STUDIES ON CERTAIN CHARACTERISTICS OF CLOSTRIDIUM CHAUVEI AND CLOSTRIDIUM EDEMATIS[1]

JOSEPH P. SCOTT

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INTRODUCTION

A knowledge of the exact biological factors involved in blackleg and blackleg-like diseases of cattle and other animals is essential in order to devise methods for controlling these diseases. The following experiments relating to cultural characteristics, pathogenicity, production of aggressive substances, immunization, and serological tests have been made in order to determine certain characteristics that may serve to differentiate *Clostridium chauvei* from allied anaerobic spore-forming rods. In these experiments thirty-six strains of *Clostridium chauvei* have been used for the more important tests, seventeen in the cultural tests and a smaller series for some of the less important determinations.

HISTORICAL RÉSUMÉ

In 1782 Chabert differentiated between anthrax and blackleg on symptomatic and pathological grounds. The causative agent of blackleg was described by Bollinger (1875) and Feser (1876). Pasteur, who by the isolation of *Bacillus butyricus* in 1861 opened up the field of anaerobic bacteriology, in 1877 isolated (Pasteur 1877) a pathogenic anaerobe, the *Vibrion septique*, from the blood of a horse and a cow. In the following year Arloing, Cornevin and Thomas (1876) reported on the artificial cultivation of *Bacterium chauvei* in chicken broth containing either glycerol

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or iron sulphate. Koch (1886) grew the *Rauschbrandbaccillus* on gelatine and potato. Liborius (1886) simplified the study of anaerobes and described eight methods of isolating and growing these organisms in pure culture. Roux (1887) used a method of growing anaerobes in sealed flasks containing slightly alkaline broth. Later he and Chamberland (1887) isolated toxin from pure cultures of the *Vibrion septique*. This toxin, when used in doses up to 40 cc. and given in two doses, produced an active immunity in guinea-pigs. Filtered muscle juices and tissue exudates proved to be more toxic, but could be used in immunization if the dosage was graduated. The following year Roux (1888) found that toxins could be produced from filtered cultures of *C. chauvei* and from tissue exudates of blackleg cases. He found that guinea-pigs immunized with these toxins or with attenuated pure cultures of the *Vibrion septique* were immune to the *Vibrion septique* but not to blackleg. Guinea-pigs similarly immunized against blackleg were immunized against both diseases.

Kitasato (1889) found that the addition of glycerol or of 1 to 2 per cent glucose to broth did not increase the growth of the blackleg organism. He used solid and liquid media to which he added fresh guinea-pig tissue. He was able to immunize guinea-pigs by the use of old and attenuated broth cultures of *C. chauvei*. The young of female guinea-pigs vaccinated while pregnant proved to be immune up to the age of fifty days. In 1890 Kitasato and Weyl used various reducing substances incorporated in agar and broth for the cultivation of anaerobes (Kitasato and Weyl, 1890). In a second paper (1890) Kitasato described the blackleg organism as a single rod and occasional pairs forming an acute angle one with another. He found that sporulation did not occur until some hours after death.

Leclainchee and Valée (1900) recognized the cultural semblances between the *Vibrion septique* and *C. chauvei*. T differentiated these organisms by the agglutination test and the use of actively and passively immunized guinea-pigs. T also produced a very virulent toxin from broth cultures *C. chauvei*.

Grassberger (1902) described two types of the blackleg organism, one non-motile and non-spore-forming, the other motile and sporulating. Shattenfroh (1903) found numerous intermediate types between the *Vibrion septique* and blackleg. In 1904 Grassberger and Shattenfroh produced a toxin from pure cultures of the *Rauschbrandbaccillus*. They found that different strains had to be given different substances for the best production of the toxin, either glucose or calcium lactate having to be supplied. They (Grassberger and Shattenfroh 1907) also produced an antitoxin by immunizing animals with this toxin. This antitoxin was used in differentiating blackleg from other diseases. It was found that tissue extracts also contained a toxin and they described two groups of *C. chauvei*, (1) a highly virulent non-toxic group and (2) a slightly virulent toxic group.

Werner (1905) differentiated the gas gangrene organisms by means of the agglutination test, using agar cultures grown in an atmosphere of hydrogen, both for immunization and as the antigen in the test.

Eisenberg (1907) produced a very toxic product by decantation and centrifugation of six-day broth cultures of *C. chauvei*. Doses of 0.005 cc. of the toxin killed guinea-pigs in a few minutes. Pfuhl (1917) described a method of growing anaerobes aerobically in liver broth. He found that liver broth absorbed oxygen and produced carbon dioxide. He reviewed the previous attempts to grow anaerobes aerobically, those of Catoni (1891) who grew the tetanus bacillus in rabbit blood, of Tarrozzi and Wryosek (1905) who used nutrient broth containing pieces of fresh meat and of Harass (1906) who used liverbrew-liverbroth and brainbrew-brainbroth media.

Von Hibler (1908) described the characteristics of fifteen anaerobic species, noting their morphological and cultural characters in detail. These organisms were grouped by means of the deep agar colony formation, the blackening of brain medium and their proteolytic characters. He found that *B. welchii*, *B. sporogenes*, blackleg and pseudo-blackleg organisms formed smooth colonies, the other anaerobes irregular or woolly colonies.

Bâlavaine (1909) discussed the results of vaccinating cattle

against blackleg. He found that vaccination might cause death from blackleg or might fail to give lasting immunity.

Foth (1909-1910) discussed the differentiation of blackleg from anthrax and from other anaerobic diseases; using the agglutination reaction he described *C. chauvei* as a single rod, while most other organisms found in similar diseases were chain-forming rods. He found that the characteristic chains or single rods were formed on the diaphragmatic surface of the liver.

Hasenkamp (1909) described a case of blackleg in a horse, diagnosing the condition after finding numerous blackleg organisms in smears from the affected tissues.

Schöbl (1919) described a method of immunizing guinea-pigs by the use of filtered blackleg exudates, or aggressins.

MacCrudden (1910) studied the proteolytic properties of *C. chauvei* and *Clostridium salus*, finding that *C. salus* produced gas, tyrosin, leucin, and trytophane, while *C. chauvei* produced little gas and tryptophane. Neither produced indol and skatol. Veillon and Masé (1910) found that the addition of potassium nitrate to agar facillitated the Veillon and Zuber deep agar colony method of isolation.

Markoff (1911) compared the organisms isolated from typical cases of blackleg with parturient blackleg and also with some chain formers. He found that the blackleg group are pathogenic for old guinea-pigs only, parturient blackleg types for both young and old guinea-pigs, and the chain formers for all animals. Differentiation could be made by means of the guinea-pig lesions, stained liver smears, toxin production, and the agglutination test. He found it possible to use the microscopic agglutination test in the examination of impure or mixed cultures.

Diederichs(1911) reported on two cases of disease in horses. One was diagnosed as pseudo-blackleg, since the organism isolated was pathogenic for guinea pigs and rabbits, the other as true blackleg, since the organism in this case was pathogenic for guinea-pigs only.

Möller (1911) found that the glycogenic content of muscles determined the extent of the lesions produced. He found that guinea-pigs fed on sugar beets developed very extensive lesions

when injected with blackleg virus, while starved animals developed only slight lesions or were refractory to the inoculation.

Wulff (1912) used the agglutination reaction to differentiate blackleg from other conditions. He disproved the theory that blackleg is a wound infection by demonstrating (1) that there are numerous cases of blackleg showing no muscle or subcutaneous lesions, and (2) that blackleg occurs when there are neither macroscopic nor microscopic wounds. In a second paper (1912) he showed that both blackleg and malignant edema organisms may be found in the bile, thus disproving von Hibler's observation on malignant edema and showing that the presence or absence of organisms in the bile is of no value for diagnostic purposes.

Hölzel (1913) grew *C. chauvei* aerobically in broth containing starch.

Lechlainchee and Valée (1913) reported on the production and use of a pure culture blackleg vaccine obtained by growing cultures at 42°C.

Grassberger and Shattenfroh (1913) stated that biological reactions are of secondary importance in the differentiation of blackleg from other diseases and that the most reliable test is the production of the toxin by the culture and the use of blackleg antitoxin to test its specificity.

Detre (1913) immunized horses by the use of pure cultures of *C. chauvei.* He showed that the serum contained the specific agglutinins and that doses of 0.005 to 0.02 cc. protected guinea-pigs.

Von Ratz (1913) found that swine were susceptible to blackleg.

Mieszner (1913) used the Abderhalden dialysis reaction for the differentiation of blackleg.

Lesage and Pommier (1913) reported on an outbreak of blackleg in sheep following lambing.

Hecht (1913) used the Ascoli thermo-precipitin reaction for the diagnosis of blackleg.

Köves (1914) studied the so called symptomatic anthrax of swine and isolated a typical Ghon-Sachs organism from the intestinal lesions. This organism produced typical muscle lesions

in inoculated hogs. Later (1918) he studied this disease in detail and compared the organisms isolated with *C. chauvei* and malignant edema cultures.

Nicolle, Cesari, and Raphael (1915) experimented with the toxins of *C. chauvei* and the *Vibrion septique* and found that the toxicity was greatly reduced at 55 degrees. They stated that the cultural and serological differences between these two organisms are slight.

Meyer (1915) isolated the Ghon-Sachs organism from symptomatic anthrax of swine.

Von Wasserman (1916) compared the gas gangrene organism with *C. chauvei*. He found that the intestinal types of gas gangrene and blackleg or bradsot were very similar.

Richter (1916) showed that parturient blackleg of cattle and blackleg of horses are mixed infections in which malignant edema organisms can usually be found. *C. chauvei* also may be found in some cases. He also described a septicemic type of true blackleg.

Conradi and Beiling (1916) used the agglutination test to divide the malignant edema organisms into two groups, these groups being transformable from one into the other by proper cultural methods.

Franklin and Haslam (1916) showed that blackleg powder and pellet vaccines are unsatisfactory. Later (1920) they reported on the isolation of a pseudo-blackleg organism from some of this material.

Goss (1917) reported on the production of blackleg aggressin and anti-serum, and later (1919) on the production of blackleg filtrate. In the following year he and Scott (1918) reported on the potency tests for these products and described a potency test based on aggressive action.

Todd (1917) reported on the gas production of *C. chauvei*, *B. edematis-maligni* Koch, and other anaerobes.

Warnecke (1917) described a disease in a young calf which resembled blackleg but from which he isolated an organism resembling *Bact. coli*.

Fürth (1917) isolated a diplobacillus from cases of human

gangrene; he agglutinated this organism by blackleg anti-sera, and identified it as *C. chauvei.*

Landau (1917) found that the results from the agglutination test and from protection tests with blackleg and malignant edema anti-sera do not coincide.

Frommeld (1917) found that blackleg cultures containing spores were killed by exposures to thirty hours of direct sunlight.

Bohler (1917) described a case of blackleg in a horse suffering from shrapnel wounds.

Jensen (1917) described 10 cases of bradshot in sheep. He found characteristic intestinal ulcers from which the organism was isolated. Feeding experiments with these cultures gave negative results. He suggested that the cadaver bacillus which was also found in the diseased animals played a part in the infection. Histological studies proved that the organism entered through the digestive tract.

Foth (1918) described the use of a new blackleg vaccine "Emphysarcol," consisting of an attenuated suspension of spores and a spore-free filtrate. The attenuated spores are injected into the tail, the filtrate into the ear. Kelser (1918) discussed the immunity produced by blackleg filtrates. Berg (1918) described a method of concentrating this toxin in vacuo.

Pfeiler (1918) used the precipitin reaction for the diagnosis of anthrax, blackleg, swinepest and other diseases.

Szasz (1918) found it inadvisable to vaccinate cattle against anthrax and blackleg at the same time. Blackleg vaccination should be given fourteen days after the anthrax vaccination.

Schmitt (1918) diagnosed as blackleg a disease in a foal from which he isolated an anaerobe. This anaerobe occurred as a single rod or in pairs forming an angle one with another.

Nitta (1918) described the Japanese method for producing blackleg filtrate.

Steinbrucke (1918) stated that the gas gangrene organisms belong to the blackleg group.

Weinberg and Séguin (1918) discussed the etiological factors of gas gangrene and clarified the classification of malignant edema organisms and the *Vibrion septique.* They did not find *C. chauvei* in war wounds.

Van Heelsbergen (1919) found that the etiological factors of gas gangrene, malignant edema and blackleg were very similar. He thought that they should be classified under one species as Grassberger and Shattenfroh had suggested. Haslam and Lumb (1919) showed that the toxicity of filtrates had no relationship to their potency.

Zeiszler (1919) showed that spontaneous blackleg, parturient blackleg, whale septicemia and reindeer pest are all primarily caused by the blackleg organism. He isolated *C. chauvei* from a few cases of human gas gangrene and found that the agglutination reaction was unreliable for the differential diagnosis of anaerobic organisms.

Ravenna (1920) described the cardiac, pulmonary and arterial lesions of blackleg and showed the disease to be a septicemia.

Graub and Zschokke (1920) found that germ-free filtrates protected cattle against 2 M.L.D. virus. The immunity produced by these filtrates was greatly increased by the use of an attenuated virus given ten days after the filtrate.

Speigl (1920) found that blackleg in sheep might be due to Foth's blackleg organism, to the Ghon-Sachs organism or to *B. welchii.*

Haslam (1920) tabulated the results following the use of blackleg aggressin. He described the methods for testing the sterility of this product and found that brain liver medium inoculations were much more reliable than animal inoculation.

Ronca (1920) observed that the digestive juices do not injure *C. chauvei.* He produced blackleg in guinea-pigs by feeding very virulent cultures. Infection was regularly produced if the organism was introduced into a loop of the intestine. In animals infected by way of the digestive tract the characteristic lesions were found in certain parts of the body.

Heller (1920) discussed the etiological factors of blackleg and allied anaerobic diseases. She found that cattle are very susceptible to infection by the blackleg group and are occasionally affected with the *Vibrion septique* group; sheep, horses, and swine are usually affected by the *Vibrion septique* group; sheep occasionally by the *Chauvei* group and horses sometimes by

the *Oedematiens* group. She found *B. welchii* infections in animals very rare.

Gochenour (1920) used the complement fixation reaction for the identification of *C. botulinus* in canned foods.

Goss, Barbarin and Haines (1921) found that *C. chauvei* does not ferment carbohydrates. They used the passive immunity produced by anti-blackleg serum as a means of differentiating the pathogenic anaerobes. They found that anti-blackleg serum agglutinated blackleg strains in dilutions of 1:800 and *Vibrion septique* strains in dilutions of 1:200.

Ronca (1921) studied the changes in the blood pictures of animals injected with blackleg virus and toxin. He found first a decided increase followed by a progressive decrease in certain types of leucocytes in animals that died; in animals that recovered, the decrease was followed by an increase in both leucocytes and erythrocytes.

Scott (1921) described the methods of producing anti-blackleg serum and showed that the potency of this product did not decrease for several years.

Hall (1922) described the various species of spore-bearing anaerobic rods. He used spore formation as the primary criterion of classification. He found that *C. chauvei* ferments sucrose but that the *Vibrion septique* does not.

Scott (1922) described the production of blackleg anti-serum, blackleg aggressin and blackleg filtrate, and later (1923) reported on potency tests for aggressin and filtrate based on the aggressive action of these products.

Leclainchee and Valée (1923) supplemented their previous work on toxins. They reported on the production of a toxin from an extract of cells obtained from a twenty-four hour culture of *C. chauvei* and on a non toxic extract made from cells in a six day culture.

Göertiller (1923) stated that both cultural and immunological tests are necessary for the proper identification of anaerobes. He found that the most satisfactory tests were the toxin-antitoxin test and the immunization of guinea-pigs.

Reddish and Rettger (1924) described several sporulating

anaerobes. Their type *C. chauvei* strain produced deep agar colonies having a dense nucleus and radiating projections. In culture media short chains were found to be not uncommon.

HISTORY OF CULTURES USED

In the course of this work 35 strains of *C. chauvei* and 5 strains of *Clostridium edematis*[2] have been studied.

C. chauvei strains

Strains 1 to 10 were isolated between 1911 and 1916 by Drs. O. M. Franklin and T. P. Haslam from blackleg material obtained from field cases in Kansas and other states. These ten strains were used to produce the serials of blackleg anti-serum which were used in the serological tests. Strains 3, 4, 8 and 9 were discarded between 1921 and 1923 due to decreased virulence or to contamination.

Strain 11 was isolated from dried muscle obtained from a case of blackleg in 1917. It was discarded in 1919. Strain 12 was isolated in 1917 from blackleg muscle. Strain 14 was obtained in 1918 by Dr. L. W. Goss of the Kansas experiment station from a vial of liquid blackleg vaccine purchased from the Pasteur Laboratories; it was discarded in 1921. Strain 15 was isolated in 1918 by Dr. L. W. Goss from dried blackleg muscle sent from the Bureau of Animal Industry, United States Department of Agriculture, Washington, D. C. It was discarded in 1921.

Strain 16 was isolated in 1918 by Dr. Goss from blackleg tissue obtained from a field case of blackleg in southern Kansas. This strain was discarded in 1920 due to decreased virulence.

Strain 17 was obtained in 1918 from the Continental Serum Company. It was discarded in 1921.

[2] The term *Clostridium edematis* has been used in this paper instead of *Vibrion septique*, in accordance with the report of the Committee of the Society of American Bacteriologists. Jour. Bact., vol. 5, no. 3, p. 22, 1920. Bergey in Bergey's Manual of Determinative Bacteriology, 1923, p. 325, uses the term *Clostridium oedematis-maligni* presumably to avoid confusion with *clostridium Cedematiens*.

Strain 18 was isolated in 1918 by Dr. Goss from a case of blackleg. It was discarded in 1921.

Strain 19 was obtained in 1919 from Dr. Haslam of the Purity Biological Company.

Strain 20 was isolated in 1919 by Dr. Goss from material obtained from a case of blackleg in Riley county. This strain was discarded in 1921.

Strain 22 was obtained in 1919 from a vial of liquid blackleg vaccine obtained from the Jensen Salsbery Laboratories. It was discarded in 1921.

Strain 23 was isolated in 1921 from blackleg material taken from a calf inoculated with culture virus strain 1.

Strain 24 was obtained as a pure culture from Parke, Davis and Company in 1921.

Strain 25 was obtained as a pure culture from Miss H. H. Heller of the Hooper Foundation, San Francisco. This strain was discarded in 1921.

Strain 26 was isolated in 1921 from a field case of blackleg; it was discarded in the fall of 1921.

Strain 27 was isolated in 1922 from a calf inoculated with culture 21.

Strain 28 was isolated in 1922 from dried blackleg muscle obtained from the Mulford Company.

Strain 29 was isolated in 1922 from dried muscle tissue from a case of blackleg sent in for diagnosis.

Strain 30 was obtained in 1922 from blackleg material sent from the Mulford Company.

Strain 31 was isolated in 1922 from a case of blackleg in a yearling calf in Riley county, Kansas.

Strain 32 was isolated in 1922 from muscle tissue sent in for diagnosis from a case of blackleg in Wakefield, Kansas.

Strain 33 was obtained as pure culture from Prof. I. C. Hall of the University of California, Berkeley, California.

Strains 34 and 35 were isolated from an outbreak of blackleg in a herd of purebred cattle in Riley county, Kansas.

Strain 36 was isolated in 1924 from a case of blackleg treated in the ambulatory clinic.

Clostridium edematis strains

Strains C.E. 1, C.E. 2 and C.E. 3 were obtained from the Lister Institute, London. These strains were labeled *Vibrion septique* types, 1, 2, and 3.

Strain C.E. 4 was obtained from Prof. I. C. Hall of the University of California.

Strain C.E. 5 was isolated in 1922 from a case of malignant edema or so-called "symptomatic anthrax" in a hog.

CRITERIA USED TO DETERMINE THE PURITY OF C. CHAUVEI STRAINS

Up to 1920 cultural and morphological reactions were considered a sufficient indication of purity. At that time, after isolation of the strain by means of the deep agar colony method, eight cultural and morphological reactions were introduced.

1. In deep agar only those strains producing small spherical or lenticular colonies with definite solid borders were considered as typical.

2. In brain liver medium typical cultures showed cloudiness and gas production in from two to four days, after which the fluid portion rapidly cleared due to sedimentation of the organisms and spores. The brain never showed any darkening and the broth, if dark on inoculation, became amber colored on clearing.

3. The culture when freshly isolated must be pathogenic for guinea-pigs. Since 1920 cultures that have lost their virulence have not been discarded but are still used for production of filtrate. Typical edematous swellings must be produced in guinea-pigs around the point of inoculation and death must follow within three days.

4. The typical single Gram-positive rods with rounded ends must be found both in culture smears and in impression preparations made from the peritoneal surface of the liver of inoculated guinea-pigs.

5. The typical blackleg odor should be obtained both from the culture media and from guinea-pigs inoculated with the culture. However, some strains of *C. chauvei* produce no noticeable odor either in guinea-pigs or in culture media.

6. Guinea-pigs immunized by inoculation of 7 units of anti-blackleg serum must withstand the administration of 5 M.L.D. of the culture, given fifteen hours after the serum.

7. Inspissated blood serum must show no changes when inoculated and incubated anaerobically for five to seven days.

8. No growth may develop in 2 per cent glucose beef infusion agar cultures.

PATHOLOGICAL LESIONS

Blackleg is an acute, infectious, but non-contagious disease of cattle, and exceptionally of other ruminants. Young cattle from six months to two years of age are found to be most susceptible. The disease is characterized by a sudden appearance of lameness, and gas production in the affected tissues, followed by prostration and death in from twelve to thirty-six hours.

The lesions produced in calves are essentially muscular and subcutaneous in character. On opening the animal the characteristic blackleg odor is first noticed and extensive subcutaneous lesions containing dark-red exudate and gas may be found. The muscles of one of the quarters are found to be greatly distended, due to accumulation of numerous small gas bubbles throughout the muscle tissue. Areas and streaks of blackened muscle are found, intermixed with lighter pink areas, giving the muscle a characteristic mottled appearance. Some muscles show no black spots although filled with gas bubbles and of a pink color. The lesions have a tendency to be limited to single muscles and do not pass through the intermuscular septa. The internal organs are fairly normal in a fresh carcass. The serous membranes are injected and a black clot is found in the heart. No marked gas formation is noticed in the intestines. The peritoneal and pleural cavities contain large amounts of a dark-red exudate.

The disease in guinea-pigs inoculated with blackleg material in the axillary space follows a characteristic course. There is first a clearly defined edematous swelling developed around the point of inoculation, which extends to the pelvis in from thirty-six to seventy-two hours. The height of the swelling is from 2 to 4 mm. If death does not take place the hair over the

lesion is shed and the skin along the median line usually opens disclosing a line of blackened muscle. If death occurs, and the animal be opened, the subcutaneous tissue are found to be full of a red exudate; there is some gas in the muscles and they are either black or pinkish in color. The serous membranes are injected, and the blood in the heart is black in color. No gas is found in the intestines unless the animal has been dead for more than twelve hours.

MODE OF ENTRANCE OF THE ORGANISM

It has usually been considered that *C. chauvei* enters the body by means of small skin abrasions. Since 1912 considerable work has been done based on this supposition. It has been shown that the infection can enter through the digestive tract, but no evidence has been brought forward to show that the organism ever enters by way of the skin.

Wulff (1912) discounted the wound infection theory on two grounds: (1) That there were numerous cases of septicemic blackleg in which no muscles lesions were found, but from which the blackleg organism was isolated, and (2) he was unable to find any case in which skin wounds could be demonstrated, although he examined hundreds of microscopic sections from blackleg lesions. In confirmation of this the following case may be cited. In the author's work, during the last year, one case of the septicemic form of blackleg was encountered in a steer eight months of age which had been vaccinated at five months of age. It was noticed to be off feed one evening; next morning it was slightly lame and was dead by noon. When examined, the carcass was very badly distended with gas; no muscle lesions could be found; the serous membranes were injected; the heart contained a black clot; and the characteristic blackleg odor was very noticeable. A pure culture of *C. chauvei* (strain 37) was isolated from this case together with an unidentified anaerobe whose connection with the disease has not yet been demonstrated. Several other cases of the septicemic type have been reported to us from material sent to the laboratory for diagnosis. From some of this material *C. chauvei* has been isolated.

Ronca (1920) produced blackleg in guinea-pigs by feeding virulent cultures and also by introducing cultures into the intestinal canal.

In a recent outbreak in Kansas the infection was traced to pigs that had fed on the carcass of an animal that had died of blackleg. These pigs walked through the manger of some steers in an isolated lot. Some of these animals developed blackleg within three days. In another case a yearling steer developed a hard swelling of the brisket within twelve hours lameness in the right hind leg developed, followed by a swelling in the same quarter and death from blackleg in forty-eight hours. This case showed that the primary lesion may be located at a considerable distance from the final muscle lesion and also that blackleg is not a true wound infection, which presupposes greater or less destruction of tissue in which the organism must develop.

In the case of the similar disease "malignant edema" of swine or so-called "symptomatic anthrax." Koves (1914) showed that intestinal lesions were produced from which the causative organism could be isolated. This organism produced the typical black muscle lesions in other swine.

ISOLATION

All strains studied have been isolated by (1) the cultural method or (2) by the guinea-pig inoculation method.

In the cultural method brain liver medium is inoculated with some of the material to be isolated, and is then heated to between 65° and 70°C. and held at this temperature for fifteen minutes. A check tube is also inoculated from unheated material, to be used in case the heated tubes prove to be sterile. After twenty-four hours incubation three or four serial dilutions are made, into freshly heated liver agar tubes at 45°C. Twenty four hours later typical colonies are picked off into brain liver medium. This is repeated once a day till at least four sets of agar dilutions have been made. The culture is then tested on agar slants for anaerobic contamination and is inoculated into a guinea-pig for preliminary identification tests.

At the same time that the brain-liver medium is inoculated,

a guinea-pig is injected with some of the material and the organism isolated from the muscle lesions and heart blood as soon as the pig dies. In both cases the strain is preferably reisolated from the test guinea-pig injected with the culture after preliminary isolation.

MORPHOLOGY

C. chauvei is a medium-sized rod with rounded ends. It is Gram-positive except in old cultures which may show Gram-negative rods. The organism is from four to six times as long as it is wide, 3 to 6 microns in length by 0.5 micron in width. In smears made from the peritoneal surface of the liver the Gram-positive rods are easily found. They are usually well scattered throughout the preparation, not more than six to ten organisms being found in any one field. Organisms containing oval sub-terminal spores may be found in preparations made from animals that have been dead for some time. Usually these spore-bearing rods are very scarce. Pairs, in which the organisms are end to end, may occasionally be found and very rarely chains of three or four elements may be seen. In slides made from culture media the picture is very similar. In young cultures spore-bearing rods are often absent. In older cultures free spores are usually present and these are frequently unstained. Paired organisms are perhaps even less common than in liver preparations. In slides made from *C. edematis* cultures there are usually a large number of rods in each field of which a relatively large number are spore-containing. Chains of two to six or more elements are fairly common. Some strains of *C. edematis*, notably type 2, usually occur in pairs in which the elements form an acute angle one with another. Chains in this strain are uncommon.

COLONY FORMATION

Surface and deep agar colony forms have been used by numerous authors as one of the important methods of differentiation between *C. chauvei* and other anaerobes.

Kitasato (1890) described the colonies formed by *C. chauvei* as having a central nucleus surrounded by numerous fine pro-

jections. Von Hibler (1908) described the deep agar colonies as lenticular or spherical in shape and having a smooth solid outline. Zeiszler (1920) describes two types of *Rauschbrand:* The surface agar colonies formed by the Kitt type being circular with a solid border or veil like, and the Foth type forming pearl button colonies having a raised center. Kitt, as quoted by Heller (1920), described the deep-agar colonies as fine, point shaped, or small, woolly spherical colonies. Heller found colonies with smooth outlines only. Goss et al. (1921) described the colonies of *C. chauvei* in 2 per cent agar as small, spherical or elliptical, and translucent, while colonies formed by *C. edematis* were woolly or fluffy. They state that in soft agar (one per cent) the colonies of *C. chauvei* may resemble those of *C. edematis.* Hall described the deep agar colonies as semi-transparent opaque spheres with or without raylike growths. Reddish and Rettger (1924) describe the deep agar colonies as being about 1 mm. in diameter and as having a solid nucleus surrounded by irregular projections radiating out in matted formation.

In 2 per cent liver agar all strains considered in this paper produced very small colonies, spherical or pin point in character. In some cases when the agar was considerably concentrated due to evaporation invisible colonies were formed and the only indication of growth was small gas bubble formation. In 1 to 1.5 per cent agar the colonies are larger and may be small circular discs or lenticular in shape. In all cases a regular smooth definite border has been noted. The colonies formed by the *C. edematis* strains studied were typically biconvex-lenticular in shape with the edges thin and flat. Outgrowths especially from the lower surface are common. In old colonies, double discs have been found. In soft agar markedly fluffy colonies are produced.

CULTURAL CHARACTERISTICS

Milk

Von Hibler (1908) found that *C. chauvei* produced a fine clot in forty-eight to ninety-six hours. Hall (1922) found that in milk containing sterile blood, coagulation may occur. Goss et al.

(1921) found that *C. chauvei* will not grow in litmus milk even when inoculated in large amounts, while *C. edematis* produces acid fairly readily. Reddish and Rettger (1924) reported that an acid clot is formed by both types.

None of the 35 strains considered in this paper produced any change in fresh, litmus, or brom-cresol-purple milk, when inoculated in amounts of from 1 to 6 loopfuls. However, when 1 mil of brain liver culture was used a slight thickening of the milk was noticed in some cases after forty-eight hours. *Clostridium edematis* strains produced coagulation in fresh milk when four to six loops of brain-liver culture were used as the inoculum.

Gelatin

Bacto gelatin containing 1 part glucose to 1000 was liquefied; while gelatin made from Gold Seal gelatin, even when made with liver broth or containing blood serum was not liquefied after two weeks' incubation in the anaerobic jar.

Coagulated serum

Coagulated serum is not affected by *C. chauvei* even after a month's incubation in the anaerobic jar.

Carbohydrate fermentation

The fermentation of carbohydrates has been studied by numerous workers. Nicolle, Cesari, and Raphael (1915) found no great differences between *C. chauvei* and *C. edematis*. Meyer (1915) also found practically no differences between the two types. However, the strain he described as *C. chauvei* (Munich) may be the same as the strain of *C. edematis* described by Hall (1922) as Meyer's *C. chauvei* Kitt, (Munich), in which case both organisms are to be considered as *C. edematis* strains. Goss et al. (1921) found that *C. chauvei* does not ferment any of the carbohydrates. These findings are shown in table 1. From table 1 it is seen that there is considerable difference in the results obtained. With the exception of salicin, in which all workers found no fermentation by *C. chauvei* and fermentation by *C. edematis*, there is a difference between at least one report and

TABLE 1

Carbohydrate fermentations as reported by various workers

CARBOHYDRATE	GOSS ET AL.		HALL		MED. RES. REPT. 39 ROBERTSON		REDDISH		PRESENT PAPER	
	Ch.	C.E.	Ch.	C.E.	Ch.	C.E.	Ch.	C.E.	Ch.	C.E.
Amygdalin	–	A								
Arabinose							–	A G	F	F
Dextrine	–	A	–				A G	A G	F	F
Dulcitol	–	A			–	–			–	F
Galactose					F	F	A G	A G	F	F
Glucose	–	A G	A G	A G	F	F	A G	A G	F	F
Glycerol	–	A	–	–	–	–	A	–	F	F
Glycogen	–	A G					–	–		
Inulin	–	A	–	–			–	–	F –	F
Lactose	–	A G			F	F	A G	A G	F	F
Levulose	–	A G	A G	A G	F	F	A G	A G	F	F
Maltose	–	A G	A G	A G	F	F	A G	A G	F	F
Mannitol	–	A	–	–	–	–			–	F
Mannose							–	A G	F –	F
Raffinose	–	A					–	–	F	F
Rhamnose							–	–	–	F
Sucrose	–	A	A G	–	F	–	A G	–	F	– F
Salicin	–	A G	–	A G	–	F	–	A G	–	F
Sorbite	–	A								
Soluble starch							–	–	–	F
Trehalose							–	A G		
Xylose	–	A					–	–	–	F
Amylum solution					–	–				

–, no fermentation; F, fermentation; A, acid; G, gas; –F, most strains do not ferment; F–, most strains ferment. Medical Research Committee Report 39 as quoted by Heller.

the others. These conflicting reports may be due to differences in the strains studied, to the use of only a few strains, or to differences in the medium used as a base for the carbohydrates.

During the past four years various liquid and solid media have been used by us for the fermentation reactions of *C. chauvei*. The liquid media, with the exception of serum water, were made sugar-free in the ordinary way, brom-cresol-purple was then added and after sterilization 1 to 2 mils of 5 to 10 per cent carbohydrate solution added. The inoculated tubes were placed in a wire rack and held in the anaerobic pressure cooker. Anaerobic conditions were produced by burning phosphorus according to the method described by Bushnell (1922).

Sugar-free infusion broth prepared in the ordinary way and to which dextrine, galactose, glycogen, glucose, glycerol, inulin, lactose, maltose, mannitol, salicin, sucrose, or starch were added, was inoculated with one loopful of a twenty-four hour brain-liver culture, placed in the anaerobic cooker and incubated at 37°C. for ten days. Of ten strains so tested one produced acid and gas in glucose, lactose, maltose, and sucrose, all other tubes remaining unchanged.

Sugar-free liver broth: Four strains were tested on the same sugars using sugar-free liver broth as a base. No fermentation of any of the sugars was produced.

Serum water

The effect of six strains of *C. chauvei* and of strains 1, 2, 4 and 5 of *C. edematis* on the 20 carbohydrates listed in table 2 was determined. No fermentation of any of the carbohydrates was produced by any of the *C. chauvei* strains. All strains of *C. edematis* produced acid and caused coagulation of the serum so that gas production by these strains could not be recorded.

Serum water agar

Serum water agar was prepared by adding 20 grams of agar to 1000 mils Hiss serum water and proceeding with the ordinary method of agar preparation. The carbohydrate solutions were added while the agar was liquid and had been cooled to 40°C.

TABLE 2

Fermentation reactions of 17 strains of Clostridium chauvei and of five strains of Clostridium edematis in Serum Media

CARBOHYDRATE	1	3	5	6	7	10	12	19	23	24	27	28	29	30	31	32	33	C.E. 1	C.E. 2	C.E. 3	C.E. 4	C.E. 5
Arabinose	A G											A G					A	A G	A G	A G	A G	A G
Dextrine	A G											A G					–	A G	A G			
Dulcitol	–											–					–	A G	A G		A G	A G
Galactose	–	A G	A G	A G	A G	A G	A G	A G	–	A	A G	A G	A G	A G	A G	–	A G	A G	A G	A G	A G	A G
Glucose	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G
Glycerol	A G	A G	A G	A G	A G	A G	A G	A G	–	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G
Inulin	A G	A G	A G	–	A G	–	–	A G	–	A G	A G	A G	–	A G	A G	–	–	A G	A G	A G	A G	A G
Inositol	–											–					–	A G	A G		A G	A G
Lactose	–	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G
Levulose	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G
Maltose	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G
Mannitol	A G	A G	A G	–	–	–	A G	–	–	A G	A G	–	–	–	–	A G	A G	A G	A G	A G	A G	A G
Mannose	A G											A G					–	A G	A G	A G	A G	A G
Raffinose	A G											A G					A	A G	A G	A G	A G	A G
Rhamnose	–											–					–	A G	A G	A G	A G	A G
Sucrose	A G	A G	A G	A G	A G	A G	A G	A G	–	A G	A G	A G	–	A G	A	A G	A G	A G	A G	A G	–	A G
Salicin	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A G	A G	A G	A G	A G
Starch	–											–					–	A G	A G	A G	A G	A G
Xylose	–											–					–	A G	A G	A G	A G	A G

A, acid production; G, gas production.

Fifteen strains of *C. chauvei* and the 12 carbohydrates listed in experiment 1 were used in this experiment. No fermentation was produced in any carbohydrate by any of the strains used. *C. edematis* strains also showed no fermentation in this medium but cultures of proteolytic anaerobes fermented most of the carbohydrates.

Sugar-free meat infusion or serum water agar plus sterile serum was found to be a very satisfactory medium for the study of fermentation reactions of both *C. chauvei* and *C. edematis*. Three sera were used (1) fresh and unfiltered serum, (2) fresh filtered serum, and (3) old filtered serum containing 0.05 per cent phenol. No marked differences were noticed in the fermentations produced in these three media.

Table 2 shows the results obtained from the fermentation tests on twenty sugars by seventeen strains of *C. chauvei* and five strains of *Clostridium edematis*. Some strains did not always give a consistent reaction and a negative reading has been recorded only when all tests showed a negative reaction.

It is seen that *C. chauvei* is much less saccharolytic than the *C. edematis* strains examined. Sucrose in one case was not fermented by *C. chauvei*. In all cases slight gas production was not counted as an evidence of fermentation unless acid was produced in the same tube.

PATHOGENICITY

Blackleg, although primarily a disease of cattle, has been reported in horses, swine and sheep. A disease of whales was reported by Nielson (1890) and by Christiansen (1920) from which an organism resembling, or identical with, *C. chauvei* was isolated.

The reports of the isolation of *C. chauvei* from horses are not conclusive. The diagnosis has usually been made on purely morphological grounds, or on morphological and guinea-pig and rabbit pathogenicity tests, as in the cases reported by Diederichs (1911). In the production of anti-blackleg serum normal horses were injected (Scott 1923) intravenously or subcutaneously with 25 to 30 mils of virulent cultures of *C. chauvei* without developing

any symptoms whatever. This must be considered conclusive evidence that horses are not susceptible to *C. chauvei*.

Reports of the isolation of *C. chauvei* from swine are occasionally met with in the literature. In these cases the diagnosis is also incomplete, being based on morphology, or on unstated cultural and other characters. On the other hand, all detailed experiments on the so-called "symptomatic anthrax" of swine (Koves, 1914 and 1918; Meyer, 1915) have shown that this disease which has symptoms very similar to blackleg is caused by a *C. edematis* type organism. This organism when injected into the muscles of hogs produces muscle lesions almost identical with those of blackleg in cattle. At the Kansas Agricultural Experiment Station two outbreaks of this disease in swine have been examined and from both a *C. edematis* type organism has been isolated.

One experiment on the susceptibility of swine has been made at the Kansas Station. A pig weighing 100 pounds was given 15 mils of virulent *C. chauvei* culture at the same time that a calf was given the same amount. The pig showed no rise in temperature and no lameness, while the calf died of blackleg within thirty-six hours. This experiment, taken together with the isolation of *C. edematis* strains in the disease of swine, shows that this disease is rare in swine. The transmission experiments reported by von Ratz (1913) were made by the use of muscle pulp, a method open to serious objection, as anaerobes other than *C. chauvei* can usually be found in blackleg muscle, especially from field cases. The material used by von Ratz was pathogenic for rabbits, which would suggest that this material was not pure blackleg.

Blackleg has been reported in sheep, the organism having been isolated in Montana during recent years. Marsh (1919, 1920) states that the disease was controlled by the use of blackleg aggressin. Goss et al. (1921) showed that sheep were susceptible to blackleg inoculations, but required rather large doses. He suggests that natural infection is rare.

Cattle are very susceptible to blackleg. Before vaccination was practiced from 10 to 20 per cent of all young stock in the

southwest died of blackleg (Norgaard, 1898). Cattle have been repeatedly shown to be susceptible to infection by the use of pure cultures of *C. chauvei*. Most of the strains used at the Kansas Station have been tested for pathogenicity on cattle. In the production of blackleg agressin, twenty-one of the strains have been used in doses of 5 to 30 mils culture virus to kill calves for the production of agressin. Cultures 1, 2, 3, 4, 5, 6, 9, 10, 12, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 27, 28, 29 and 33 have all produced typical blackleg in cattle. In a number of these cases *C. chauvei* has been reisolated (strains 23 and 27).

Guinea-pigs are quite susceptible to blackleg. Their susceptibility has, however, been found to vary considerably. This variation may be seen in the routine tests for potency of powder vaccine. In this test three guinea-pigs are given 3 mgm. vaccine, three are given 5 mgm., three 7.5 mgm., one 15 mgm., one 25 mgm. and one 40 mgm. It is often found that the guinea-pig receiving 25 mgm., one receiving 5 mgm., and one receiving 3 mgm. die and all others on this test show no lesions whatsoever.

In determining the M.L.D. of a blackleg culture from 3 to 6 guinea-pigs are given graduated doses of the culture. The dose taken as the M.L.D. in this work must kill all guinea-pigs given this or larger doses. Some of the guinea-pigs in such a test receiving less, may, and often do die but these are not considered in determining the M.L.D.

It has been repeatedly found, in attempts to immunize guinea-pigs, and in tests to determine whether there was a relationship between the dose given and the swelling produced, that guinea-pigs receiving $\frac{1}{4}$ or $\frac{1}{5}$ M.L.D. would die while animals getting $\frac{3}{4}$ M.L.D. would live without showing any lesions.

The condition of the animals has considerable effect on their resistance. From 1917 to 1919 all guinea-pigs used at the Kansas Station were purchased from commercial concerns; since 1919 they have been obtained from the Animal Husbandry Department of the Kansas Agricultural College. Up to 1922 they were fed the ordinary ration of oats and bran. Since that time a mixture of alfalfa meal, tankage, bone ash, and mineral salts has been added. The health of the guinea-pigs has been greatly

improved and their resistance to blackleg has been increased. Previous to 1922 the average M.L.D. calculated from 52 tests on approximately 200 guinea-pigs was 0.3 mil. In the period from 1922 to 1924 the M.L.D. calculated from 21 tests on 150 pigs was 0.42 mil or 50 per cent greater. The calf pathogenicity of the cultures was practically the same, 10 to 20 mils culture being sufficient to kill healthy calves during both periods, thus showing that there was an increase in resistance in the guinea-pigs and not a decrease in virulence in the cultures used.

An experiment was started in 1919 in coöperation with Prof. H. L. Ibsen of the Department of Animal Husbandry by which it was hoped to find a race of guinea-pigs of more uniform susceptibility. Animals which had survived inoculations with *C. chauvei* cultures were mated and their offspring tested for blackleg resistance, approximately the same dosage of virus being used as had been used on their parents. This experiment was carried on until 1923 at which time 137 guinea-pigs had been reared and tested. No marked difference in resistance to blackleg could be demonstrated.

Rabbits have proved to be immune to all the strains of *C. chauvei* that have been used. Cultures have been injected both intravenously and subcutaneously in doses of 0.5 to 1.5 mil.

White rats injected with 0.1 mil of cultures 3 and 12 showed no symptoms or lameness. This dose killed guinea-pigs.

White mice have been reported as being susceptible to blackleg, and recently these animals have been used in the determination of the toxicity of the Foth and Kitt types of the organism (Kojima, 1923).

SEROLOGICAL REACTIONS

The macroscopic agglutination test has been used to study the relationship between some of the strains of *C. chauvei* and *C. edematis*. An attempt was also made to classify the three following cultures of *C. chauvei* which showed atypical cultural reactions: Strain "Reddish," obtained from Prof. G. F. Reddish of the University of Virginia, strain "Berg," from the Berg Biological Company, and strain "Lister" from the Lister Institute,

London. All these strains grow quite readily on 2 per cent glucose infusion agar. Slight morphological differences were noted, especially as to the number of organisms, pair and spore formation. In this they differed from typical strains of *C. chauvei.*

Rabbits were immunized to strains 1 and 33 of *C. chauvei*, strains 1, 3, 4 and 5 of *C. edematis* and strains "Reddish," "Berg," and "Lister." The rabbits were given from three to five injections of twenty-four to forty-eight hour brain-liver cultures intravenously or subcutaneously. Strains C.E. 2 and C.E. 5 produced a toxin that killed the first rabbits given these cultures. This toxin was destroyed in later experiments by heating the cultures to 65°C. for ninety minutes. The rabbits immunized against C.E. 2 died of other causes before immunity was developed. One serial of anti-blackleg serum 46 was used. This is a serum produced from horses immunized against strains 1 to 10.

Five dilutions of serum were made in one mil saline. The antigen in the form of twenty-four hour brain-liver cultures was added in doses of 1 mil to each tube. Duplicates of each dilution were made and a control containing no serum was used to assist in the reading of the test and to prevent the reading of auto-agglutination as a positive reaction. The reaction was read after two to six hours incubation and again after ten hours at room temperature. The final dilutions of serum were 1:40, 1:80, 1:240, 1:480 and 1:960.

Table 3 shows the agglutinations obtained.

From these data it is seen that the *C. chauvei* sera agglutinated *C. chauvei* strains in a fairly high dilution. *C. edematis* strains were agglutinated in dilutions of 1:80 at the most. The three atypical strains were agglutinated in dilutions of from 1:40 to 1:240. In one case no agglutination was produced, thus indicating that these strains are not identical with the type used as standard *C. chauvei.* The agglutinations by the *C. edematis* sera show that strain 5 is a type 1 culture, and also that the three atypical *C. chauvei* strains are not the C.E. 1 or C.E. 3 type cultures. If there are only three serological types of *C. edematis* it might be possible to identify C.E. 4 as a type 2 culture. Also

the three atypical *C. chauvei* are not *C. edematis* types. The cultural reactions of strain "Lister" which produced acid in all carbohydrates, using serum water as a base, indicate that it was closely related to *C. edematis* culturally. Serologically it is seen that these three strains are neither *C. edematis* nor *C. chauvei* strains of the type described in this paper.

TOXINS

The production of toxins by *C. chauvei* has been reported by numerous authors since Roux in 1888 reported on the immuniza-

TABLE 3

Agglutination reactions of C. chauvei and C. edematis strains

ANTIGENS	HORSE SERUM 46	RABBIT ANTI-SERA								
		1	33	C.E. 1	C.E. 3	C.E. 4	C.E. 5	CHAUVEI LISTER	CHAUVEI REDDISH	CHAUVEI BERG
1	240	*480*	80	0	0	0	0	40	0	0
10	240	480	960					80	0	0
24	240	480	960	0	0	40	0	80		
33	240	480	*960*	0	0	40	0	80	40	40
C.E. 1	40	40	40	*480*	80	40	480	240	40	0
C.E. 2	40	40	80	40	40	80	80	80	40	80
C.E. 3	0	40	80	40	*480*	80	0	80	40	240
C.E. 4	0	40	80	40	80	*240*	80	40	80	40
C.E. 5	0	80	80	960	80	80	*960*	80	40	80
Lister	240	240	240	80	40	40	40	*240*	240	40
Reddish	80	40	0	80	80	240	0	240	*480*	240
Berg	80	80	40	40	80	240	80	40	240	*240*

Rabbit anti-serum C.E. 2 agglutinated the strains in the following dilutions. C. chauvei 1, 40; 10, 40; 24, 40; 33, 40; C. edematis 1, 80; 2, *960* 3, 80; 4, 480; Lister 240; Reddish 80; Berg 80.

tion of guinea-pigs by means of filtered broth cultures of *C. chauvei* and tissue exudates from cases of blackleg. Leclainches and Valée (1900, 1923) found that cultures of *C. chauvei* produce toxins that kill guinea-pigs in a few hours. Grassberger and Schattenfroh (1904) and Eisenberg (1907) also found powerful toxins in blackleg cultures. Eisenberg, however, used centrifugation instead of filtration for the production of his toxin.

Kelser (1918) stated that toxic filtrates produced greater immunity in calves than non-toxic filtrates. Eichorn (1918) found that blackleg filtrate is toxic. Haslam and Lumb (1919) showed that the protective powers of filtrate had no relationship to its toxicity.

In the production of blackleg aggressin and blackleg filtrate at the Kansas Experiment Station a routine safety test on guinea-

TABLE 4

The non-toxic nature of blackleg filtrates and aggressins

GUINEA PIG		PRODUCT	METHOD OF INJECTION	DOSE	RESULTS		
Number	Weight				1 day	2 days	3 days
	grams			*mils*			
305	500	Filtrate	Subcutaneous	15	OK	OK	OK
306	500	Filtrate	Subcutaneous	25	OK	OK	OK
307	400	Aggressin	Subcutaneous	15	OK	OK	OK
308	450	Aggressin	Subcutaneous	23	2X	1X	OK
380	250	Non-phenolized aggressin 1	Subcutaneous	1	OK	OK	OK
381	250	Non-phenolized aggressin 1	Subcutaneous	2	OK	OK	OK
430	300	Non-phenolized aggressin 2	Subcutaneous	3	OK	OK	OK
431	300	Non-phenolized aggressin 2	Subcutaneous	6	OK	OK	OK
490	400	1 day filtrate	Subcutaneous	5	2X	OK	OK
491	375	2 day filtrate	Subcutaneous	5	OK	OK	OK
492	300	3 day filtrate	Subcutaneous	5	1X	OK	OK
493	400	6 day filtrate	Subcutaneous	5	OK	OK	OK
494	400	7 day filtrate	Subcutaneous	5	OK	OK	OK
533	400	10 day filtrate	Intracardially	1	OK	OK	OK
537	300	10 day filtrate	Intracardially	1	OK	OK	OK
538	300	10 day filtrate	Intravenously	0.5	OK	OK	OK

1X, slight swelling; 2X, moderate swelling; 3X, large swelling.

pigs is made. Two guinea-pigs are given 7 mils of the product subcutaneously. In all cases where this test has been made on material shown to be sterile by cultural tests on brain liver medium the guinea-pigs have shown practically no swellings. In a few cases where the cultural test and the guinea-pig test were started simultaneously the guinea-pigs have died. In all

these cases the material was shown to be contaminated and in several cases *C. chauvei* has been reisolated from these guinea-pigs. Large doses, 15 to 25 mils of filtrate and aggressin, have been injected into guinea-pigs without producing any lesions, as shown in table 4.

A series of tests on unphenolized filtrates and aggressins was also made; no lesions were produced by these products. A series of flasks were then inoculated with *C. chauvei* 33. After twenty-four hours incubation, one flask was taken, the fluid poured off and filtered through small Mandler filter candles. This was repeated on the second, third, sixth and seventh days. In the case of the first filtrate a guinea-pig was given 5 mils filtrate, and a culture tube containing brain liver medium was inoculated with the same amount (5 mils). The guinea-pig was dead the next morning and the culture tube showed cloudiness and gas. Both were shown to contain *C. chauvei*. This filtrate was refiltered and together with the other daily filtrates was tested for sterility before injecting other guinea-pig. Table 4 shows that these filtrates produced no lesions in guinea-pigs, except in the case of filtrates 1 and 3 which were contaminated by a few organisms; this was shown by growth of the brain liver test after three days incubation. It was found that sterile filtrates were much more easily obtained from old cultures that had auto-agglutinated than from cultures containing vegetative cells. Non-phenolized filtrates of fresh exudates and muscle juices obtained from calves that had died of spontaneous blackleg were also shown to be non-toxic (table 4). Intravenous and intracardiac injections of filtrate were also shown to be non-toxic.

AGGRESSINS

The substances found in filtered muscle juices and exudates of blackleg lesions (aggressins), or in filtered cultures of *C. chauvei* (filtrates) have been shown to be non-toxic (table 4). These products are shown to be true aggressins by the fact that (1) the addition of small amounts of these products will activate non-lethal doses of blackleg virus, (2) the addition of small amounts of these products to small amounts of avirulent washed cultures

of *C. chauvei* will produce typical blackleg lesions in guinea-pigs, (3) the addition of small amounts of these products to *C. chauvei* cultures will produce death in guinea-pigs passively immunized against blackleg.

Table 5 shows that when a small amount of aggressin was added to 1/10 M.L.D. (0.07 mil) blackleg culture, death of the guinea-pig was produced in two days, and when 1 mil filtrate was added to 1/5 M.L.D. death was produced in four days. These experiments show that sublethal doses of blackleg culture virus are activated by small amounts of the products of growth of *C. chauvei*, either in the calf as in the case of the aggressin, or in culture media as in the case of filtrate.

TABLE 5

The aggressiveness of blackleg aggressin and filtrate when added to $\frac{1}{10}$ and $\frac{1}{5}$ M.L.D. C. chauvei culture

GUINEA-PIG		PRODUCT	DOSE	CULTURE	DOSE M.L.D.	RESULTS			
Number	Weight					1 day	2 days	3 days	4 days
			mil						
389	275	Aggressin	0.75	33	1/10	OK	D		
710	300	Filtrate	1.0	33	1/5	OK	2X	3X	D

2X, moderate swelling; 3X, large swelling; D, death.

The same reaction has been shown to occur in cattle (Scott, 1923). Several cases of death from blackleg in calves treated with blackleg filtrate have been reported to the Kansas Agricultural College. It was found that these calves had been vaccinated with blackleg powder vaccine some weeks before. The disease in every case developed a few days after the use of the filtrate in the region of the neck where the vaccine had been injected, thus showing the aggressiveness of this product.

If cultures of *C. chauvei* are washed by centrifugation in salt solution so as to free the cells from all the products of growth an avirulent product remains (table 6). If small amounts of this washed culture be added to small amounts of filtrate or aggressin a virulent product results. Table 6 shows that by washing cultures of *C. chauvei* three times in salt solution and diluting the resulting packed cells in salt solution up to the original volume

an absolutely avirulent product results. The large doses may produce slight swellings due to the large amounts of cells to be absorbed; these swellings are more in the nature of absorption swellings than inflammatory swellings. The M.L.D. of culture 28 was 0.2 mil before washing and after centrifugation three times in salt solution a dose of 15 mils or the equivalent of 75 times the M.L.D. is see to be avirulent, this amount only producing a slight absorbtion swelling in guinea-pig 441.

TABLE 6

Avirulence of washed culture

GUINEA-PIG		WASHED CULTURE	DOSE	RESULTS			
Number	Weight			1 day	2 days	3 days	4 days
			mils				
441	600	28	15	1X	2X	OK	OK
442	600	28	7	OK	OK	OK	OK
330	500	3	10	OK	OK	OK	OK
830	700	2	15	1X	1X	OK	OK

1X, slight swelling; 2X, moderate swelling.

TABLE 7

Aggressiveness of aggressin and filtrate in the presence of washed culture

GUINEA-PIG		WASHED CULTURE		PRODUCT	DOSE	RESULTS		
Number	Weight	Number	Dose			1 day	2 days	3 days
			mil		*mil*			
119	300	28	0.3	Aggressin	0.3	D		
126	350	28	0.3	Filtrate	0.3	3X	D	

3X, large swelling; D, death.

It is therefore seen that the addition of the products of growth of *C. chauvei* will reactivate the washed culture.

When 0.03 mil of washed culture 28 shown to be avirulent in doses of 15 mils (table 6) was added to blackleg aggressin or filtrate and guinea-pigs were injected with these mixtures they died of typical blackleg as shown in table 7. This demonstrates that the products of growth of *C. chauvei* contain an aggressive substance.

If guinea-pigs are passively immunized by the injection of anti-

blackleg serum and fifteen hours later a mixture of *C. chauvei* culture and filtrate or aggressin is injected, an aggressive reaction will be produced resulting in the death of the guinea pig from blackleg as shown in table 8. This reaction has been called the Neutralization reaction.

Table 8 shows that amounts of anti-blackleg serum sufficient to protect a guinea-pig against at least 15 M.L.D. of *C. chauvei* culture virus, were neutralized by the injection of a mixture of 0.3 mil blackleg filtrate and aggressin with 1.5 M.L.D. *C. chauvei* culture fifteen hours later. This shows that these products contain an aggressive substance. Guinea-pig 684, given anti-blackleg serum, was protected against 16 M.L.D. of the same culture when given unmixed with aggressin or filtrate.

TABLE 8

Neutralization reaction

GUINEA-PIG		SERUM		PRODUCT	DOSE	DATE	VIRUS	DOSE M.L.D.	DATE	RESULTS		
Number	Weight	Dose	Date							1 day	2 days	3 days
		mil			*mil*							
394	250	0.15	12/5	Aggressin	3	12/6	28	1.5	12/6	3X	3X	D
398	250	0.15		Filtrate	3		28	1.5		3X	3X	D
684	200	0.15		.			28	16.0		½X	OK	OK

3X, large swelling; D, Death.

The neutralization reaction shown in table 8 forms the basis for the neutralization potency test for blackleg filtrate and aggressin (Goss and Scott, 1918). In the neutralization test a series of guinea-pigs are given a known amount of anti-blackleg serum, the usual dose being 15 anti-blackleg units (one anti-blackleg unit of serum is that amount which will protect a guinea-pig against one M.L.D. of blackleg virus). Fifteen hours later these guinea-pigs are given from 1 to 3 M.L.D. blackleg culture virus mixed with graduated doses (1, 2, 3, 4 and 5 mils) of blackleg filtrate or aggressin. Some of these guinea-pigs will die or show marked lesions. The guinea-pig dying from the smallest dose of filtrate or aggressin determines the strength of the prod-

uct. This amount will contain 15 minus 2 (dose of serum minus dose of virus), or 13 aggressive units from which the aggressive units in a 5 mil dose can be calculated.

The reaction in table 7 showing the activation of the avirulent washed culture is made use of in the washed culture potency test for blackleg filtrate and aggressin (Scott, 1923). In the washed culture test the smallest amount of washed culture that is activated by small amounts of filtrate or aggressin (0.3 to 0.7 mil) is taken to be the unit of washed culture. This unit is called the "potential" M.L.D. In determining the aggressive strength of filtrate or aggressin by means of the washed culture test a series of guinea-pigs are given one potential M.L.D. of washed culture mixed with graduated doses (0.15, 0.25, 0.3 and 0.4 mil) of filtrate or aggressin. The smallest amount of these products that activates the washed culture determines the strength of that product. This amount is called one aggressive unit.

In comparing filtrates and aggressins by means of these two tests five important points have been brought out.

1. The aggressive strength of various serials of filtrate and aggressin, as tested by these methods, was fairly constant and the strengths, as determined by several repetitions of the same test, were also very close. In table 9 a comparison of several such serials has been made. Serials numbered, strain 1 to 32, are filtrates made from stock cultures of *C. chauvei* having these numbers.

Table 9 shows that there is not much difference in the amount of aggressive substance produced by different strains of *C. chauvei*. It also shows that the neutralization and washed culture tests give very nearly the same readings for the different products tested and that successive tests do not vary to any marked extent. The average of 47 neutralization tests gives a reading of 16.35 aggressive units and the 38 washed culture tests on the same 27 products a reading of 15.53 aggressive units. Filtrate "28" was made from a virulent culture of this organism having an M.L.D. of 0.2 mil and filtrate "avirulent 28" was made from a culture of this strain having an M.L.D. of 0.9 mil. In both cases the M.L.D. was determined from the culture before filtration. It is

seen that the filtrates from both the virulent and avirulent cultures have an aggressive strength of 16.5 aggressive units.

2. The aggressiveness of filtrates made from cultures of virulent and avirulent strains shows no marked differences, which indicates

TABLE 9

Comparison of aggressiveness of filtrates and aggressins

	NEUTRALIZATION TEST				WASHED CULTURE TEST		
	1st test	2nd test	3rd test	4th test	1st test	2nd test	3rd test
Filtrate 1195	21.5				16.65		
Filtrate 1196	13.0	16.5			12.5		
Filtrate 197	13.0	16.5			12.5	12.5	
Filtrate 298	13.0				12.5	16.5	
Filtrate 299	10.0	16.5			10.0		
Filtrate 310	16.5	16.5			20.0		
Filtrate 101	13.0	16.5			20.0		
Filtrate 102	16.5	16.5			14.28	16.55	
Strain 1	13.0	13.0			14.65	16.65	
Strain 2	13.0				12.5		
Strain 3	13.0				12.5		
Strain 5	16.5				10.0		
Strain 6	21.5				12.5		
Strain 10	13.0	16.5	16.5		16.65	16.65	16.65
Strain 12	16.5	16.5			14.28		
Strain 19	16.5	16.5			33.33		
Strain 23	16.5	21.65			14.28		
Strain 24	21.65	21.65			16.65		
Strain 27	32.5				16.65	16.65	16.65
Strain 28	13.0	16.5	16.5		16.65	14.28	
Strain 29	13.0				9.0		
Strain 30	13.0				12.5		
Strain 31	16.5	16.5			16.65		
Strain 32	16.5				16.65		
Avirulent 28	13.0	16.5	16.5	16.5	16.65	16.65	14.28
Aggressin 207	13.0				16.65		
Aggressin 208	21.5	21.5			16.65		
	47 tests Average, 16.35				38 tests Average, 15.53		

that the lethal part of a virulent culture is in the cells and not in the products of growth.

Table 10 shows the aggressiveness of filtrates prepared from

cultures having different M.L.D. values. Each culture was first tested to determine its M.L.D. and was then filtered. Strain 28 had an M.L.D. of 0.2 in one case and 0.9 in the other. It

TABLE 10

Comparison of aggressiveness of virulent and avirulent strains of C. chauvei

AVIRULENT STRAINS			VIRULENT STRAINS		
Number	M.L.D.	Aggressive strength (A. U.)	Number	M.L.D.	Aggressive strength (A. U.)
	mil			*mil*	
1	>1	14.5	28	0.2	16.5
2	>1	13.0	29	0.3	13.0
12	>1	16.5	32	0.2	16.5
28	0.9	16.5			

TABLE 11

The action of heat on aggressive substances

PRODUCT	PRODUCTION TESTS		UNHEATED TESTS		HEATED AT 60°C.					
					15 minutes		30 minutes		60 minutes	
	Neutralisation A. U.	Washed culture A. U.	Neutralisation A. U.	Washed culture A. U.	Neutralisation A. U.	Washed culture A. U.	Neutralisation A. U.	Washed culture A. U.	Neutralisation A. U.	Washed culture A. U.
	1922		*1923*							
Fil. 102.....	16.5	14.28	16.5	16.65	21.65	33.33	21.65	—	32.5	32.33

PRODUCT	PRODUCTION TESTS 1920–1922		UNHEATED TESTS 1923			HEATED AT 60° FOR 60 MINUTES	
	Neutralisation A. U.		Neutralisation A. U.		Washed culture A. U.	Neutralisation A. U.	Washed culture A. U.
Agg. 205.....	21.65		21.65	16.5	20.0	32.5	33.33
Agg. 206.....	21.65		21.65	21.65	20.0	32.5	33.33
Agg. 209.....	21.65		21.65	21.65	20.0	32.5	33.33
Fil. 1195.....	21.65		21.65		20.0	32.5	27.0
Fil. 1196.....	13.0	16.5	13.0		20.0	24.5	33.33
Fil. 298.....	12.5	16.5	21.65		16.5	16.5	22.0

is seen that the virulence of the cultures does not materially affect the aggressive strength of the filtrate produced. It is also seen that one strain does not have a constant M.L.D.

3. The potential M.L.D. of the washed culture was found to be proportional to the M.L.D. of the unwashed culture. It was found to be from 0.05 to 0.2 mil greater than the corresponding M.L.D. The same amount of agressive substance was required to activate the potential M.L.D. of cultures having a high virulence as for those having a low virulence. Washed cultures made from avirulent strains may have a potential M.L.D. but this could not be determined. The cells of these avirulent cultures therefore contain little or no lethal substance but as has been seen (table 10) they produce as much aggressive substance as the virulent strains.

4. The action of heat on the aggressive substances increases their activity. This point is referred to in Karsner and Ecker's text, "The Principles of Immunology," who cite Bail's work as authority.

TABLE 12

Hydrogen-ion concentration of filtrate before and after the application of heat

FILTRATE	pH BEFORE HEATING	pH AFTER HEATING 60° FOR ONE HOUR
280	6.6	6.5
101	5.5	5.6
108	6.1	6.2
1195	5.4	5.5

Table 11 shows that heating blackleg filtrates or aggressins at 60°C. for one hour increases the aggressiveness of these products.

This action is not due to changes in the hydrogen-ion concentration, as is shown by table 12 which gives the pH reading before and after heating four serials of blackleg filtrate. One, filtrate 1195, was the same as that tested in table 11.

5. The keeping qualities of blackleg filtrate and aggressin were found to be high. Three aggressins produced in 1920 having an aggressive strength of 21.5, 13.0, and 16.25 aggressive units had in 1923 an aggressive strength of 13.0, 16.25, and 21.65 aggressive units respectively. Filtrate 1, having an aggressive strength of 13.5 in 1918 had an aggressive strength of 9 in 1923, and after being held in the incubator for forty weeks had a strength of 26.0 aggressive units.

SPECIFICITY OF AGGRESSIVE SUBSTANCES

The aggressive substances found in filtrates made from cultures of *C. chauvei* and *C. edematis* are essentially non-specific as far as can be determined by guinea pig tests. In some cases where the dose of virus and of aggressive substance and the resistance of the guinea-pig are favorable, specificity can be demonstrated as shown in table 13.

TABLE 13

Specificity reactions of aggressive substance

GUINEA-PIG		VIRUS	DOSE M.L.D.	FILTRATE	DOSE	RESULTS		
Number	Weight					1 day	2 days	3 days
					mil			
549	325	C.E. 1	1/10	C.E 1	0.75	OK	OK	OK
550	350	C.E. 1	1/10	C.E. 2	0.75	OK	OK	OK
551	375	C.E. 1	1/10	C.E. 3	0.75	OK	OK	OK
552	300	C.E. 1	1/10	Ch. 33	0.75	OK	OK	OK
553	325	C.E. 2	1/10	C.E. 1	0.75	OK	OK	OK
554	375	C.E. 2	1/10	C.E. 2	0.75	OK	OK	D
555	350	C.E. 2	1/10	C.E. 3	0.75	OK	OK	OK
556	325	C.E. 2	1/10	Ch. 33	0.75	OK	OK	OK
634	250	C.E. 3	1/5	C.E. 1	0.75	OK	OK	OK
635	225	C.E. 3	1/5	C.E. 2	0.75	OK	OK	OK
636	300	C.E. 3	1/5	C.E. 3	0.75	1X	1X	1X
637	350	C.E. 3	1/5	Ch. 33	0.75	OK	OK	OK
671	325	Ch. 33	1/4	C.E. 1	1.25	3X	D	
672	275	Ch. 33	1/4	C.E. 2	1.25	3X	D	
673	275	Ch. 33	1/4	C.E. 3	1.25	3X	D	
670	300	Ch. 33	1/4	Ch. 33	1.25	1X	OK	OK

The nonspecific reaction is also seen in the fact that filtrates of *C. edematis* strains will activate *C. chauvei* virus in the neutralization test.

Table 13 shows that in the use of *C. edematis* Type 1 culture no aggressiveness was produced by any filtrates. In the case of *C. edematis* Type 2 culture true specificity was shown by filtrate *C. edematis* 2 which was the only filtrate to produce a reaction in the guinea-pigs C.E. 2 culture. The test on culture *C. edematis* 3 also shows specificity but the test using *C. chauvei* 33 shows a non specific reaction. The filtrates of *C. edematis*

1, 2 and 3 contained enough aggressive substance to activate the culture virus of *C. chauvei*, while the filtrate of *C. chauvei* 33 did not contain sufficient aggressive substance to produce more than a very slight reaction in any case.

The finding that the aggressive substance in blackleg aggressins and filtrate is non-specific when tested on guinea-pigs and the fact that filtrates and aggressins produced from typical strains of *C. chauvei* produce a lasting immunity in cattle but not in guinea pigs, while filtrates produced from atypical strains or from other organisms do not produce these results, leads to the supposition that the aggressive substances may not be the immunizing agents.

IMMUNITY

Immunization of cattle against blackleg has been carried on since Arloing Cornevin and Thomas (1887) introduced the powdered muscle tissue or Lyons backleg vaccine. Leclainchee and Valée (1913) introduced the liquid spore vaccine which is produced by growing cultures of *C. chauvei* at temperatures of 42°C. In 1916 Franklin and Haslam (Goss, 1917), working at the Kansas State Agricultural College introduced blackleg aggressin, which is produced by filtering the muscle juices and exudates obtained from a case of blackleg. In 1917 Goss and Scott (Goss, 1919) developed the artificial aggressin, or culture filtrate, produced by filtering pure cultures of *C. chauvei.*

The results from the use of powder vaccine are not satisfactory. The immunity produced is of rather short duration and very susceptible calves may be given blackleg. The liquid spore vaccine has proved to be very satisfactory. Blackleg filtrate and aggressin produce a high degree of immunity (Scott, 1923) which protects the animals during their period of greatest susceptibility (from six months to two years of age), if these products are given at the age of five or six months of age. Vaccination with powder vaccines reduced the losses from 10 to 20 per cent to 1 per cent (Norgaard 1898). Vaccination with blackleg filtrate and aggressin have reduced the losses to 1 in 10,000 or less.

Immunization of guinea-pigs by means of blackleg muscle virus, *C. chauvei* cultures, serum virus mixtures and filtrates and aggressins does not give uniform results. Immunization with cultures and muscle virus may perhaps give slightly greater immunity than when filtrates or aggressins are used. Of 138 guinea-pigs injected with from 2 to 7 mils blackleg aggressin and ten days to two weeks later with 1 to 2 M.L.D. *C. chauvei* culture, 70, or 50.7 per cent died. Of 270 guinea-pigs injected with 2 to 7 mil blackleg, filtrate and ten days to two weeks later with 1 to 2 M.L.D. *C. chauvei* culture, or muscle virus, 128, or 47.4 per cent died. These experiments show that the immunity produced by the use of these products is very low. This immunity does not always exceed the limits of natural variation in resistance of guinea-pigs. It is possible that all the pigs that died were animals which had a very low initial resistance and that their resistance was increased by the injection of these products but not to a degree sufficient to protect them against the test dose.

The cross immunization tests showing immunization of guinea-pigs by whole cultures of *C. chauvei* and *C. edematis* (table 14a) show that strain *C. chauvei* 33 immunized guinea-pigs against itself but not against *C. edematis* strains 1, 2, or 3. The *C. edematis* strains protected guinea pigs against themselves and also showed some protection against strains not used in the immunization. Immunization by the use of culture filtrates (table 14b) shows irregular protection.

In these experiments with culture immunization it is seen that guinea-pigs immunized by the injection of culture *C. chauvei* 33 were only protected against this culture but not against *C. edematis* strains 1, 2 or 3. *C. edematis* 1 protected a guinea pig against itself and against *C. edematis* 2. *C. edematis* 2 protected against *C. edematis* 1 but not against itself. *C. edematis* 3 protected guinea-pigs against itself and against *C. chauvei* 33. In the series of tests using filtrates of these cultures it is seen that filtrate of *C. chauvei* culture 33 protected against *C. edematis* 1 and 3 but not against itself. *C. edematis* 1 filtrate protected guinea-pigs against the culture itself but not against the other

TABLE 14

Cross immunization tests

GUINEA-PIG		IMMUNIZATION CULTURE	DOSE	DATE	TEST CULTURE	DOSE M.L.D.	DATE	RESULTS		
Number	Weight							1 day	2 days	3 days
					(a)					
			mil							
354	325	Ch. 33	0.35	1/18	Ch. 33	1.3	2/2	OK	OK	OK
355	300	Ch. 33	0.35	1/18	C.E. 1	1.3	2/2	3X	D	
356	300	Ch. 33	0.35		C.E. 2	1.3		D		
357	300	Ch. 33	0.35		C. E. 3	1.3		D		
350	250	C.E. 1	0.1		Ch. 33	1.3		3X	D	
349	300	C.E. 1	0.1		C.E. 1	1.3		OK	OK	OK
375	250	C.E. 1	0.025	1/20	C.E. 2	1.3		OK	OK	OK
376	250	C.E. 1	0.025		C.E. 3	1.3		D		
361	300	C.E. 3	0.1	1/18	Ch. 33	1.3		2X	1X	OK
363	300	C.E. 3	0.1		C.E. 1	1.3		3X	D	
378	250	C.E. 3	0.025	1/20	C.E. 2	1.3		OK	D	
379	250	C.E. 3	0.025		C.E. 3	1.3		OK	OK	OK
364	300	C.E. 2	0.025		C.E. 1	1.3		2X	1X	OK
366	250	C.E. 2	0.025		C.E. 2	1.3		OK	OK	D
382	250	C.E. 2	0.01	1/23	C.E. 3	1.3		D		
					(b)					
		FILTRATE								
601	300	F.C.E. 1	4	3/29	C.E. 1	1	4/7	1X	OK	OK
602	275	F.C.E. 1	4		C.E. 2	1		D		
603	300	F.C.E. 1	4		C.E. 3	1		D		
604	275	F.C.E. 1	4		Ch. 33	1		3X	D	
605	275	F.C.E. 2	4		C.E. 1	1		2X	D	
606	275	F.C.E. 2	4		C.E. 2	1		D		
607	300	F.C.E. 2	4		C.E. 3	1		D		
608	300	F.C.E. 2	4		Ch. 33	1		3X	D	
609	225	F.C.E. 3	4		C.E. 1	1		OK	OK	OK
610	300	F.C.E. 3	4		C.E. 2	1		D		
547	375	F.C.E. 3	5		C.E. 3	1		D		
612	300	F.C.E. 3	4		Ch.33	1		3X	D	
613	225	F.Ch. 33	4		C.E. 1	1		OK	OK	OK
614	250	F.Ch. 33	4		C.E. 2	1		D		
615	250	F.Ch. 33	4		C.E. 3	1		OK	OK	OK
616	250	F.Ch. 33	4		Ch. 33	1		D		

strains. *C. edematis* 2 filtrate did not protect guinea pigs against any cultures used. *C. edematis* 3 filtrate protected only against *C. edematis* 1 culture.

Similar results were obtained in a series of tests on the immunization of guinea pigs by means of filtrates injected in doses of 5 to 10 mils given in six injections over a period of seven days.

From these experiments it is seen that immunization of guinea-pigs by means of whole cultures does not give satisfactory results, and that immunization of guinea-pigs by means of

TABLE 15

Passive immunization of guinea-pigs

GUINEA-PIG		SERUM	DOSE	DATE	CULTURE	DOSE M.L.D.	DATE	RESULTS		
Number	WEIGHT							1 day	2 days	3 days
			mil							
251	200	46	0.07	11/28	Ch. 33	5	11/29	OK	OK	OK
252	200	46	0.07		C.E. 4	5	11/29	D		
253	150	46	0.07		C.E. 1	5		D		
254	250	46	0.07		C.E. 2	5		D		
708	350	46	0.05	5/5	C.E. 3	3	5/6	D		
709	250	46	0.05		C.E. 5	3		D		
703	300	C.E. 5	1.25	5/5	C.E. 1	1.5	5/6	OK	OK	OK
704	250	C.E. 5	1.25		C.E. 2	1.5		D		
705	350	C.E. 5	1.25		C.E. 3	1.5		D		
706	350	C.E. 5	1.25		C.E. 4	1.5		D		
711	300	C.E. 5	1.25		C.E. 5	1.5		2X	3X	D
712	300	C.E. 5	1.25		Ch. 33	1.5		D		

2X, slight swelling; 3X, large swelling; D, death.

filtrates is even less satisfactory. In neither case is the immunization of sufficient regularity to be used as a basis for differentiation of strains.

Immunization of guinea-pigs by the use of anti-sera on the other hand, gives uniform results, if sufficient serum is given to protect the guinea pigs against at least 2 M.L.D. of culture. In this way the variation in susceptibility of the guinea-pigs is minimized. Table 15 shows a series of passive immunization tests.

Anti-blackleg serum 46 was produced from horses immunized

against strains 1 to 10. The anti-*C. edematis* serum was a rabbit serum produced by the injection of whole cultures of *C. edematis* 5 intravenously and subcutaneously. Serum 46 protected guinea pigs against *C. chauvei* strain 33 but not against any of the *C. edematis* strains. Serum 46 has also been used to immunize guinea-pigs against most of the other strains of *C. chauvei* and has protected guinea-pigs against these strains in every case. The serum produced from a rabbit immunized against *C. edematis* 5 was rather weak, protecting guinea-pigs only against 1.5 M.L.D. (1.25 mil). Guinea-pig 711 was immunized against C.E. 5 by the use of anti-serum. This guinea-pig died on the third day following the test dose of C.E. 5. This shows the importance of having enough latitude between the expected protective strength of the serum to be used and the test dose of the virus. Guinea-pig 703 immunized against C.E. 5 and tested with *C. edematis* strain 1 showed no swellings. This shows that C.E. 5 is a type 1 strain and confirms the results found in the agglutination reaction (table 3) which also showed that *C. edematis* 5 was a type 1 strain.

SUMMARY

1. It has been shown that anti-blackleg serum from horses protected guinea-pigs against *C. chauvei* strains isolated from typical cases of blackleg disease. These strains did not grow in 2 per cent glucose infusion agar.

2. *C. chauvei* strains were shown to be typically single Gram-positive rods, producing occasional oval subterminal spores. *C. edematis* strains were seen to be typically chain forming types showing numerous filamentous rods. Some strains of *C. edematis* produced a predominance of paired organisms, in a number of which the elements formed a sharp angle one with another.

3. *C. chauvei* strains were shown to be much less active biochemically than the *C. edematis* strains, *C. chauvei* only fermenting carbohydrates in the presence of unheated serum, while the *C. edematis* strains fermented carbohydrates in a greater number of media. *C. chauvei* ferments a restricted number of

carbohydrates only while the *C. edematis* types ferment most carbohydrates.

4. The pathogenicity of the cultures of *C. chauvei* for guinea-pigs was not constant. This was partly due to dfferences in resistance of individual guinea-pigs. Changes in culture media apparently did not affect the pathogenicity of *C. chauvei* for guinea-pigs to any marked extent, but the use of a feed rich in vitamines and mineral salts increased the resistance of the guinea-pigs. *C. edematis* type strains were more pathogenic for guinea-pigs than *C. chauvei* strains and were also in some cases highly pathogenic for rabbits and white rats.

5. There was found to be no direct relationship between the pathogenicity of *C. chauvei* cultures for guinea-pigs and their pathogenicity for calves.

6. The agglutination tests showed positive serological differences between the types studied.

7. Both tissue extract and culture filtrates were shown to be non-toxic in doses up to 25 mils.

8. An aggressive substance was demonstrated in both the tissue extract and culture filtrates. This aggressive substance was increased in potency by the action of heat.

9. The absolute specificity of the aggressive substance could not be demonstrated, cross aggressin reactions being apparent in many cases.

10. The amount of aggressive substance produced by a virulent strain was found to be no greater than that produced by an avirulent strain.

11. Active immunization of cattle by means of attenuated cultures, and especially by the use of filtrates and aggressins has been demonstrated, but the active immunization of guinea-pigs against both *C. chauvei* and *C. edematis* was shown to be uncertain.

12. Passive immunization of guinea-pigs by means of antisera proved to be specific and gave a highly reliable method of differentiation.

13. The results obtained by passive immunization reactions confirmed the findings obtained in the agglutination test.

14. Two potency tests based on the production of aggressive substance in cultures of *C. chauvei* and in the tissues of calves affected with blackleg are described.

15. It is shown that the lethal substance is found in the cells of *C. chauvei* and that this lethal substance can not produce death in the absence of products of growth. The amount of this substance present in any given culture determines its virulence.

16. The eight criteria of purity described in connection with the isolation of *C. chauvei* strains were shown to be sufficient evidence of the identity of these strains.

17. The study of three atypical *C. chauvei* strains indicates the possibility of there being two types of *C. chauvei*, such as have been described by Zeiszler and Kojima.

18. The *C. chauvei* type described in this paper appears to be the most prevalent. Anti-sera, filtrates and aggressins produced from this type have been shown to prevent blackleg in all parts of the United States, Mexico, Central America and in South Africa.

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