers times  $10^{-7}$ .

\*\* Dead mice/total mice injected.

mice I.M. using a 0.2 ml dose with a final concentration of 2.5% calcium chloride. The results of these injections are summarized in Table VII and indicate that the organisms from 10-hr growth were more pathogenic. Bacterins were made from dilutions of this preparation.

Table VII. Lethality titrations of vegetative cell preparations

Growth in Hours	*Dilutions							
	10 <sup>1</sup>	10 <sup>2</sup>	10 <sup>3</sup>	10 <sup>4</sup>	10 <sup>5</sup>	10 <sup>6</sup>	10 <sup>7</sup>	10 <sup>8</sup>
6 hr	** 10/10	10/10	10/10	10/10	7/10	2/10	0/10	0/10
10 hr	10/10	10/10	10/10	10/10	10/10	10/10	10/10	10/10

\* Dilutions were prepared from vegetative cell suspensions that had a Macfarlane nephelometer reading of 8.

\*\* Dead mice/total mice injected.

Preparation of Whole Culture Bacterins.

Whole culture production. Several tubes of standard growth medium were inoculated with strain 1732 of C. sordellii. These tubes were incubated anaerobically at 37 C, one test tube was removed every 4 hrs, starting at 6 hrs post-inoculation.

Whole culture titrations. Cultures from the test tubes were diluted in the same broth medium that the cultures were grown in making 10-fold

dilutions. The dilutions were injected I.M. into 10 white Swiss mice per dilution with a final concentration of 2.5% calcium chloride.

No end point was reached and the 6, 10 and 14-hr incubation periods were not significantly different. The 18-hr culture had a definitely lower end point and was not included in a second trial. The results of the second trial are summarized in Table VIII and show that the whole culture was most pathogenic after a 10-hr incubation period. Bacterins were subsequently prepared from cultures incubated 10 hrs.

Table VIII. Lethality titration of whole culture C. sordellii

Growth in Hours	*Dilutions							
	10 <sup>4</sup>	10 <sup>5</sup>	10 <sup>6</sup>	10 <sup>7</sup>	10 <sup>8</sup>	10 <sup>9</sup>	10 <sup>10</sup>	10 <sup>11</sup>
6 hr	** 10/10	10/10	10/10	9/10	5/10	4/10	1/10	0/10
10 hr	10/10	10/10	10/10	10/10	10/10	3/10	3/10	0/10
14 hr	10/10	9/10	10/10	8/10	4/10	2/10	1/10	0/10

\* Dilutions were made from strained whole cultures.

\*\* Dead mice/total mice injected.

Comparison Studies of Immunizing Ability of C. sordellii Products.

Several C. sordellii products were produced that were suspected of having immunizing ability against homologous or heterologous challenge

with C. sordellii toxin or spores. Three of these products were toxoids produced with different concentrations of lyophilized toxin. Three were spore bacterins prepared with different concentrations of spore suspensions. Two were bacterins prepared from 2 different concentrations of washed vegetative cells and 2 were bacterins prepared from 2 different concentrations of whole cultures. Commercially prepared aluminum hydroxide precipitated whole culture bacterins were also used. These products were acquired from the National Animal Disease Laboratory in Ames, Iowa and designated by the code numbers B9425 and D9764.

Toxin challenge determinations. Animals vaccinated with various products were challenged with one M.L.D. of toxin. The M.L.D. was determined in mice given 2-fold dilutions of toxin. The results of these titrations and a comparison between I.P. and I.V. injections are summarized in Table IX.

It was observed that the M.L.D. of the toxin when given I.P. was  $5 \times 10^{-3}$  mg. The M.L.D. by the I.V. route was greater than  $5 \times 10^{-3}$ . The I.P. route was selected as the route of administration of the toxin challenge.

Spore challenge determinations. It was decided to challenge at or near the M.L.D. Calculations were made from titrations recorded in Table VI. The selected challenge dose of the spore suspension was  $5 \times 10^{-7}$  mg.

Table IX. Intraperitoneal and intravenous titrations of toxin challenge

Repetitions	Toxin in mg					
	$5 \times 10^{-3}$	$2.5 \times 10^{-3}$	$1.25 \times 10^{-3}$	$6.25 \times 10^{-4}$	$3.13 \times 10^{-4}$	$1.56 \times 10^{-4}$
#1 IP	* 5/5	5/5	4/5	2/5	0/5	0/5
#2 IP	5/5	5/5	4/5	0/5	0/5	0/5
#3 IP	5/5	4/5	4/5	0/5	ND	ND
#4 IP	5/5	4/5	2/5	0/5	ND	ND
#5 IP	5/5	4/5	2/5	1/5	ND	ND
Total	25/25	22/25	16/25	3/25	0/10	0/10
#1 IV	5/5	4/5	3/5	1/5	0/5	0/5
#2 IV	5/5	3/5	3/5	0/5	0/5	0/5
#3 IV	5/5	5/5	2/5	2/5	ND	ND
#4 IV	4/5	5/5	3/5	0/5	ND	ND
#5 IV	5/5	1/5	1/5	0/5	ND	ND
Total	24/25	18/25	12/25	3/25	0/10	0/10

\* Dead mice/total mice injected.

ND indicates that the repetition was not done.



First animal protection trial. Potential immunizing products in 0.5 ml amounts were injected S.C. into the right rear flank of mice. Ten days after the initial vaccination, a second 0.5 ml injection was administered into the opposite flank of one-half of the vaccinated animals. Eleven days after the second vaccination, one-half of the single-vaccinated animals and one-half of the double-vaccinated animals were challenged with toxin. The other half of the single-vaccinated and double-vaccinated animals were challenged with spore suspension. The experiment using toxin challenge was terminated at 24 hrs, and the experiment using spore challenge was terminated at 72 hrs. Results of the first animal protection test are given in Table X. It will be noted that against an LD<sub>90</sub> spore challenge, the toxoids, 2 of the spore bacterins, the washed vegetative cell bacterins and the whole culture bacterins produced no significant protection. The spore bacterin prepared with  $1.25 \times 10^{-4}$  mg of spores appeared to produce some immunity as did the commercially prepared aluminum hydroxide precipitated whole culture bacterins.

Against the M.L.D. toxin challenge there was no significant protection except with the toxoid prepared with 0.5 mg per dose of toxin, which seemed to produce some protection in single- and double-vaccinated groups of mice.

The first animal protection experiment produced no valid results because many variable reactions were obtained.

Table X. First animal protection experiment.

Immunizing Product	Spore challenge		Toxin challenge	
	1 vaccination	2 vaccinations	1 vaccination	2 vaccinations
* T-10	** 6/7	4/5	6/6	7/7
T-100	7/8	5/5	5/8	6/8
T-1000	6/7	7/8	7/7	7/8
S-10	7/7	7/7	7/8	6/6
S-100	2/4	4/5	5/5	4/4
S-1000	4/5	4/5	5/5	7/7
V-3	8/8	7/7	8/8	7/7
V-8	8/8	7/7	8/8	7/7
WC-3	10/10	7/7	8/9	8/8
WC-8	8/9	6/7	9/9	7/7
B9425	9/10	6/10	10/10	10/10
D9764	8/10	9/9	9/10	10/10
Controls	9/10		10/10	

\* T-10, T-100 and T-1000 denote 10 M.L.D., 100 M.L.D. and 1000 M.L.D. toxoids respectively. S-10, S-100 and S-1000 denote 10 M.L.D., 100 M.L.D. and 1000 M.L.D. spore bacterins respectively. V-3 and V-8 denote bacterins prepared from  $10^{-3}$  and  $10^{-8}$  dilutions of vegetative cells respectively. WC-3 and WC-8 denote bacterins prepared from  $10^{-3}$  and  $10^{-8}$  dilutions of whole cultures respectively. B9425 and D9764 denote coded commercially prepared bacterins.

\*\* Dead mice/total mice injected.

Second animal protection trial. A repeat test was initiated with various alterations. First because there were several post-vaccination, but pre-challenge deaths, the pH of immunizing products was examined and found to be either quite acid or in some cases very basic. The pH of all products was adjusted to between 6.8 and 7.4. This reduced the amount of non-specific pre-challenge deaths. Secondly as there appeared to be no difference in immunity between single- and double-vaccinated animals, only one vaccination was administered and challenge was given 21 days post-vaccination. Thirdly because it was believed the previous experimental mice had been overchallenged, the challenge was reduced to an approximate LD<sub>80</sub> that corresponded to  $2.5 \times 10^{-3}$  mg of toxin and to  $10^{-7}$  mg of spore suspension.

Also included in the second experiment was a heterologous spore suspension ( $5 \times 10^{-7}$  mg) challenge acquired from the National Animal Disease Laboratory and identified as "#72 spore challenge". Results of a comparison between the 2 spore challenge materials are given in Table XI.

Table XI. Comparison of 2 spore suspension challenges

Spore Challenge	Spore suspensions in mg				
	$10^{-6}$	$5 \times 10^{-7}$	$10^{-7}$	$5 \times 10^{-8}$	$10^{-8}$
1732 Spore	* 5/5	5/5	3/5	1/5	0/5
#72 Spore	5/5	2/5	2/5	1/5	0/5

\* Dead mice/total mice injected.

These results were used to estimate the desired challenge dose for the second animal protection test.

Also included in the second animal protection experiment were 2 freshly prepared immunizing products. One was a replicate of the toxoid prepared with 0.5 mg toxin and the other a spore bacterin containing 100 times the concentration used in previous spore bacterins. Results of the second animal protection experiment are summarized in Table XII.

It can be seen from the results of the second animal protection experiment that with the homologous spore challenge, there may have been some protection with V-8 vegetative cell bacterin and T-100' toxoid.

Against the 90% toxin challenge, it appeared as if some protection was produced with the T-100 toxoid, the commercially prepared D9764 bacterin, and the 2 homologous whole culture bacterins.

All products tested excepting the T-10 toxoids, the S-1000' spore bacterin and the V-3 vegetative cell bacterin appeared to produce some protection against the low level heterologous spore challenge.

The results of the second animal protection test were inconclusive and there was very poor correlation between the 2 animal protection tests, thus a third animal protection test was necessary.

Third animal protection trial. In the first 2 experiments, it appeared that of the toxoids, the T-100 was the most effective. In the third trial the other toxoids were not studied. Also any protection that was produced with spore bacterins was produced with the S-100 preparation so the others were eliminated from this trial. The spore

Table XII. Second animal protection experiment

Immunizing Product	Challenges		
	1732 Spore	Toxin	#72 Spore
* T-10	7/8/10	9/10	1/9
T-100	8/10	#4/10	#0/8
T-1000	9/10	10/10	#0/9
S-10	10/10	9/10	#0/10
S-100	9/10	9/10	#0/7
S-1000	10/10	10/10	1/9
V-3	8/10	10/10	2/10
V-8	#5/10	8/10	#0/10
WC-3	9/10	#7/10	#0/8
WC-8	9/10	#6/10	#0/9
B9425	10/10	8/10	ND
D9764	10/10	#4/10	ND
** T-100'	#7/10	9/10	#0/10
*** S-10 <sup>5</sup>	9/10	8/10	#0/9
Controls	10/10	9/10	2/10

\* See Table X for denotation of products.

\*\* T-100' denotes second production of 100 M.L.D. toxoid.

\*\*\* S-10<sup>5</sup> denotes spore bacterin produced using 100,000 M.L.D. spore suspension.

7 Dead mice/total mice injected.

# Indicates the test groups that may have some protection.

bacterin prepared with 100,000 M.L.D.s was retested because it had been tested only once previously. With the vegetative cell bacterin and the whole culture bacterin, the V-8 and WC-8 were the only ones used because they seemed to offer slightly more protection than the other vegetative cell and whole culture bacterins. A different commercially prepared whole culture bacterin, C2135 was substituted for B9425 which was used up.

In the third trial an endeavor was made to use an LD<sub>50</sub> challenge dose. For the toxin this was  $1.5 \times 10^{-3}$  mg per dose. The spore challenges were retitered and the LD<sub>50</sub> calculated from this titer.

The challenge dose for the homologous spore suspension was  $2 \times 10^{-7}$  mg and the dose for the heterologous, #72 spore challenge, was  $4 \times 10^{-7}$  mg. The results of the third animal protection test are summarized in Table XIII.

Double-vaccinations as used in the first animal protection test were employed in an attempt to induce increased protection. Against the LD<sub>50</sub> toxin challenge, protection was observed with the commercial vaccine, D9764, the T-100 toxoid, the S-100 spore bacterin and the vegetative cell bacterin, all from the double-vaccinated group. Some protection may have been achieved with D9764 and the vegetative cell bacterin with only one vaccination.

Against the homologous spore challenge, which turned out to be an LD<sub>20</sub> challenge, protection may have been achieved with the vegetative cell bacterin, S-100 spore bacterin and the commercial D9764 whole culture

Table XIII. Third animal protection experiment.

Immunizing Product	Challenges					
	Toxin		Spore		#72 Spore	
	** one	two	one	two	one	two
* T-100	*** 6/9	#1/9	1/8	2/9	2/8	2/9
S-100	4/9	#1/9	#0/8	1/9	1/7	#0/9
V-8	3/8	#1/8	#0/8	2/9	3/8	1/9
WC-8	6/8	6/10	2/9	1/9	3/9	#0/10
D9764	3/10	#1/10	#0/10	1/9	2/10	#0/10
S-10 <sup>5</sup>	5/8	ND	2/8	ND	1/7	ND
G2135	7/10	6/10	4/10	1/9	4/10	#0/10
Controls	5/10		2/10		2/10	

\* See Tables X and XII for description of products.

\*\* Indicate the number of vaccinations.

\*\*\* Dead mice/total mice injected.

# Indicates the test groups that may have immunity.

bacterin with one vaccination. There appeared to be no significant protection with any of the products when they were injected twice into mice at 10-day intervals.

Against the LD<sub>20</sub> challenge of the heterologous #72 spore suspension, single-vaccinated animals produced no protection but in the double-vaccinated group, there appeared to be some protection produced by the S-100 spore bacterin, the whole culture bacterin and the 2 commercial products.

Experimentation with a variable challenge. From the previous experiments it was learned that if the location of the immunizing antigen was to be determined or if any information as to its efficacy was to be disclosed, further experimentation would have to be done.

The bacterin or toxoid from each of the various groups that appeared to demonstrate any possible protection was selected to be injected into mice and then challenged with a variable dose of toxin.

The trial was initiated by injecting 5 groups of approximately 40 mice each with the 5 selected products. The 0.5 ml vaccinations were made S.C. into the right rear flank. Eleven days later, each of the 5 groups were divided into 4 separate subgroups and each subgroup was challenged with a 0.1 ml dose of toxin I.P. The amount of challenge was selected by calculating the LD<sub>50</sub> which was approximately  $1.5 \times 10^{-3}$  mg of toxin. Serial dilutions were prepared in such a manner that 2 of the challenge doses were greater than the LD<sub>50</sub> and 2 were below the LD<sub>50</sub>. To present another interpretation of the data, since all of the products



were challenged with serial 2-fold dilutions of the toxin, the LD<sub>50</sub> was calculated for all of the products by the Reed-Muench method and compared to the LD<sub>50</sub> of unvaccinated controls. An arbitrary rating of effectiveness consisted of estimating the per cent difference of the LD<sub>50</sub> of the product to the unvaccinated controls. The results of this experiment are summarized in Table XIV, from which it can be seen that essentially no protection was produced by any of the products.

Heat inactivation experiment. It was learned from previous experimentation that there appeared to be no immunity produced by formalinized products. Another method of inactivation of C. sordellii was indicated as an alternative. Heat inactivation trials were done by exposing actively growing culture to 80 C and 90 C for 30 min, transferring the heat treated cultures to new medium, incubating for 18 hrs, harvesting and vaccinating 40 mice per temperature group with 0.5 ml S.C. Twenty-four hrs after vaccination, all of the mice were dead and it was decided that this particular strain of C. sordellii could not be inactivated by heat under these conditions.

Attempt to induce immunity with sublethal doses of toxin. From prior results, it was apparent that there was a possibility that mice would not produce protective antibodies against C. sordellii. An experiment was devised to shed some light on this possibility by injecting 100 mice with a sublethal or low lethal dose of toxin. The dosage of  $6.25 \times 10^{-4}$  mg was selected as on previous titrations this dose would produce 10% or less lethality.

Table XIV. Animal protection trial against variable toxin challenge.

Immunizing Product	Toxin challenge in mg				Protection	
	$5 \times 10^{-3}$	$2.5 \times 10^{-3}$	$1.25 \times 10^{-3}$	$6.25 \times 10^{-4}$	*LD <sub>50</sub>	**P
T-100	*** 10/10	7/8	2/10	1/7	1.725	none
S-100	9/9	6/8	2/9	1/9	1.804	none
V-8	6/7	7/9	4/9	0/10	1.712	none
WC-8	9/10	3/8	4/9	1/10	2.061	12.5%
D9764	8/10	3/10	8/10	0/10	2.006	10.1%
Controls.	8/10	10/10	1/9	3/10	1.804	

\* All LD<sub>50</sub> values are times 10<sup>-3</sup>.

\*\* P indicates the amount of protection in per cent.

\*\*\* Dead mice/total mice injected.

Within 24 hrs post-injection 5 mice were dead and 95 survived for 5 days. Five days after the first injection, 40 mice were selected to be challenged at a later date. The other 55 were given a second injection of  $10^{-3}$  mg of toxin. Within 24 hrs 8 of the 55 mice were dead, the other 47 survived. Five days after the second injection, 40 mice were selected from the double-injected group and challenged along with the 40 mice that had received only one injection. The challenge that was used was a sequence of four 2-fold dilutions, 2 calculated to be greater than the  $LD_{50}$  and 2 below the  $LD_{50}$ . In order to make a comparison with the formalinized products, evaluation of the sublethal toxin vaccinations were made in the same way as the variable toxin challenge experiment. The results of this experiment are given in Table XV.

It can be seen that significant protection was produced by vaccinating mice with low lethality doses of toxin. The per cent protection calculated by taking the percentage difference between the  $LD_{50}$  of the controls and the  $LD_{50}$  of the vaccinated animals gives an evaluation of the amount of protection produced. It was learned from this experiment that mice can be immunized against lethal doses of C. sordellii toxin.

At the completion of the 24 hrs, which was the length of time that toxin challenged experiments were maintained, the surviving mice were retained for serological studies. An unusual phenomenon was observed; that is deaths of test animals at 48 and 72 hrs post-challenge. These deaths are recorded in Table XVI.

Table XV. Sublethal toxin induced immunity experiment.

Injection Group	Toxin challenge in mg				Protection	
	$5 \times 10^{-3}$	$2.5 \times 10^{-3}$	$1.25 \times 10^{-3}$	$6.25 \times 10^{-4}$	** LD <sub>50</sub>	*** P
Single	* 7/10	3/10	0/10	0/10	4.875	61.5%
Double	3/10	2/10	0/10	0/10	5.710	67.1%
Controls	9/10	8/10	2/10	1/10	1.875	

\* Dead mice/total mice injected.

\*\* All LD<sub>50</sub> values are times 10<sup>-3</sup>.

\*\*\* P indicates the amount of protection in per cent.

Table XVI. Post-challenge deaths of experimental mice.

Challenged Groups	Hours Post Challenge				
	*24	48	72	96	12 days
<u>Single</u>					
5 X 10 <sup>-3</sup>	** 7/10	9/10	9/10	*** 10/10	10/10
2.5 X 10 <sup>-3</sup>	3/10	7/10	10/10	10/10	10/10
1.25 X 10 <sup>-3</sup>	0/10	3/10	3/10	3/10	3/10
6.25 X 10 <sup>-4</sup>	0/10	1/10	1/10	1/10	1/10
<u>Double</u>					
5 X 10 <sup>-3</sup>	3/10	6/10	10/10	10/10	10/10
2.5 X 10 <sup>-3</sup>	2/10	5/10	9/10	*** 10/10	10/10
1.25 X 10 <sup>-3</sup>	0/10	0/10	0/10	0/10	*** 2/10
6.25 X 10 <sup>-4</sup>	0/10	3/10	3/10	3/10	3/10

\* Usual duration of a toxin challenge experiment.

\*\* Dead mice/total mice injected.

\*\*\* Animals recorded dead were sacrificed for serological studies.

To further test for antibody production, serum was obtained from a rabbit that had been injected with 2.5 mg of toxoid preparation 5 and 15 days previous to bleeding and from mice that received  $6.25 \times 10^{-4}$  mg of toxin 15 days previous to bleeding,  $10^{-3}$  mg of toxin 10 days previous to bleeding and  $5 \times 10^{-3}$  mg of toxin 5 days previous to bleeding. These sera were diffused through gel against  $3.75 \times 10^{-3}$  mg of toxin,  $3.75 \times 10^{-3}$  mg of toxoid and 1% peptone. After 24 hrs, no antigen-antibody precipitation was observed. The cells of the gel-diffusion plate were emptied and fresh samples placed in them. No precipitation was observed even after 48 hrs.

A repeat gel-diffusion experiment was done using serum from mice that had been injected with toxin 22 days, 17 days and 12 days previous to bleeding. This serum was diffused against 0.01875 mg of toxin, 0.00375 mg toxin, 0.00375 mg toxoid and 1% peptone. There was no precipitation observed even after 72 hrs.

A third gel-diffusion experiment was done using serum from a rabbit that had been vaccinated with 2.5 mg of toxoid 22 and 12 days previous to bleeding. This serum was concentrated from 25 ml to 10 ml by lyophilization. The concentrated serum was diffused against 0.01875 mg toxin, 0.00375 mg toxin, 0.00375 mg toxoid and 1% peptone. After 24 hrs there was a very faint line of precipitation with the concentrated serum diffusing against the concentrated, 0.01875 mg, toxin. There was no other reaction observed.

## DISCUSSION

This investigation was undertaken with the intent of studying the immunizing antigens of C. sordellii. From a review of the literature, this author believed that an effective immunizing product could be made and that some information as to the efficacy of this product might become evident.

Ever since the first isolation of C. sordellii, (Sordelli, 1922) it was known that filtrates of supernatant fluids obtained by growing this organism in broth cultures were lethal to laboratory animals. In the research of Meleney, Humphreys and Carp (1927) and Hall, Rymer and Jungherr, (1929), it was learned that C. sordellii antiserum produced a passive immunity in mice against injections of toxin. Meisel and Rymkiewicz (1958) reported that endospores of proteolytic clostridia produce antibodies when injected into rabbits that would agglutinate not only spore antigens but also H and O antigens. This was supported when Nishida, Tamai and Yamagishi (1964) reported that the ability of C. sordellii to sporulate was closely associated with its toxigenicity. Walker (1963) observed that there are vegetative cell antigens in C. sordellii. In 1942, Vawter worked with whole cultures of C. sordellii and stated that "the formalinized cultures were antigenic", although he did not elaborate nor produce any data to substantiate this statement. The commercial products that are on the market are whole culture bacterins that have been precipitated with aluminum hydroxide.

In order to clarify the mechanisms of immunity associated with C. sordellii, the following products were evaluated: toxoids, bacterins prepared from a washed spore suspension, bacterins prepared from a washed vegetative cell suspension, whole culture bacterins and commercial aluminum hydroxide precipitated whole culture bacterins.

The in vivo method of evaluating immunity of vaccinated animals was selected. Mice were used as the experimental animals because previous work was done using this species, (Meleney et al, 1927; Stewart, 1938; Lillie, 1938). The experimental animals were challenged with lyophilized toxin and spores of C. sordellii. Toxin was used because it is lethal (Saissac, Renee, and Raynaud, 1951) and because it could be obtained in a form that would permit reproducible titers. Spore suspensions were used because it was learned that they were lethal to mice and because it was believed that this may be the form initiating C. sordellii infections in the field.

From the 3 animal protection experiments that were done it was concluded that none of the formalinized products prepared nor commercial bacterins induced significant protection against challenge materials. It appeared in some instances that some of the products might have been producing immunity, but in no instance was there consistent protection.

Since no conclusions could be derived from the constant challenge experiments, it was postulated that possibly some information could be gained from challenging these products against serial 2-fold dilutions of toxin and comparing the LD<sub>50</sub> of each product to the LD<sub>50</sub> of unvaccinated



controls. It was learned that there was no protection produced by the toxoid, spore bacterin or vegetative cell bacterin. The whole culture bacterins did have a higher LD<sub>50</sub> than the challenge controls but this was not believed significant.

Having concluded that none of the formalinized products stimulated immunity, animals were injected with sublethal doses of toxin and challenged with serial 2-fold dilutions of toxin. The protection that was observed from this experiment demonstrated that mice will produce antibodies when stimulated with unaltered toxin. Significant protection was observed against LD<sub>50</sub> or less amounts of toxin, but only minimal response was produced against the more severe challenges.

The surviving animals from toxin immunity assays were retained after the usual 24-hr duration for serological studies. During the second 24-hr post-challenge period, every group of experimental mice except one had animals that died. In the third 24-hr post-challenge period, a few more animals died. This had not been observed in previous experiments or when toxin titrations were done. Five control animals also died after the first 24 hrs post-challenge, but they all died within 30 hrs, and could be considered as part of the initial assay. Animals that died 72 hrs post-challenge may indicate the probability of dissociation of the antigen-antibody complex with resultant free lethal toxin.

No precipitation was observed on agar gel-diffusion plates using toxoid vaccinated rabbit serum and toxin vaccinated mouse serum against toxin and toxoid antigens. When the toxoid vaccinated rabbit serum was

concentrated to approximately one third of its original volume and the toxin was concentrated to 500 times the M.L.D., there was a faint line of precipitation. This information supports the theory of low antibody avidity reported by Glenny et al (1932) and Velich et al (1960).

The conclusion can be made that the formalinized products prepared by this investigator as well as the commercially prepared formalinized products did not produce immunity in mice. A generalization cannot be made as to what the results might be with other strains of the organism, other species of experimental animals or other methods of product preparation. But this information does indicate the need for further work in these areas.

It can also be concluded that the immunizing antigen is, in all probability, the toxin produced by C. sordellii. Further studies are necessary to determine if the dissociation of the antigen-antibody complex that was observed could be eliminated by extending the duration of time between vaccinations and challenge or if this was a phenomenon of enzyme action on the antibodies by the proteolytic enzymes produced by the organism (Smith, 1955).

Although this investigation has left many unanswered questions, it has determined the location of the immunizing antigen of C. sordellii and given some insight into some of the problems of in vivo antibody production. Also it is believed by this author that this work has opened some avenues for future investigation.

## SUMMARY

This study was designed to determine what antigen or antigens of C. sordellii would stimulate an immune response in mice.

Several types of immunizing products were selected for the experiment. A toxoid was prepared after selecting a medium and some conditions apparently necessary for optimum production of toxic culture fluids. A toxin was then prepared by precipitating broth culture supernatant fluids with ammonium sulfate and lyophilizing this precipitate. Some of the dried toxin preparation was dissolved in 1% peptone water and inactivated with formalin.

A spore bacterin was produced by obtaining heavy growth of spores on solid medium, separating the spores from the medium and vegetative cells by a series of washings, lyophilizing the washed spore suspension, suspending the dried spores in sterile saline and inactivating with formalin.

A vegetative cell bacterin was produced by growing C. sordellii cultures, harvesting them by a series of washing prior to sporulation, and producing a vegetative cell suspension which was inactivated with formalin to give a vegetative cell bacterin.

A whole culture bacterin was produced from liquid cultures containing vegetative cells, spores and toxins. These were harvested at their most pathogenic stage and inactivated with formalin to give a whole culture bacterin.

Also used in this study were commercially prepared whole culture bacterins that had been precipitated with aluminum hydroxide.

The immunizing ability of the above products was studied by a series of experiments consisting of vaccinating mice with the potential immunizing products and challenging with lethal doses of toxin or spores of C. sordellii. It was concluded from the results of these experiments that none of the formalinized products developed sufficient antibodies to protect mice from a lethal challenge.

Further studies were initiated replacing the formalinized products with sublethal doses of toxin. It was concluded that antibodies were produced in mice from the low level toxin stimulus, that a high level of immunity did not develop, that in this particular experiment the antibodies had a low avidity and that there may have been dissociation of the antigen-antibody complex.

It appears that the toxin produced by the C. sordellii is the antigen which will produce an immunogenic response. Future studies are necessary on methods of inactivation of toxin and the role of proteolytic enzymes on stability of the antigen-antibody complex.

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