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ANTISEPTIC CHARACTERISTICS OF VIBRIO FEXUS

by

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A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Veterinary Science to the School of Graduate Studies University of Toronto 1956
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INTRODUCTION

*Vibrio fetus* infection has become recognized as one of the principal infectious causes of reduced reproductive efficiency in cattle and sheep. The disease is manifest in cattle by infertility, abortion and sterility. Serious losses in many herds are caused by the reduced milk yield and decreased calf crop. After the initial onset of the disease in a herd, undetected early abortions, apparent failure to conceive from three or more services and off-cycle return to estrus usually causes greater economic losses than detected abortions.

Bovine vibriosis has been reported in many countries, e.g., in Britain by McFadyean and Stockman (41), Lawson and MacKinnon (36); in United States by Smith et al. (64, 66, 68), Carpenter (17), Walsh and Marsh (77), Ryff (62), and many others; in Australia by Hindmarsh (26); in South Africa by Snyman (71) and Canham (16); in Denmark by Thomsen (72); in Sweden by Olson (44); in Germany by Geindler (25); in Japan by Kasahima et al. (33); in the Netherlands by Sjollema et al. (63); in Canada, the disease has been reported in Ontario by Bain (1, 2, 3, 4, 5), and by Rossell (60, 61); in British Columbia by Myhrihan and Stovell (43), and in the Maritime Provinces by Frank (22).

*Vibrio fetus* infection has been reported in human females by Vincent, Dumas and Fricard (75) and again by Vincent, Delarue
and Hebert (74). Weissman-Netter et al. (76) report a case of *Vibrio fetus* infection in a human male. The cases were characterized by an acute septicemia and were characterized by blood culture. It usually caused abortion in pregnant human females.

Laboratory tests are required to differentiate vibriosis from brucellosis, trichomoniasis, infection with pyogenic bacteria and other causes of abortion and breeding troubles. Isolation of *Vibrio fetus* is desirable to make a positive diagnosis. However, this is often impossible because of the presence of other bacteria and because of the failure of some strains to grow on primary isolation. Once the organism has been isolated it is necessary to differentiate it from other vibrio species, especially if it is isolated from sources other than the aborted fetus.

Agglutination tests on blood serum and/or vaginal mucus are used as aids in diagnosis. Platridge et al. (49) have outlined the requirements for an agglutination test for vibriosis as (1) selection of cultures that are specifically agglutinable, (2) methods of maintaining the specific agglutinability of the culture selected, (3) cultural methods of growing large volumes of cells and (4) information on the significance of the reactions obtained. The greatest single difficulty in the use of the agglutination test is the production of a suitable antigen.

Some reports in the literature indicate that the various strains of *Vibrio fetus* form a serologically homogenous group while other reports represent this species as serologically heterogenous.
Many workers in this field have used bovine sera, while others have used hyperimmune rabbit sera, to study the antigenic characteristics of this organism. The bovine sera was usually obtained from infected animals or from animals inoculated subcutaneously with a bacterin prepared by suspending the organism in formalinized saline. Hyperimmune rabbit sera was obtained, as a rule, by intravenous injection of the organism. The purpose of this paper is to describe the results of research on the antigenic structure of various strains of *Vibrio fetus* and to compare the antibody response in bovine and rabbit sera produced by intravenous and by subcutaneous inoculation.
Vibriosis was first associated with abortion in sheep by McFadyean and Stockman (41). They reported an average abortion rate of 23.2 per cent and an average sterility rate of 10 per cent in flocks of sheep with vibrio infection. Seven head of cattle were infected with exudate from aborting ewes either with or without the addition of pure cultures of the organism. In two instances, the mode of introduction of the "virus" was by the mouth, in the rest by intravenous inoculation. Two of the five given intravenous inoculation aborted and in one of these "vibrios" were detected. Vibrios were also found in the fetuses of cows that aborted in two herds, one in Ireland and one in Wales in 1911.

In 1918, Smith (64) isolated a spirillum from 14 cases of abortion in cattle. He stated that the abortion was probably due to interference with the placental circulation and the injurious action of the spirillum was limited to the fetal membranes. The organism grew only under reduced oxygen tension and laboratory animals were refractory. Smith compared his spirillum with the vibrio described by McFadyean and Stockman and was unable to demonstrate any essential difference between the two organisms.

Smith and Taylor (70) reported that 21 of 22 strains of \textit{Vibrio fetus} studied were serologically identical. This conclusion was based upon agglutination tests with four specific antisera prepared by intraperitoneal injection of living organisms into rabbits.
The remaining strain was somewhat different but it belonged to the same group on the basis of its agglutination affinities. Recently isolated strains were agglutinated less readily and to lower titer by rabbit antisera than were strains maintained for some time in the laboratory. This publication undoubtedly is the basis for the belief that only insignificant variations are found among strains of *Vibrio fetus*. However, it must be pointed out that all of these strains were isolated from one large cattle herd and therefore probably are not a good representation of the population. It is of interest that one of the photomicrographs in Smith and Taylor's article shows what is called "some capsular substance, unstained, sheathing the long forms of the organism". This negative-staining is not a good method of demonstrating capsules and it may well be an artifact due to fixing and staining.

Smith (65) was able to produce disease of the fetal membranes in 2 of 4 cows injected intravenously with cultures of *Vibrio fetus*.

The possibility of using an agglutination test to aid in the diagnosis of vibriosis in cattle was suggested by Smith et al. (66). They found that the blood serum of three cows which aborted from vibriosis agglutinated *Vibrio fetus*. In the same report it was noted that a strain was isolated from an aborted fetus which failed to agglutinate with serum from the cow. However, a titer of 1:320 was obtained using this serum with an antigen prepared from another strain. The theory that agglutinability of recent isolates
was low or absent and that it rises under artificial cultivation was shown not to be true in all cases. Some recent isolates gave high agglutination titers.

Intravenous injection of *Vibrio fetus* cultures by Smith (67) succeeded in infecting 5 out of 6 cows. Two cases aborted and four were killed at varying periods after the inoculation. Five of the six showed lesions of the fetal membranes and *Vibrio fetus* was demonstrated in the membranes and exudate.

In another paper, Smith and Orcutt (69) describe experiments on strain interrelations. Two strains isolated from the liver of calves and five fetal strains were investigated. Cross agglutination tests with sera prepared by injection of living cultures indicated a common vibrio factor while cross agglutination tests with sera prepared from heated cultures and reciprocal absorption tests between calf and fetal strains showed distinct calf and fetal group factors. Reciprocal absorption tests between fetal strains and between calf strains suggested some individual strain factor which is heat labile. Contrary to the conclusions of his former investigations, it was concluded that vibrios are of a complex antigenic nature.

Barger (6) reported two cases of bovine abortion induced by *Vibrio fetus*. He found that an agglutination test shortly after abortion gave a titer of 1:500 but a subsequent test six months later was entirely negative.

Plastridge and Williams (47) observed that a high incidence
of positive reactors to the agglutination test for <i>Vibrio fetus</i> infection may or may not be accompanied by a high abortion rate, depending upon the stage of gestation of the animals at the time of the positive reaction and whether or not the positive-reacting animals have been infected previously. It was concluded that the agglutination test is of value in diagnosing abortion due to <i>Vibrio fetus</i> infection in a herd of cattle; that abortions due to <i>Vibrio fetus</i> usually occur during the fifth, sixth and seventh month of pregnancy and that <i>Vibrio fetus</i> infection in cattle tends to be self-limiting because of the transitory nature of the infection and a tendency for the initial infection to increase resistance to subsequent exposure.

Brocklehurst (14) examined 255 blood samples that were submitted for routine brucellosis testing and reported that 7 percent gave significant titers with <i>Vibrio fetus</i> antigen. No cross agglutination between <i>Vibrio fetus</i> and <i>Brucella abortus</i> was found.

Four strains of <i>Vibrio fetus</i> isolated from aborted ovine fetuses were studied by Blakenore and Gladhill (10). They describe two colonial variants, one being comparatively large (2-3 mm.) and the other minute, but they were unable to obtain a pure growth of either form. Antigens were prepared for each type of colony but agglutination tests showed that neither of these colonial variants was connected with the loss of flagellar antigen. Their "H" sera was prepared by rabbit inoculation with merthiolated antigen. The "O" antigens for use in the production of agglutinating sera were
prepared by heating the organism in absolute alcohol for 30 minutes at 56°. After removing the alcohol, the organisms were resuspended in saline solution and the immunization was carried out as for the "H" sera. It was found that while there was evidence of antigenic relationship between the flagellar antigens, the "O" antigen of the four strains were completely heterologous. This observation, in their opinion, confirmed the view that tests carried out against a single laboratory strain are of little value and that the agglutination test could not be recommended for the routine diagnosis of vibrionic abortion in sheep.

Mastridge, Williams and Petrič (50) reported studies in which vibrionic abortion had been detected in 18 cattle herds by bacteriological examination and in 29 herds by serological tests only. A comparison of the agglutinin titer of serum from 20 cows aborting infected fetuses with those of 52 cows aborting fetuses in which *Vibrio fetus* was not found suggested that titers of less than 1:200 should be classed as negative, 1:200 as suspicious and 1:400 or higher as positive. Of 26 cows that became positive during the last half of gestation, 42 per cent aborted; of 38 cows that were positive and became negative by the fourth month of gestation, 7 per cent aborted. This report also noted that a lowered conception rate was observed in five herds following the occurrence of vibrionic abortion. The fact that the bull might be implicated in the disease was first suggested in this article when it was found that high agglutination titers (1:400 to 1:1600) occurred in five of six bulls
originating from two of seven herds which were using natural service.

That early abortion (50 to 90 days after conception) appeared to be more common in *Vibrio fetus* infection than in brucellosis was noted by Plastridge and Williams (43). In this report it was stated that the incidence of infection, as indicated by the agglutination test and abortion, appears to reach a peak soon after the start of infection and to gradually decrease thereafter until infection either disappears entirely or a new cycle of infection is started among the young heifers. About 75 per cent of the animals that reacted positively to the agglutination test become negative in less than six months. Apparently agglutinins tend to appear shortly before and at the time of abortion and disappear soon afterward.

Evidence that a herd condition recognized in the Netherlands for many years as "enzootic sterility" was caused by *Vibrio fetus* was presented by Sjollenaar, Stegenga, and Terpstra (63). They state that the disease in cattle is venereal in character and is transmitted by both natural and artificial service. The infection causes a catarrhal cervicitis; consequently infected cows fail to conceive or the fertile ovum soon dies. It was postulated that since the organism enters by way of the genital tract, agglutinins may be formed locally and only appear at times in the blood stream. Agglutinins were demonstrated in the vaginal mucus of many infected cows. *Vibrio fetus* was isolated from the semen of two bulls and breeding experiments proved they were disseminating the infection. The blood serum of one of the bulls had an agglutination titer of 1:50 but
agglutination tests on the semen plasma gave negative results.

An improved method of preparation of Vibrio fetus agglutination antigen was developed by Plastridge, Williams and Roman (51). This antigen was prepared on thiol agar medium with added glutathione. The antigen thus prepared showed less tendency to auto-agglutinate than antigen prepared from cells grown in the semisolid medium formerly used.

It was suggested by Roberta, Gilman and Larsen (58) that, in cattle, since the agglutination test is not reliable in individual cases, it is most valuable as an indication of the presence of infection in a herd rather than as an indication of disease in an individual animal.

Levi (37) in work on the agglutination test in vibrionic abortion of sheep found that there was a partial antigenic relationship among strains rather than complete antigenic identity. He postulated that sheep naturally infected with different vibrio strains may unmask certain common antigens which artificially immunized animals are unable to do. However, in a large number of cases (24 of 49) the sera of infected ewes failed to react significantly when tested against strains other than the one with which they were infected. In summary, Levi states that the results obtained indicated that the agglutination test could not be recommended as a routine method of diagnosis in vibrionic abortion of sheep.

Wilson and McDaniel (90), investigating the possible causes of abortion in cattle previously vaccinated with Brucella...
aborted strain 19, found that Vibrio fetus was present in 7 of 23 aborted fetuses obtained from 20 herds in Berkshire. Because of the difficulties in growing the organism and in the technique of the agglutination test they postulate that this 30 per cent infection rate may be considerably lower than the true incidence of the organism.

Working with nine strains of Vibrio fetus which had been isolated from cattle, Robertstad (50) showed that some strains cross react serologically with Brucella abortus. He carried out various tests to determine the antigenic variation of these strains but all strains reacted similarly. Applying the neutral acriflavine test, developed by Braun and Bonestall (13) for brucella, Robertstad showed that his cultures of Vibrio fetus gave a type of reaction typical of antigenically mucoid cultures. Various other tests, including the triphenyl tetrazolium chloride dye test of Levine and Garber (38) gave reactions typical of smooth type colonies. Agglutination tests with rabbit sera, produced by intravenous inoculation, showed that the strains were serologically heterogeneous.

Flaetridge et al. (49) published an extensive review of vibriosis in cattle. They state that some strains selected and used for antigen production had undergone changes in agglutinability when maintained for 6 to 12 months in soft agar medium. These strains showed increased agglutination with normal serum without a proportionally higher titer with positive serum, thus decreasing the range between negative and positive sera to a point where these cultures
could not be used in preparing antigen for routine use. The strain selected for antigen production should yield no agglutination or incomplete agglutination in 1:25 dilutions of normal sera and agglutinate in known positive bovine sera in dilutions of 1:100 upward. They concluded that, in general, when the titer of normal animals, with respect to the antigen used, is taken into consideration, the agglutination test is valuable in the diagnosis of bovine vibriosis.

The same review points out that agglutination tests require the selection of strains suitable for antigen production, a reserve supply of lyophilised cells of the antigen strain and check tests on each lot of antigen. Tests with heated and unheated *Vibrio fetus* cultures indicate that "O" agglutinins are present in normal serum and "H" and "O" agglutinins are present in positive serum. They also conclude that both the blood serum and vaginal mucus agglutination tests should be used in diagnosis because in some cases the vaginal mucus test is positive in animals that react negatively to the blood test and in other cases the blood is positive, especially in recent infection, and the mucus test is negative.

Lawson and MacKinnon (36) reviewed the work done in Great Britain up to 1952. In their opinion, the serum agglutination test for *Vibrio fetus* was of little value as a diagnostic agent but the vaginal mucus agglutination test was a valuable aid to diagnosis provided the sample for testing was not collected at or around estrus.

In France, Gallut (23) carried out serological studies on 10 strains of *Vibrio fetus, 4 isolated from human cases and 6 isolated
from animals. Antisera were produced by intravenous inoculation of living cultures into rabbits. He postulated the presence of six different antigenic factors—true antigens and haptens—to account for the variations present in the ten strains. Of the 10 strains studied, ten different antigenic types, without relationship to their human or animal origin, were found.

Immunochromatographic and serological studies on these strains were then carried out by Gallut (24). Two fractions were separated by phenol extraction. One of the fractions was polysaccharide in nature and antigenic in rabbits. It was strain specific when tested serologically against serum produced against itself and that prepared with whole, live organisms. Gallut concluded that it corresponded to the somatic "O" antigen whose heterogeneity it possessed. The second fraction was protein in nature and was identical in 9 of the 10 strains studied. He concluded that this is the common antigen shared by all *Vibrio fetus* strains and that it exists only in a "truly masked" form. It is demonstrable only by laborious and time consuming manipulation of the organism but, despite this fact, Gallut suggests that this fraction seems suitable to furnish a diagnostic test for *Vibrio fetus*.

In presenting evidence on the reliability of serological diagnostic tests for *Vibrio fetus* Plastridge and Easterbrooks (45) stated that titers of four times the average normal serum titer or higher should be classed as positive. This report states that agglutinin titers obtained indicated that the organisms present in
different herds were serologically similar.

In an investigation of agglutinins in vaginal secretions in bovine vibriosis, Hughes (30) concluded that the vaginal mucus titers indicate a past or present infection but that the absence of a titer does not indicate absence of infection. The tampon test of vaginal secretions seemed to be useful as an adjunct to the serological agglutination test, when correlated with the clinical history and cultural studies, in reaching a herd diagnosis.

Price, Poelke and Faber (53) carried out cross agglutination tests on 34 strains of *Vibrio fetus* against 25 antisera prepared by the intravenous injection of living cultures into rabbits. The strains used were isolated from bovine and ovine fetuses, bovine vaginal mucus, semen, uterus, porcine tissue and undetermined bovine sources. Antigen for agglutination testing was heated at 100° for 30 minutes. Most of the bovine and ovine strains were found to be fairly closely related antigenically but three strains isolated from vaginal mucus, semen and a uterus were markedly different. The porcine vibrio also seemed to be unrelated. The authors concluded that the serologically distinct strains were probably non-pathogenic and not true *Vibrio fetus*. It was advised that a strain to be used as an antigen should be tested against a large number of bovine and rabbit antisera and should be agglutinated to a fairly high titer by all test antisera.

That *Salmonella pullorum*, *Vibrio fetus*, and *Bacillus* species are antigenically related was determined by Morse, et al. (42).
Pasteurella tularensis and Vibrio comma share antigenic components with Brucella species but not with Vibrio fetus. The effect of these relationships upon the various diagnostic agglutination tests was unknown.

Firehammer and March (21) suggested a modification of Huntone's "hormone" medium for the cultivation of the organism to prepare antigens for agglutinin detection. They state that for antigen production, strains should be selected in which the short spirillar forms and motility can be maintained.

The serological relationship of 23 ovine and 3 bovine strains of *Vibrio fetus* were investigated by Marsh and Firehammer (39). As indicated by agglutinins produced by the intravenous injection of living organisms into rabbits the ovine strains fall into four types while the bovine strains were of a fifth type. The majority of ovine strains were of one type which was sharply differentiated from the other four types but two of the less common ovine types showed some relationship to the bovine type.

Hoff (27) carried out inoculation experiment with an ovine strain of *Vibrio fetus* and was able to infect pregnant guinea-pigs. Thus a laboratory animal suitable for pathogenicity tests was found. Cattle were susceptible to this strain. He produced a typical vibrionic sterility in one heifer by intravaginal inoculation of the organism.

An experiment by Ristic *et al.* (56) using intravaginal *Vibrio fetus* infection in guinea-pigs produced blood serum agglutinin
response similar to that of cattle, while oral infection resulted in higher titers. Although the findings in guinea-pigs cannot be applied directly to the other species, they suggest that genital *Vibrio fetus* infection may be of a local nature involving chiefly uterine tissue changes without sufficient general body reaction to stimulate formation of blood serum agglutinating antibodies to any marked extent.

*Vibrio fetus* abortion in three sheep herds was described by Hoff and Kaldahl (28) and the three strains isolated were morphologically and culturally identical to cattle strains. In these out-breaks, homologous antigen gave a much higher titer than heterologous antigen used in the serum agglutination test. They concluded that, on the whole, the agglutination test is not a particularly good diagnostic adjuvant.

Birns and Fincher (9) in tests involving 384 head of cattle in 31 dairy herds found that the results of the bacteriological examination and the agglutination tests, using two strains of *Vibrio fetus*, showed no significant correlation. They concluded that the only test in which a positive diagnosis of *Vibrio fetus* infection could be made was by the isolation of the organism through bacteriological examination.

In a review of bovine vibriosis in Denmark, Rasbeck (54) stated that the vaginal mucus agglutination test was regarded as being sufficiently reliable to identify the infection in suspicious herds. As a method of testing the individual animal, however, it
was subject to some uncertainty.

Bovine vibriosis in Sweden was reviewed by Boyd and Lagerlof (54). An experiment in which the main diagnostic method was the agglutination test on vaginal mucus was conducted involving 90 herds totalling over 3,000 cattle. It was pointed out that the possible sources of error in this test may be:

"1. variation in the sensitivity of the antigen;
2. agglutination produced by mucus from cows which have not been infected by Vibrio fetus;
3. non-specific reactions of samples from infected animals on account of differences between Vibrio fetus organisms causing animal infections and those used for the preparation of the antigen."

Extensive tests had shown that the antigen had not become more sensitive during the 20-month period of the test. However, they did find that non-specific positive reactors were "not very rare".

Serological examination of 21 strains of Vibrio fetus which had been isolated from the semen and prepuce.washings of 21 bulls from various parts of Denmark was carried out by Juhler (31, 32). Antisera for both flagellar plus somatic and somatic antigens were prepared by the intravenous inoculation of rabbits. The flagellar plus somatic antisera differentiated the 21 strains into four distinct serological groups: a main group consisting of 18 strains and three smaller groups each represented by only one strain. The
analysis of the cell body antigen showed the relationship between
then to be so slight that the somatic antigen was considered specific
for each individual strain. The flagellar antigen, on the other
hand, was considered to be responsible for the fairly high inci-
dence of cross reactions obtained within the main group.

In a paper presented at the same meeting as the one men-
tioned above, Kudik and Elidar (78, 79) presented evidence for the
presence of a "K" antigen in *Vibrio fetus* similar to that observed
in *Escherichia coli*. Antisera was produced by the intravenous in-
oculation of the organism into rabbits. It was noted that the dif-
ference between flagellar and somatic agglutination was not as great
with *Vibrio fetus* as is the case with the flagellated enteric organ-
isms where somatic agglutination is the predominant phenomenon and
precedes flagellar agglutination in time of appearance. The contrast
between the two types of clumping ("H" and "anti-H" and "O" and
"anti-O") was not as sharp as in other motile bacteria. Bacterial
suspensions heated to destroy "H" antigen were agglutinated in the
same sera as were the "H" plus "O" antigen but at a lower titer.
In one case, however, no agglutination occurred. This serum gave a
significant reaction with an antigen prepared by autoclaving (120°C)
for 2 hours and the author concluded that an envelope antigen of
Kauffman's "K" type, inhibiting "O" agglutination, was present in
the unautoclaved suspensions. By means of slide cross-agglutination
tests using antisera prepared against "O" autoclaved antigen and,
utilizing live cells as antigen for the test, a common "K" antigen
was demonstrated. All five strains studied contained "K" antigen but
the content of this factor varied quantitatively. The "K" antigen or
capsule factor could be demonstrated by the Quellung reaction. How-
ever, once again the negative staining technique of demonstrating
capsules was employed and the objection to this is pointed out above.
Other workers have not been able to demonstrate the "K" antigen.

Kiggins (34) investigated the flagellar and somatic antigens
of 10 Vibrio fetus strains of bovine origin and 6 of ovine origin.
The agglutinins produced by both antigens were greater in rabbits
than in cattle. The "O" fraction was found to be non-antigenic in
cattle. Bovine antisera was produced by subcutaneous inoculation of
formalinised suspensions of the organism while the rabbits were in-
oculated intravenously with the same suspension. In the 10 bovine
strains, two distinct serological types, based on "H" and "O" antigens,
were found while the 6 ovine cultures showed three types of "H" an-
tigen and two of "O" antigen. Reciprocal agglutinin absorption tests
failed to show any distinct cross reactions between the various
strains. It was demonstrated that antigens made from subcultures
of individual colonies of a strain of Vibrio fetus may show a wide
variation in agglutinability. In cross-agglutination tests involv-
ing several other species of bacteria it was shown that 3 out of 16
cultures of Vibrio fetus were agglutinated by Brucella abortus anti-
sera but Brucella abortus was not agglutinated by any of the 16
Vibrio fetus antisera.

Casorso (19) found that the agglutinability of different
strains of *Vibrio fetus* by known positive serum varied considerably. He also confirmed the report of Kiggins that individual colonies tend to vary in their agglutinability from that of the original strain but he found that this variation tends to revert to the reaction of the original strain on subsequent colony isolation.

Thouvenot and Floret (73) describe a vibrio isolated from bull semen and the vaginas of cattle which may be mistaken for *Vibrio fetus*. This organism was found to be unrelated to *Vibrio fetus*.

Using an electron microscope to study the morphology of *Vibrio fetus*, Rhodes (55) showed that young cultures had a homogeneous cytoplasmic matrix surrounded by a "cell wall". In older cultures the cytoplasm had a granular appearance and long forms of the organism occurred. Coccoïd forms also occur in older cultures and in cultures grown under unfavourable conditions. Saline suspensions of cultures containing many coccoïd forms were found to be auto-agglutinable. This article contains some very good electron photomicrographs of the various forms of the organism.

In a comparative study of agglutinin response in vaginal mucus and blood serum from 10 cows which had aborted, Eistic, Sanders and Tyler (57) showed that blood serum of infected animals tended to exhibit maximum titer during the first three weeks following abortion, after which the titer gradually receded. The antibodies persisted in vaginal mucus over a longer period than they did in blood serum. The conglutination complement absorption test described by Hole and Coombs (29) was applied and antibodies in seven of
the sera were demonstrated. However, the conglutination test did not reveal antibodies in sera found negative in agglutination tests.

In a further report on their investigations, Price, Foeilsa and Faber (40) used 16 strains of *Vibrio fetus* from bovine and ovine origin and utilized suspensions of heated (100° for 2 hours) cells as "O" antigen. One of the bovine isolates failed to be agglutinated by homologous or heterologous antisera and another, an ovine fetal strain, failed to be agglutinated by heterologous antisera. When unheated antigen had been used for immunization and agglutination, this bovine strain showed cross reactions with the majority of strains. Results obtained from cross agglutination and reciprocal absorption tests with the remaining 14 strains indicated that the somatic antigens of the bovine strains were of four different types and tentative antigenic formulae were suggested. Eight strains were found to be identical, five showed varying absorption patterns and one ovine strain was serologically unrelated. Various biochemical tests were carried out. It was found that none of the strains produced acid in carbohydrate media and the only H2S producers were three serologically unrelated strains which were probably not true *Vibrio fetus*.

Marsh and Tunnicliff (40) conducted agglutination tests on sera from a large number of sheep which were infected with *Vibrio fetus*. They found agglutinin titers were usually present in the serum in the acute stages of the infection but in most cases the titers decreased rapidly after expulsion of the fetus. Ninety-five per cent of the aborting ewes were negative to the agglutination
test before the next breeding season. They concluded that the agglutination test has only a limited application in the practical control of vibriosis of sheep.

Kiggins et al. (35) showed that Vibrio fetus infection does not interfere with the agglutination test for brucellosis but Brucella abortus infection may interfere with the agglutination test for vibriosis if the Vibrio fetus antigen is made from strains which possess antigenic components shared by Brucella abortus.

Working with a large number of vibrio type organisms isolated from aborted fetuses and the reproductive tract of cows Bryner and Frank (15) showed two distinct types of vibrios. One was a catalase-positive, \( \text{H}_2\text{S} \)-negative organism which was thought to be true Vibrio fetus and the other was a catalase-negative, \( \text{H}_2\text{S} \)-positive organism. These catalase-negative strains also show different growth habits in that on deep-stab culture in semisolid thioli medium they grow in the depth of the medium whereas the catalase-positive strains grow only near the surface of this medium.

Twenty-five catalase-positive and 12 catalase-negative strains and their rabbit antisera were compared by cross agglutination tests. No significant cross reactions occurred between catalase-positive and catalase-negative vibrios and the latter group formed two distinct antigenic types. The inference drawn from these findings was that since catalase-negative vibrios were never recovered from aborted fetuses nor from herds with endemic sterility (except in association with catalase-positive strains) they represent a
species other than *Vibrio fetus*.

In heifers experimentally infected with *Vibrio fetus*, Plastridge et al. (46) found that following cervical exposure a four to twelve week interval was required for the development of significant levels of agglutinins in the blood and vaginal mucus. The appearance of agglutinins in the blood serum was independent of agglutinin formation in the vaginal mucus and vice versa. Thus, both blood serum and vaginal mucus should be tested for agglutinins in the diagnosis of vibriosis.

Fifty-seven strains of vibrio organisms from various sources were studied by Biberstein (7, 3). Antisera were produced for 51 strains by the intravenous injection of formalinised cultures into rabbits. Forty-eight of the strains were catalase-positive and cross agglutination tests on these revealed close serological relationship between 44 of them. Four serological types of the catalase-positive organism were shown and were designated as types I, II, III and IV. In 9 catalase-negative strains two serotypes were found; 7 strains occurred in one and 2 in the other. Agglutinin absorption tests using 18 absorbed sera showed that "the multiplicity of serological patterns among the species is largely due to haptenes rather than complete antigens".

Complement fixation tests were carried out utilizing:

1. washed bacterial suspensions,
2. heat-extracted antigens (100° for 40 minutes),
3. autoclave-extracted antigens (120° for 60 minutes).
4. acid-extracted antigens.
5. alkali-extracted antigens.
6. sonic-vibrated antigens.

The results indicated that the existence of a common *Vibrio fetus* antigen "remained dubious".

Biberstein carried out indirect hemagglutination tests on several strains of *Vibrio fetus* using sheep red blood cells. Unheated antigen was unable to modify sheep red blood cells but antigen heated at 100° for two hours caused an amount of sensitivity with the same degree of specificity as the agglutination test. By heating the antigen at 130° for one hour both the supernatant and the residue was capable of modifying sheep erythrocytes and the specificity was in the same order as the agglutination and complement fixation tests although some strains had no effective modifying antigen.

Precipitation tests carried out on the supernatant from sonic-vibrated antigen gave erratic results while agglutination tests on the cellular residue showed no sign of a breakdown of type specificity.

These various tests failed to demonstrate any species specific antigen for the 57 strains studied or even for the 48 catalase-positive strains. It was suggested that serological variant strains be maintained to use for the serological confirmation of infection which cannot be obtained with the standard type I strain antigen. It was confirmed that catalase-negative strains
which produce \( \text{H}_2\text{S} \) fail to cross react with strains of *Vibrio fetus* of known pathogenicity.
A. Cultures

1. Source of cultures - Sixteen strains of *Vibrio fetus* derived from aborted fetuses and bovine genital tracts and their excretions were studied serologically. The source and date of the original isolation, and herd history is presented in table I. Most of the strains were isolated in Ontario but strains 10, 110, 149 and 1969 were isolated in the United States and were obtained through the courtesy of Dr. Biberstein of Cornell University.

2. Maintenance of cultures - Stock cultures of *Vibrio fetus* were maintained in semisolid thiol medium consisting of thiol medium with 0.3 per cent agar added. Subcultures were made at intervals of two to four weeks. The subcultures were incubated at 37°C for 48 hours in jars containing 10 per cent CO₂, then removed from the jars and stored in the dark in a 25°C incubator. Lyophilized cultures of some strains were also prepared and were maintained at refrigerator temperature.

B. Antigen Preparation

1. General - The method of preparation of antigen was essentially that of Piastridge *et al.* (49).

    Liquid thiol medium, without added agar, dispensed in five inch screw-cap culture tubes, was inoculated from the stock

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* Bacto Thiol Medium, Difco Laboratories, Detroit, Mich., U.S.A.
Table I

Strains of Vibrio Tested

<table>
<thead>
<tr>
<th>Strain</th>
<th>Source</th>
<th>Location of Isolation</th>
<th>Date Isolated</th>
<th>Herd History</th>
</tr>
</thead>
<tbody>
<tr>
<td>1903</td>
<td>Bovine fetus</td>
<td>Ontario</td>
<td>1950</td>
<td>Abortion</td>
</tr>
<tr>
<td>54</td>
<td>Bovine vaginal mucus</td>
<td>Ontario</td>
<td>1954</td>
<td>Infertility</td>
</tr>
<tr>
<td>8</td>
<td>Bovine vaginal mucus</td>
<td>Ontario</td>
<td>1954</td>
<td>Infertility</td>
</tr>
<tr>
<td>1033</td>
<td>Bovine fetus</td>
<td>Ontario</td>
<td>1954</td>
<td>Abortion and infertility</td>
</tr>
<tr>
<td>6</td>
<td>Bovine vaginal mucus</td>
<td>Ontario</td>
<td>1954</td>
<td>Infertility</td>
</tr>
<tr>
<td>T</td>
<td>Bovine vaginal mucus</td>
<td>Ontario</td>
<td>1954</td>
<td>Infertility</td>
</tr>
<tr>
<td>1887</td>
<td>Bovine fetus</td>
<td>Ontario</td>
<td>1954</td>
<td>Abortion and infertility</td>
</tr>
<tr>
<td>1717</td>
<td>Bovine fetus</td>
<td>Ontario</td>
<td>1950</td>
<td>Abortion</td>
</tr>
<tr>
<td>H</td>
<td>Bovine vaginal mucus</td>
<td>Ontario</td>
<td>1956</td>
<td>Infertility</td>
</tr>
<tr>
<td>S-3</td>
<td>Bovine vaginal mucus</td>
<td>Ontario</td>
<td>1955</td>
<td>Infertility</td>
</tr>
<tr>
<td>D</td>
<td>Bovine vaginal mucus</td>
<td>Ontario</td>
<td>1954</td>
<td>Infertility</td>
</tr>
<tr>
<td>L</td>
<td>Bovine vaginal mucus</td>
<td>Ontario</td>
<td>1955</td>
<td>Infertility</td>
</tr>
<tr>
<td>149</td>
<td>Unknown</td>
<td>Connecticut, U.S.A.</td>
<td>1949</td>
<td>Unknown</td>
</tr>
<tr>
<td>10</td>
<td>Bovine semen</td>
<td>Indiana, U.S.A.</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
<tr>
<td>110</td>
<td>Bovine semen</td>
<td>Indiana, U.S.A.</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
<tr>
<td>1969</td>
<td>Bovine semen</td>
<td>New York, U.S.A.</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
</tbody>
</table>
culture with a heavy inoculum and incubated for 48 hours at 37° in 10 to 20 per cent CO₂. Seed cultures were prepared from this subculture by inoculating another tube of liquid thioli and incubating as above.

Roux flasks containing 150 to 175 ml. of solid medium composed of one liter of thioli plus 25 g., agar plus 0.5 g., glutathione were prepared. The flasks containing the media were sterilized in an autoclave at 120° for 20 minutes. After hardening in a horizontal position, the flasks were inverted and remained at room temperature for 24 to 48 hours before use in order to remove excess moisture from the media. The water of condensation was removed from the flask and 30 to 40 sterile glass beads were placed into the flask.

The cell-containing layer of a seed culture was removed utilizing a 2 ml. pipette and was dispensed onto five or six small areas on the solid media. One tube of seed culture was used for each flask. The flasks were kept in a horizontal position for two or three hours to prevent the inoculum from spreading until the solid media had absorbed most of the moisture from the inoculum. The flasks were stoppered with a single-hole rubber stopper and 15 to 20 per cent CO₂ was added from a commercial CO₂ cylinder. The flask was placed in a 37° incubator and, at the end of two to three hours, was inverted so that the media was uppermost in the flask.

After 48 hours' incubation, masses of growth were present
in the seeded areas. This growth was spread over the entire surface by gently tipping the flask and allowing the beads to roll over the culture and media surface. The flask was then reincubated in the inverted position for an additional 48 hours.

The resulting growth was harvested by adding 20 ml. of normal saline solution to the flask and again gently tipping the flask to allow the beads to loosen the growth from the solid media. The cell suspension was collected in sterile six inch screw-cap tubes and the cells were concentrated by centrifugation at 2600 r.p.m. for 40 minutes. The supernatant was discarded, the cells washed once in normal saline solution, resuspended in a small volume of normal saline solution and kept at 5°C.

A gram-stained smear was made from the cell suspension from each flask. At each subculturing and with the final antigen, the culture was streaked onto a 5 per cent sheep-blood agar plate which was incubated aerobically at 37°C for 48 hours. Any culture or antigen giving rise to growth on these plates was discarded and any antigen which showed contamination on stained smear was discarded.

2. Somatic plus flagellar (H + O) antigen — The prepared antigen after being checked for purity was pooled and stored in tightly stoppered containers in a concentration adjusted to about tube #16 McFarland's nephelometer. Immediately before use in agglutination tests, the antigen was diluted to the required concentration.

All centrifugation in this experiment was carried out in a No. 531 conical head of a size 2 International Centrifuge.
The density of all dilutions of antigen utilized in these tests were standardized spectrophotometrically.

3. Somatic (0) antigen - Organisms used in the preparation of somatic antigen were prepared as described above. One hundred ml. amounts of the cell suspension, adjusted to about tube #16 McFarland's nephelometer, was heated at 100° for 2 hours in an autoclave in order to destroy flagellar antigens. After heating, the flasks were tightly stoppered and stored at 5° until the antigen was used for agglutination tests.

C. Hyperimmune Sera

1. General - Hyperimmune sera were prepared in both cattle and rabbits by both subcutaneous and intravenous inoculation. Adult rabbits were used throughout. Calves ranged in age from two weeks to about five months. Prior to inoculation each animal was bled and the serum harvested and stored.

Antigen for immunization, in all cases, was used at a concentration equal to tube #16 McFarland's nephelometer. For each strain of organism one rabbit and, as far as possible, one calf were inoculated intravenously and one rabbit and one calf were inoculated subcutaneously. Six injections were given to each animal at three or four day intervals.

2. Rabbit sera - In rabbits the intravenous injections were administered into the marginal ear vein and the subcutaneous injection, into the dorsal thoracic area. The same amount of antigen was given regardless of the route utilized. The injections were
given in the sequence 1, 1, 1.5, 1.5, 2 and 2 ml. Some bleedings were carried out by exsanguination from the jugular vein but it was found to be more satisfactory to bleed directly from the heart.

3. Cattle sera - In calves, the intravenous injections were administered into the jugular vein and the subcutaneous injections into the lateral neck region. Ten ml. amounts of antigen were given in each injection. Bleeding was done from the jugular vein.

4. Harvesting of sera - Blood was collected six days after the last injection and was allowed to stand for several hours at room temperature, then stored in the refrigerator overnight. As soon as a firm clot had formed, the tube was rinsed. The blood was centrifuged at 2,000 r.p.m. for 30 minutes and the serum collected into luer-rod tubes and immediately frozen and stored at minus 20° until used. As pointed out by Price, Poelman and Faber (52) repeated thadings and refreezings failed to cause any significant drop in titer for several months when antisera were stored in this manner. When required for testing, the sera were allowed to thaw by standing at room temperature.

D. Cross Agglutination Tests

1. Antigens - For agglutination tests, the stored antigen was diluted with normal saline solution and standardized to equal approximately tube #2 McFarland's nephelometer. Whenever possible, all sera were tested at the same time against the same batch of pooled antigen for any given strain.
2. Dilution scheme - A series of eleven agglutination tubes were set up for each hyperimmune serum to be tested. One ml. of diluted antigen was dispensed to each tube except the first, which received 2 ml. To the first tube (2 ml. antigen) 0.03 ml. of serum was added and the serum and antigen were mixed thoroughly. One ml. of the serum-antigen mixture was transferred to the next tube utilizing a one-ml. syringe. This suspension was mixed thoroughly by drawing the solution into the syringe and ejecting it forcibly several times. One ml. of this mixture was then transferred to the next tube and the process was repeated for all eleven tubes. This gave a series of doubling dilutions of serum in antigen ranging from 1:25 to 1:25,600.

3. Controls - A saline-antigen control tube containing only the diluted antigen was set up for each test. A serum control was set up for each serum tested against each antigen consisting of pre-injection serum in doubling dilutions from 1:25 to 1:200.

4. Incubation - All agglutination tests were placed in the 37° incubator for 48 hours. The tests were read at this time and then maintained at room temperature for an additional 24 hours and re-read.

5. Reading - The tests were read by removing the tubes from the rack and examining them in indirect light against a black background for clearing of the solution. Gentle agitation was applied to stir up the deposited cells and to note the nature of the deposit. The degree of agglutination was recorded as
$3^+ =$ Complete agglutination with clear supernatant.

$2^+ =$ Nearly complete agglutination but some cloudiness of the supernatant.

$1^+ =$ Some agglutination but incomplete and with a cloudy supernatant.

Trace reactions were recorded but were not considered in the interpretation of the test.

**B. Agglutinin Absorptions**

1. **Antigen** - Antigen for use in agglutinin absorptions was prepared as above (B) and obtained from the stock of flagellar plus somatic antigen. Sixty ml. of the antigen (standardized to tube #16 McFarland's nephelometer) was centrifuged at 2600 r.p.m. for 40 minutes and the supernatant discarded.

2. **Absorption procedure** - The serum to be absorbed was removed from the freezer and allowed to thaw. Two ml. of serum was pipetted into the tube containing the packed cells and mixed thoroughly. The cell-serum mixture was transferred to a 15 ml. centrifuge tube and placed in the $37^\circ$ incubator overnight. This mixture was then centrifuged at 3000 r.p.m. for 40 minutes. The clear serum was collected and re-stored at minus $20^\circ$ until tested. The absorbed serum was tested against the absorbing antigen and if agglutination occurred in any dilution, the absorption procedure was repeated.

Since only packed cells were used for absorption, the resulting serum was considered to be undiluted.
3. Testing - The absorbed sera were tested using the procedure outlined above (d) against a number of flagellar plus somatic antigens. Controls of saline, preinjection serum and preabsorbed hyperimmune serum were used with each antigen tested.

F. Indirect Hemagglutination Tests
Attempts were made to demonstrate capsular material by the technique employed with Pasteurella multocida as reported by Carter (13).

1. Preparation of extracts - Antigen was grown on Roux flasks as described above (b) and the cells were washed off each flask with 13 ml. of normal saline solution. A 4 ml. amount of this cell suspension was heated at 56° for 30 minutes in an attempt to remove any capsular material present. The organisms were then separated by centrifugation and the supernatant transferred to another tube.

2. Preparation of erythrocytes - Human type O red blood cells were obtained through the courtesy of local hospitals. The cells were stored in the original transfusion bottles at 5° and, as required, the blood was drawn off aseptically with a syringe. The blood cells were washed three times with six volumes of normal saline solution. The packed cells from the last washing were stored in the refrigerator until used.

3. Sensitization of erythrocytes with extract - To each bacterial extract consisting of approximately 3 ml., there was added 0.2 ml. of packed erythrocytes. After thorough mixing, the extracts
and cells were placed in a 37° incubator for two hours. The red blood cells were then separated by centrifugation and washed once with 10 ml. of normal saline solution after which sufficient saline solution was added to give a one per cent suspension.

4. Hyperimmune sera - Homologous antisera for each strain of organism was prepared as above (6) by the intravenous inoculation of living Vibrio fetus organisms. Complement was inactivated by heating at 56° for 30 minutes. All sera were then absorbed by the addition of 0.2 ml. of packed, washed, human cells to each 1.5 ml. serum. After incubation for several hours, the cells were removed by centrifugation.

5. Procedure - Twofold serial dilutions of immune sera were made in saline in 12 ml. diameter tubes. The dilutions employed ranged from 1:10 to 1:320. A 0.4 ml. amount of the one per cent suspension of treated red blood cells were added to the tubes containing 0.4 ml. of the serum dilutions. Two control tubes were used: (1) 0.4 ml. of one per cent suspension of treated cells plus 0.4 ml. of normal saline solution and (2) 0.4 ml. of 1:5 dilution of the serum used plus 0.4 ml. of one per cent suspension of erythrocytes.

The racks of tubes were shaken vigorously then left at room temperature for approximately two hours, at which time a reading was taken. The tubes were then placed in the refrigerator and a second reading was made the following morning. A positive reaction,
as described by Carter, consisted of marked agglutination, while a negative test showed no evidence of clumping.

6. **Catalase Test**

A modification of the catalase test described by Bryner and Frank (15) was used to determine the catalase activity of the various strains.

1. **Cultures** - The cultures to be used for the catalase test were obtained by subculturing the stock culture into liquid thiol medium and incubating in 10 to 20 per cent CO₂ at 37° for 48 hours. If a good growth of cells was not obtained, the strain was subcultured again and reincubated as before. This process was repeated until a good growth of cells was produced.

2. **Reagent** - A commercial hydrogen peroxide solution* containing 3 per cent H₂O₂ was used undiluted in these tests.

3. **Equipment** - A 100 x 13 mm. culture tube with a rubber stopper which had a thin central section was utilized. A 20 gauge needle was inserted through the thin central portion of the rubber stopper.

4. **Procedure** - The cell-containing layer of the thiol culture was measured and pipetted into the culture tube. An equal volume of hydrogen peroxide solution was added and the tube immediately plugged with the rubber stopper, pierced by the needle. Mixing was obtained by inverting the tube a few times. The inverted tube was placed in a wire rack over a sink and allowed to

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* Hydrogen Peroxide (10 Volumes, 3% H₂O₂), Stevens.
stand for 20 minutes.

No attempt was made to measure the volume of liquid displaced by gas an Bryner and Frank had done. They considered that cultures displacing five mm. or less of liquid were catalase-negative while those displacing more than this amount were catalase-positive but they used the entire culture instead of just the cell-containing layer as was utilized in the present study.

II. Acriflavine Test

A modification of the acriflavine test as proposed by Braun and Bonestell (13) was carried out on the cultures studied.

1. Culture - Seed cultures were prepared as for antigen production (B-1). A large (4 mm.) platinum wire loopful of the cellular layer of the seed culture was spread over a 5 per cent sheep-blood agar petri plate. The plates were incubated in 15 to 20% CO₂ at 37° for 72 hours.

2. Reagent - Neutral acriflavine was made up in a 1:1000 aqueous solution and stored in a brown bottle in the refrigerator. A fresh solution was made up each week when required.

3. Procedure - A gram-stained smear was made of the growth on the plate. A drop or two of sterile saline was placed on a clean glass slide. A number of colonies were picked from the plate and a smooth suspension of cells in saline was obtained. A 4 ml. loopful of the acriflavine solution was mixed with this

Acriflavine (Neutral) NF, National Aniline Division, Allied Chemical and Dye Corp.
suspension and the results were observed directly and under low magnification.

An attempt was made to relate the results obtained to the morphology of the organism observed on gram-stained smear and to the antigenic characteristics of the strain.
RESULTS

A. Antigen

The preparation of Vibrio fetus antigen is beset with many difficulties. It was found that any given strain of the organism varied greatly in the amount of growth obtained within the 48-hour incubation period. Many strains grew inadequately on the original subculture from the stock culture and repeated serial subculturings at 48-hour intervals were needed to rejuvenate the strain. In some cases it was found expedient to culture the organism on 5 per cent sheep-blood agar plates, then subculture back to thiol from the growth thus obtained.

In spite of the fact that numerous cultures of the organism were maintained at all times and that vigorous attempts were made to reclaim the more laggard strains, three of the strains failed to survive for the duration of the experiment. However, no great difficulty was encountered in maintaining most of the strains under test.

B. Reactions to Immunization

No deleterious effects attributable to the injection of the organism were observed in rabbits.

In calves no apparent reaction occurred to subcutaneous inoculation of the antigen except for a transitory dullness and anorexia in a few instances which lasted for several hours after injection.
Intravenous injection of some of the strains caused allergic or anaphylactic reactions in some calves and three calves died, apparently as a result of the inoculations.

1. Calf #18, a 4 month old male Ayrshire, had received three intravenous injections of vibrio strain 1969 without showing any marked reaction. After administration of the fourth injection, the animal was left unattended for two and a half hours at which time it was found dead. A frothy exudate was present in the nostrils and the animal was slightly bloated. Unfortunately, circumstances prevented a necropsy of the animal.

2. Calf #22, a one week old male Ayrshire, was given one intravenous injection of Vibrio fetus, strain 1717 antigen. Thirty minutes later it began to show dyspnea and uneasiness. An intramuscular injection of 0.75 ml. of 1:1000 solution of epinephrine, diluted to 10 ml. with sterile normal saline solution, was given. Within a few minutes, the animal's condition improved. Two hours later, the calf was very dull and showed rapid, shallow breathing. The animal was massaged for several minutes and then forced to exercise for a few minutes and a marked improvement occurred. However, its condition slowly degenerated and five hours after the injection, when the animal could not rise, a second injection of epinephrine —0.5 ml. of 1:1000 solution diluted in 7 ml. of sterile normal saline solution—was given. This was followed by massage of the animal and once again amelioration was observed. As the effect of the stimulant decreased, the animal regressed rapidly and died about
six hours after the antigen was injected. A post-mortem examination was carried out.

**Necropsy findings:** The cornea of the right eye was slightly opaque. The lungs were markedly edematous. The spleen and heart although normal in size presented subcapsular and subpericardial petechial hemorrhages. The adrenals were slightly enlarged and hyperemic.

**Histopathological examination:** The spleen was congested and had subcapsular hemorrhages. The interlobular branches of the portal vein of the liver were distended with blood. Marked congestion, mild alveolar and marked interstitial edema were present in the lungs. A few interstitial hemorrhages were seen in the kidney cortex. The adrenal glands were congested and had interstitial hemorrhages in the cortex and medulla.

**Bacteriological cultures from various tissues failed to reveal any pathogenic organisms.**

2. Calf #26, a six week old male Ayrshire, was injected once intravenously with *Vibrio fetus* strain 1717. Because of the reaction and death of calf #22, which had been injected with the same antigen, the normal dosage of antigen was reduced by one-half; the 10 ml. volume was made up with sterile normal saline solution and 0.5 ml. of 1:1000 epinephrine was mixed with the antigen. About 15 minutes after the injection the animal was showing marked dyspnea. Before another stimulant could be obtained, the calf died.

**Necropsy findings:** The lungs were very edematous and the
trachea and bronchi, filled with a frothy exudate. Other tissues appeared normal.

Histopathological examination: The spleen was slightly congested with a separation of cellular constituents in some areas indicative of edema. The lungs had a marked alveolar and interstitial edema with hemorrhage into some alveoli and bronchi. The kidney showed a degeneration and necrosis of some of the proximal tubules with hemorrhage into some areas of degeneration. The heart was essentially normal but some separation of muscle fibers indicated edema.

Bacteriological examination of the tissues failed to demonstrate any significant pathogenic organisms or to recover Vibrio fetus.

C. Cross Agglutination Tests

1. Flagellar plus somatic ("H" + "O") antigens - The results of the cross agglutination tests involving 14 antigens and 53 antisera are summarized in Table II. Several antigens showed some reaction in the pre-injection serum and corrections were applied for these reactions. The table records only complete agglutination but partial agglutination did occur in some cases.

The highest titers were obtained in sera produced by intravenous inoculation of rabbits. There was a marked difference between the titers produced in rabbits depending on the method of inoculation—the subcutaneous route giving rise to much lower titers than the intravenous route. In cattle the titers in the
### Table II

**Cross Agglutination Reactions of 14 Vibrio "H + O" Antigens Tested Against 53 Antisera**

**Legend**

Numbers denote the reciprocal of the highest dilution of sera causing complete agglutination.

- **Not tested.**

1. Hyperimmune serum prepared by intravenous injection of antigen into cattle.

2. Hyperimmune serum prepared by subcutaneous injection of antigen into cattle.

3. Hyperimmune serum prepared by intravenous injection of antigen into rabbits.

4. Hyperimmune serum prepared by subcutaneous injection of antigen into rabbits.
sera produced by different routes of inoculation did not differ to the same extent and in many instances the titer produced by subcutaneous inoculations equaled that produced by intravenous injections.

As pointed out by Smith (65, 70) and later by Riberstein (7) some antigens were agglutinated to higher titer by heterologous sera than by homologous serum. For example (table II) antigen 7 was agglutinated by the homologous antiserum 7,2 to a titer of only 1:50 while the same antigen was agglutinated by the heterologous serum 5,2 to a titer of 1:400. Some sera which had a low titer when tested against the homologous antigen agglutinated heterologous antigen to a higher dilution. For example (table II) antiserum 7,2 agglutinated the homologous antigen 7 to a titer of 1:50 but this same antiserum agglutinated antigen 1903 to a titer of 1:400. These phenomena are generally ascribed to differences in the "antibody stimulating" properties of the antigen, the agglutinability of the antigen and the potency of the immune sera.

Another phenomenon noted was unilateral reactions. In such cases, an antigen was agglutinated by heterologous antiserum while the homologous antiserum failed to react with the corresponding heterologous antigen. For example (table II) antigen 3 was agglutinated by antiserum 1903,3 to a titer of 1:800 while antiserum 3,3 failed to agglutinate antigen 1903 in any dilution tested.
Two antigens, strains $D$ and $L$, showed a marked tendency toward auto-agglutination and therefore were not used as antigen for agglutination tests although antisera were produced against both these strains.

The strains studied were divided into two main groups by the presence or absence of the enzyme catalase. Antigenic differences were demonstrated between these two types.

(a) Catalase-positive strains — Nine of the 13 catalase-positive strains fell into one, more or less, closely related group serologically. Two of the other strains, 1887 and 110, varied somewhat in their reactions and were not considered to be of the same group. One strain, 1717 failed to react in significant titer to any sera except the homologous sera and the anti-1717 sera failed to agglutinate any other vibrio strains in significant dilutions. Strain II, a very recent isolate from bovine vaginal mucus, was agglutinated in relatively high titer by most anti-vibrio sera produced against both catalase-positive and catalase-negative strains. A marked prozone was noted in some tests and was especially marked with antigen II.

(b) Catalase-negative strains — Two of the three catalase negative strains were nearly identical serologically. One of these, strain S-3 was isolated from bovine vaginal mucus in Ontario while the other, strain 10, was isolated from bovine semen in Indiana, U.S.A. The third catalase-negative strain was not antigenically related to the other two.
None of the catalase-negative vibrios showed any significant serological relationship to the catalase-positive strains. It is of interest that the catalase-negative strain 5-3 was isolated from the same herd as was the catalase-positive, main-group strain 54.

2. Somatic antigens - Cross agglutination tests were carried out using 14 heated antigens and the 53 antisera. The reciprocals of the highest dilution showing complete agglutination are given in table III.

In nearly every test a marked reduction in titer occurred when compared to the tests with "H" + "O" antigen. This reduction was most marked in sera showing high titers in the complete-antigen tests.

Many of the heated antigens tended to settle out of solution quite rapidly and the tests were difficult to read. Observation of the form of the settled antigen and gentle agitation were employed to differentiate between true agglutination and the normal sedimenting of the cells.

Although many partial reactions occurred in low titers, in no case did the antisera produced by the subcutaneous inoculation of calves cause complete agglutination of the "O" antigen of catalase-positive vibrio strains. However, in several instances the antisera produced by intravenous inoculation of calves gave as high a titer with heated antigen as was obtained with unheated antigen. For example, antiserum 149,1 (tables II and III) gave
Table III
Cross Agglutination Reactions of 14 Vibrio "O" Antigens
Tested Against 53 Antisera

Legend
Numbers denote the reciprocal of the highest dilution of
sera causing complete agglutination.

-- Not tested.

1. Hyperimmune serum prepared by intravenous injection of living
antigen into cattle.

2. Hyperimmune serum prepared by subcutaneous injection of living
antigen into cattle.

3. Hyperimmune serum prepared by intravenous injection of living
antigen into rabbits.

4. Hyperimmune serum prepared by subcutaneous injection of living
antigen into rabbits.
a titer of 1:50 with both "H" + "O" and with "O" antigen 110
while antiserum 149,2 showed a 1:50 titer with "H" + "O" antigen
and no titer against "O" antigen 110.

The rabbit sera also showed a decreased titer when tested
against "O" antigen as compared to the tests with "H" + "O" an-
tigen. However, the sera produced by subcutaneous injection of
rabbits did cause complete agglutination of some of the heated
catalase-positive antigens. In a few such cases no reaction oc-
curred with the serum produced by intravenous inoculation. For
example (table III) with strain 5 antigen tested against anti-
sera 1033,3 and 1033,4, the antisera 1033,4 retained a titer of
1:50 against "O" antigen while antisera 1033,3 did not show any
titer against this antigen.

(a) Catalase-positive strains - The heated antigens retained
the same general antigenic relationship to one another as did
the unheated.

Strain 1717 "O" antigen showed some reaction with the other
catalase-positive sera, thus indicating that this strain is re-
lated to the other catalase-positive vibrio through its somatic
antigen.

The reactions of strain H after heating showed greatly re-
duced titers but the antigen still reacted with sera produced
against both catalase-positive and catalase-negative strains.

(b) Catalase-negative strains - In general the reactions of the
catalase-negative strains were unchanged from the tests with
unheated antigen except for a reduction of titer. However, strain 1969 showed several cross reactions with antisera produced against some of the catalase-positive strains, thus indicating that it contains somatic antigens common to these strains.

D. Agglutinin Absorption Tests

Several methods of agglutinin absorption were attempted but the one found most satisfactory was the technique described above. This method of measuring and handling the antigens was found to be easily reproducible and gave consistent results.

The absorbed sera were tested by cross agglutination tests with ten representative strains of vibrio including both catalase-positive and catalase-negative strains. The results are summarized in table IV.

These agglutination test results provide added evidence of the close antigenic relationship between strains 149, 1303, 54, 5 and 1033 and the partial relationship between these strains and strains 1837 and T. The catalase-negative strains were again proven to be serologically unrelated to the catalase-positive strains. Strain 8-3 failed to remove the agglutinins from anti-3 serum but did remove all demonstrable agglutinins from anti-10 serum thus proving it to be closely related to strain 10 and indicating it is not related to strain 8.

E. Indirect Hemagglutination (HIA) Tests

Six strains representing both catalase-positive and
### Table IV

**Cross Agglutination Tests with Absorbed Sera**

<table>
<thead>
<tr>
<th>Antigens</th>
<th>Absorbed with</th>
<th>Pre</th>
<th>Post</th>
<th>Pre</th>
<th>Post</th>
<th>Pre</th>
<th>Post</th>
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</table>

**Legend:**
- Pre = pre-absorption hyperimmune serum
- Post = postabsorption hyperimmune serum

Figures indicate the reciprocals of the highest dilution of serum at which complete agglutination occurred.
catalase-negative organisms were tested by this technique against homologous antisera produced by intravenous injection into rabbits. This serum was chosen because it showed the highest titer in the agglutination tests, therefore it was felt that it would have the greatest chance of showing reaction in the IHA test. However, no evidence of sensitization of human type O erythrocytes was demonstrated.

F. Catalase Tests

All strains of the organism utilized in these tests were examined for catalase activity. All strains were catalase-positive except three.

It was felt that, to be significant, the presence or absence of the enzyme, catalase, should be qualitative rather than quantitative and that the assignment of a catalase number, as proposed by Bryner and Frank (15), to each culture implies a degree of precision which is probably not justified by the test. With the technique employed, the catalase-positive strains caused a complete or almost complete expulsion of the liquid from the inverted tube within minutes after mixing the hydrogen peroxide and culture. Catalase-negative strains failed to show any significant displacement of liquids from the tubes.

All *Vibrio fetus* isolated from aborted fetuses proved to be catalase-positive. Two of the catalase-negative strains were isolated from bovine semen and the third, from bovine vaginal mucus.
Table V

Classification of 16 Vibrio Strains by Catalase Activity and Antigenic Type

<table>
<thead>
<tr>
<th>Strain</th>
<th>Catalase Activity</th>
<th>Antigenic Type</th>
<th>Bieberstein's Classification</th>
</tr>
</thead>
<tbody>
<tr>
<td>149</td>
<td>+</td>
<td>Main group</td>
<td>Catalase-positive Type I</td>
</tr>
<tr>
<td>1306</td>
<td>+</td>
<td>Main group</td>
<td>Catalase-positive Type I</td>
</tr>
<tr>
<td>54</td>
<td>+</td>
<td>Main group</td>
<td>Catalase-positive Type I</td>
</tr>
<tr>
<td>S</td>
<td>+</td>
<td>Main group</td>
<td>Catalase-positive Type I</td>
</tr>
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<td>1023</td>
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<td>Catalase-positive Type I</td>
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<td>+</td>
<td>Main group</td>
<td>Catalase-positive Type I</td>
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<tr>
<td>T</td>
<td>+</td>
<td>Main group</td>
<td>Catalase-positive Type I</td>
</tr>
<tr>
<td>1887</td>
<td>+</td>
<td>Slight variant</td>
<td></td>
</tr>
<tr>
<td>110</td>
<td>+</td>
<td>Slight variant</td>
<td>Catalase-positive Type IV</td>
</tr>
<tr>
<td>1717</td>
<td>+</td>
<td>Variant</td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>+</td>
<td>Unclassified</td>
<td></td>
</tr>
<tr>
<td>S-3</td>
<td>-</td>
<td>Main catalase-negative group</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>-</td>
<td>Main catalase-negative group</td>
<td>Catalase-negative Type I</td>
</tr>
<tr>
<td>1969</td>
<td>-</td>
<td>Variant</td>
<td>Catalase-negative Type II</td>
</tr>
</tbody>
</table>
Table V shows the classification of the various strains by antigenic and catalase activity.

C. Acriflavine Tests

The reactions observed with *Vibrio fetus* were somewhat different from those which occurred with *Brucella abortus* variants as described by Braun and Bonestell (13). Tests on old cultures of the organism gave a rough or mucoid type of reaction in which very coarse granules or stringiness appeared. Since 72 hours was the shortest incubation period which gave sufficient cells to carry out the test, all cultures were tested after this period of incubation. It was impossible to obtain a suspension of cells of sufficient density by picking a single colony, therefore a number of colonies were fished from the plate and examined in one suspension.

Reactions typical of the smooth-colony type as described by Braun and Bonestell were seen. When the acriflavine was added, the cells remained almost completely in suspension with no evidence of clumping. This type of reaction was observed with the catalase-negative strains and with the recently isolated strain II. Gram-stained smears of the catalase-negative organisms showed that most cells were the short forms and had a less-pronounced spiral than the other strains (Fig. 1). Strain II appeared as short curved rods occurring single and in very short chains (Fig. 2).

Another reaction obtained was the immediate development of a fine granularity in the cell suspension similar to the $S^R$
type of Braun and Bonestell. The cells tended to clump into small aggregates. This was the reaction observed with most strains of the main antigenic group of catalase-positive organisms. Gram-stained smears showed a mixture of forms consisting mostly of short forms but some intermediate-length organisms were present (Fig. 3).

A third type of reaction consisted of the formation of a coarse granularity which appeared shortly after the addition of acriflavine. The cells massed into large clumps and settled out of suspension. This type of reaction was found most commonly with catalase-positive cells which were not of the main serological type. On smear the organism appeared to be fairly straight and mostly of the short form with an occasional long chain (Figs. 4 and 6).

The findings are summarized in table VI and the various reactions observed are compared with the morphology of the organism as demonstrated by gram-stained smear. Although not subjected to the acriflavine test, the morphology of strain 1717 was typical of strains which gave the coarse granular reaction (Fig. 5).
### Table VI

**Acriflavine Reactions of Vibrio Strains**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Reaction</th>
<th>Morphology on Stained Smear</th>
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</thead>
<tbody>
<tr>
<td>149</td>
<td>finely granular</td>
<td>single organisms and short chains, pronounced spiral</td>
</tr>
<tr>
<td>1806</td>
<td>finely granular</td>
<td>single organisms and short chains, pronounced spiral</td>
</tr>
<tr>
<td>54</td>
<td>finely granular</td>
<td>single organisms and short chains, spiral</td>
</tr>
<tr>
<td>8</td>
<td>coarsely granular</td>
<td>short chains, slight spiral</td>
</tr>
<tr>
<td>T</td>
<td>coarsely granular</td>
<td>short and long chains, no spiral</td>
</tr>
<tr>
<td>1837</td>
<td>coarsely granular</td>
<td>short and long chains, no spiral</td>
</tr>
<tr>
<td>H</td>
<td>smooth, no clumps</td>
<td>single organisms and very short chains; curved</td>
</tr>
<tr>
<td>S-3</td>
<td>smooth, no clumps</td>
<td>single cells and short chains, relatively straight</td>
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<tr>
<td>10</td>
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<td>single cells and short chains, relatively straight</td>
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<tr>
<td>1969</td>
<td>smooth, no clumps</td>
<td>single cells and short chains, relatively straight</td>
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STATISTICAL ANALYSIS OF SELECTED DATA

The data obtained from the agglutination tests of homologous "H" and "O" antigen and antisera were tested to determine if the difference in titers in cattle and rabbit antisera produced by intravenous and by subcutaneous inoculations was significant. The individual titers, the means, the standard deviations and standard errors of the means are shown in table VII.

Since the arithmetic means and standard deviations of these groups of data tend to be equal, in most cases the standard deviation is larger than the mean, the distribution of the titers does not fit the normal curve. The measurements of titer were not increased by a common increment but were increased proportionally, therefore, it is probable that the geometric mean is closer than the arithmetic mean to the true mean.

The mean of the titers of rabbit antisera produced by intravenous inoculation plus or minus two standard errors of the mean \((\overline{x} \pm 2\frac{s}{\sqrt{n}})\) is greater than the means of the other three antisera plus or minus two standard deviations of these means. Thus, under the conditions of this experiment, the rabbit antisera produced by intravenous inoculation was significantly higher in titer than the rabbit antisera produced by subcutaneous inoculation or the cattle antisera produced by either route of injection of antigen.

The other three mean values plus or minus two standard
Table VII
Antibody Titters of Antisera to Vibrio Organisms

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<th>Rabbit Antisera</th>
<th>Cattle Antisera</th>
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<td></td>
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<td>839.2</td>
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<tr>
<td>Standard Error of Mean</td>
<td>1869.9</td>
<td>232.7</td>
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deviations of the mean are all well within range of one another. This indicates that, in this experiment, there was no marked difference among the antibody titers in rabbit antisera produced by subcutaneous inoculation, cattle antisera produced by subcutaneous inoculation and cattle antisera produced by intravenous inoculation.
DISCUSSION

The unpredictable factors of the antigenicity of the organism, antigenic sensitivity and the antibody response of the animal undergoing immunisation must all be carefully considered in the interpretation of serological experiments. In many instances, the sensitivity of the antigen to agglutination rather than its complement of individual factors accounts for great titer differences as well as for such phenomena as unilateral and paradoxical reactions.

It is of interest that in the severe allergic reactions which occurred in calves being inoculated intravenously, the antigens were of serological types other than the main *Vibrio fetus* type. One animal was injected with strain 1969, which is a catalase-negative strain, and the other two were injected with strain 1717 which is catalase-positive but antigenically distinct from the main group. In the first instance, death was probably due to a classical anaphylactic reaction. Since the animal had received three injections prior to the fatal one, these may have sensitised it to the antigen.

The other two animals both succumbed to the first injection of antigen in spite of the administration of epinephrine and both showed reactions and post-mortem lesions typical of an acute allergic reaction.
The interpretation of quantitative differences in cross agglutination reactions requires a good deal of caution. Antigen sensitivity to agglutination can account for antibodies being detected in unabsorbed sera by one strain and not by another, very similar, strain. Higher titers with heterologous than with homologous sera can be accounted for by quantitative differences in the antigens. As pointed out by Biberstein (7), a strain may contain an antigen or haptene in such small quantities that while it is capable of stimulating antibodies against this factor it does not react with these antibodies to an observable degree. Another strain which possesses larger amounts of this factor will be completely agglutinated by such antibodies. When viewed in this light, the variations and apparent contradictions in the cross agglutination titers lose much of their significance.

Undoubtedly qualitative differences in antigenic structure do exist in this species. Consistent and striking differences in serological behavior have been demonstrated in the results obtained from the cross agglutination tests. Price, Poelman and Faber (53) proposed that *Vibrio fetus* could be divided into five antigenic groups depending on the presence or absence of five different somatic antigens which occurred in varying combinations. *Vibrio fetus* of bovine origin fell into one or the other of four of these types while one ovine strain was serologically unrelated. In an earlier publication (52) dealing with both flagellar and somatic antigens, the same authors stated that many variations
appear to be quantitative rather than qualitative in nature.

Biberstein (7) concluded that the catalase-positive vibrio could be divided into four types antigenically. The main serological type contained 44 of the 47 catalase-positive strains while the remaining three strains each represented distinct types. Nine catalase-negative strains were divided into two groups: one contained seven strains and the other, two. Type strains were selected for each separate type.

Strain 149 is a representative of Biberstein's main type and in the present study it was found that this type was similar to many strains isolated in Ontario. This indicates that the main type of *Vibrio fetus* in both countries is antigenically similar and makes up the majority of the population of *Vibrio fetus* strains which have been isolated.

Strain 110 was the type species of Biberstein's catalase-positive type IV and one strain isolated here was closely related to this type. In the cross agglutination tests carried out in this experiment, strain 110 showed a good deal more cross reaction than is indicated in Biberstein's results.

Two other Ontario isolates which were catalase-positive and antigenically unrelated, were demonstrated. Cultures of Biberstein's catalase-positive types II and III were not included in this study and the antigenically unrelated strains isolated in Ontario may be representatives of these types or they may be distinctive types.
Of the catalase-negative vibrios, strain 10 was the type species of Biberstein's catalase-negative type I and one Ontario isolate was very closely related to this type, strain 1969 was Biberstein's catalase-negative type II but no Ontario isolate was related to this organism.

The cross agglutination tests using heated antigen tended to confirm the serological and physiological groupings found in the tests utilizing complete antigens. Low titers were obtained with some strains not showing cross reactions in the test with unheated antigen. These results indicate that the strains may have common somatic but not flagellar antigens. The catalase-negative strain 1969 showed cross reaction with some antisera of catalase-positive origin but no significant titers were observed.

Vibrio fetus usually possesses only a single polar flagellum but in some cases it may be bipolar. Because of the non-flagellate condition Wildik and Hildar (78) concluded that the "H" antigen is present in relatively small amounts as compared to the "O" antigen and consequently an antisera prepared by immunization with an intact organism should produce, predominantly, an "O" type antibody. The contrast between the "H" and "O" type of clumping as seen in Vibrio fetus are not as distinct as in other motile organisms. However, the comparison of titers to "H" + "O" and "O" antigen obtained in these tests indicates that the major agglutinin response to Vibrio fetus organisms is due to flagellar rather than somatic antigens. On the other hand, it is possible
that heating the organism at 100° for 2 hours may cause a partial
denaturation of somatic antigens as well as the complete destruct-
tion of flagellar components.

Numerous attempts to demonstrate capsular substance on
representative strains of the organism by various staining tech-
niques and by extraction (IHA tests) failed to show any evidence
of capsular material. However, capsular substance and "K" antigen
may be present without a visible capsule being demonstrable. The
two reported demonstrations of capsular substance—Smith (70),
Hildik and Hildar (73)—were both based on an unstained area around
the organism and, as pointed out earlier, this may be an artefact.
For positive identification, a capsule should be stained and thus
demonstrated around the organism.

The importance of "H" antigen as a major factor in cross
reactions substantiates the findings of Juhler (32) and Kiggins
(34). It was postulated by Kiggins that "O" antigen was non-
antigenic in cattle but the present investigation shows this to
be incorrect. The "O" antigen is antigenic in both cattle and
rabbits but the route of inoculation has a pronounced effect on
antibody response to this component. While no anti-"O" antibody
was produced in cattle by subcutaneous injection and only a very
low titer produced in rabbits using this inoculation route, both
these species did produce demonstrable anti-"O" titers when the in-
travenous route of inoculation was used.

In agglutinin absorption tests, Ristic, Sanders and
Tyler (57) concluded that all antigens used in this study were homologous as indicated by their capacity to absorb completely all demonstrable agglutinins. The sera used were of low titer (the highest being 1:160) and were obtained from naturally occurring cases of the disease. Bibarstein (7) carried out agglutinin absorption experiments which suggested a large number of subtypes within his catalase-positive type I strains. The tests in the present study serve to strengthen the evidence for the division of the organism into the groups suggested by the cross agglutination test results. Although some evidence of subtypes was found, it was not considered to be significant.

The isolation of a common Vibrio fetus antigen was reported by Gallut (24). This phenol-extracted substance from serologically heterologous vibrio strains failed to react with sera prepared against even the homologous strain of living organisms. However, when antisera were prepared against the extract from each strain this antisera reacted freely with all factors both homologous and heterologous. The extract was thought to be the common vibrio antigen which existed in a "masked" form. A masked antigen is usually considered to be one which will not react with homologous antisera in vitro until it is unmasked. An example of this is the somatic antigen of an encapsulated organism. When living bacterial antigens are injected into rabbits antibodies against the masked antigens are formed. The findings of Gallut do not conform with the commonly accepted definition of a "masked" antigen.
since it failed to react with rabbit sera prepared against the living organism. The extraction processes to which the vibrio were subjected to isolate this factor were rather strenuous and it is possible that this may not be a true vibrio antigen but an artefact caused by extraction. In any event, the process probably cannot be considered as a routine diagnostic aid because the factor probably would not react with sera obtained from infected animals.

Utilizing numerous techniques, Biberstein (3) found that experiments in which a breakdown of type specificity occurred revealed that the resulting cross reactions were not confined to *Vibrio fetus* strain but extended to catalase-negative vibrio and even to "normal" sera. The breakdown of type specificity generally lead to destruction of antigen specificity so that the resulting preparation could not be relied upon to confine its reactivity to *Vibrio fetus* antibodies.

The results of the present experiments are similar to those of both Price, Poole and Faber (52, 53) and Biberstein (7) in that one antigenic type of *Vibrio fetus* predominated. However, the assignment of a type and the setting up of antigenic type species for single strains of the organism may be somewhat academic and premature at this time. It is becoming recognized that most true *Vibrio fetus* form one main antigenic type. The remainder should merely be classed as variant types until at least a significant number of strains are shown to be related, at which time a distinct type with a type species should be recognized.
For practical purposes, the antigen used in routine diagnostic agglutination tests should be derived from a strain within the same serological type. Antigen for the variant strains should be available for agglutination tests to confirm infection in animals which show clinical symptoms but whose sera fail to react with the standard antigen.

In general, the antibody titers produced by the intravenous inoculation route in rabbits were markedly higher than those produced in cattle inoculated in the same manner and were significantly higher than those produced in rabbits or cattle by subcutaneous inoculation. No significant quantitative difference was shown between sera produced in rabbits by subcutaneous inoculation and in cattle by intravenous or by subcutaneous injection.

Qualitative differences were shown between sera produced in cattle by intravenous and subcutaneous inoculation. While sera produced by intravenous injection frequently showed reactions with somatic \textit{Vibrio fetus} antigen, sera produced by subcutaneous inoculation failed to produce complete agglutination of this "O" antigen in any titer tested. Thus Kiggins' failure to demonstrate "O" antibody in cattle is probably due to the selection of the subcutaneous rather than the intravenous route of inoculation.

Early literature in bacteriology refers to "dissociation" of cultures but this effect has now been shown to be due to genetic mutation and selection - Braun (12). Spontaneous mutations affecting antigenic characteristics may occur independent of changes in
colonial morphology and physiological behaviour. If these antigenic mutants are, in themselves, selectively advantageous or are combined with selectively advantageous genetic factors, the mutant type may increase proportionally in the population until it is the predominant type or, possibly, until it entirely displaces the "normal" parent antigenic type. The possibility of these variant types displacing the normal population may be much greater in stock culture, where the organism is maintained under relatively unfavourable conditions, than it would be in the natural habitat of the organism.

The acriflavine test has been applied to various gram-negative species to demonstrate antigenic variation in the species. Antigenic-variant types can be demonstrated by the acriflavine test which are not accompanied by changed colonial or physiological characteristics. Some variation in reaction was demonstrated in the Vibrio fetus strains. However, before any significance can be attached to these changes, which are largely quantitative and not qualitative in nature, the test should be applied to a large number of cultures.
SUMMARY

1. Sixteen strains of vibrios of bovine origin were tested for antigenic relationships using 53 antisera prepared by intravenous and by subcutaneous inoculations of living organisms into cattle and into rabbits.

2. Each strain was classified according to its catalase activity.

3. Cross-agglutination tests were performed, testing 14 antigens against 53 antisera utilizing both "H" + "O" and "O" antigen. Of the 13 catalase-positive strains, nine formed one antigenically similar group; two, although not identical with the main group did show some cross reaction; one did not show any significant cross reactions; and one, a very recent isolate, reacted with nearly all antisera and was therefore antigenically untypable. Two of the three catalase-negative strains were practically identical and the third was antigenically distinct.

4. Agglutinin absorption tests confirmed the antigenic similarity of members of the main group and the antigenic differences of variant strains and catalase-negative strains.

5. Strains subjected to the indirect hemagglutination test failed to react with the technique employed.

6. Acidflavine tests showed some variation in reaction, but before any significance can be attached to these reactions, the test should be applied to a significant number of cultures of Vibrio fetus.
7. Statistical analysis on data from agglutination test titers using "H" + "0" antigen showed:

(a) A significant increase in titer in rabbit sera prepared by intravenous injection over that prepared by subcutaneous injection in rabbits and that prepared in cattle by intravenous and by subcutaneous inoculation.

(b) No significant difference in titer among the antisera produced by subcutaneous inoculation of rabbits and that produced by intravenous and by subcutaneous inoculation of cattle.

8. While no quantitative difference between the titers obtained with "HI" + "0" antigen was shown in cattle antisera produced by intravenous and by subcutaneous inoculations, a qualitative difference was demonstrated by the use of "0" antigen. Complete agglutination was not caused by any antisera produced by subcutaneous inoculation of cattle with true Vibrio fetus "0" antigen but complete agglutination was caused by antisera produced by the intravenous route of injection.
FIG. 1. *Vibrio* strain S-3. Single cell and short, straight chains. Gram stained smear, approximately 1000X.

FIG. 2. *Vibrio fetus*, strain H. Single, curved organisms. Gram stained smear, approximately 1000X.
FIG. 3. *Vibrio fetus*, strain 1808. Single organisms and short chains, pronounced spiral. Gram stained smear, approximately 1000X.

FIG. 4. *Vibrio fetus*, strain 1. Mixed short and long chains, relatively straight. Gram stained smear, approximately 1000X.
FIG. 5. _Vibrio fetus_, strain 1717. Short chains, quite straight. Gram stained smear, approximately 1000X.

FIG. 6. _Vibrio fetus_, strain 2. Short, relatively straight chains. Gram stained smear, approximately 1000X.
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