

A STUDY OF A BACTERIUM ABORTUS
INFECTED UDDER



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By

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INTRODUCTION

No disease, except tuberculosis, is of such great loss to the cattle industry of the United States as is infectious abortion. Abortions in cattle are generally caused by one organism, Bacterium abortus (Bang).

It has been estimated by the Bureau of Animal Industry of the U. S. Department of Agriculture (1) that the national annual loss from this disease is \$20,000,000.

Bact. abortus in cattle is found mainly in the udder and reproductive tract. Schroeder and Cotton (2) state that the udder is the favorite habitat of Bact. abortus. They further state that the udder is the only habitat of this organism in non-pregnant cows, except in recently parturient cattle.

Numerous investigations have shown that certain organisms produce certain pathological changes upon invasion into the tissues. The pathological changes may be seen both macroscopically and microscopically. Bact. tuberculosis is known to produce lesions in udders which are infected with this organism. One of the purposes of this study is to determine whether Bact. abortus can pro-

duce macroscopic or microscopic lesions in the udder, such as a catarrhal condition, areas of induration, abscess formation, cell infiltration or any other.

REVIEW OF LITERATURE

That abortion disease is caused by an infectious agent was the view held early in the nineteenth century. A book expressing this view was published in England in 1807 (3), and in this widely read book, "The Complete Farmer", the following occurs: "It is considered certainly contagious, and when it happens the abortion should be immediately buried and the cow kept as widely apart as possible and not receive the bull that goes with them." It was not, however, until the science of bacteriology began to develop that the contagious nature of abortion was generally recognized.

Zundel, Styrl, Franck, Roloff, Brauer and Johnne were early workers in the abortion field and made some valuable contributions.

Nocord 1885 (4) concluded that abortion was due to a bacterial infection of the fetus and placenta, but he was unable to designate the specific organism. Bang (5) and his assistant, Stribolt, in 1895 discovered the specific organism and demonstrated its relation to the disease. It

was not, however, until 1907 that Bang fulfilled Koch's laws.

During the period from 1837 to 1900, and later, numerous investigators studied the histology of the mammary gland and the process of milk secretion. Early workers found cells in the alveoli of the mammary gland, but did not ascertain their presence in secreted milk. No particular attention was paid to the presence of cells in milk until Stokes and Wegefarth (6) called attention to the presence of leucocytes in market milk. Stokes and Wegefarth distinguished these from the epithelial cells by their nuclei. Their method of examining milk was to centrifuge a given quantity. Then a constant amount of slime was secured by means of a platinum loop, smeared on the slide, dried, stained and examined. Milk was declared unfit for use when more than five leucocytes were found per field. Although the method of counting and the numerical standard suggested by these two men is of little value, still their work attracted attention by showing the universal presence of cells in milk and raised the question of their sanitary significance.

This method of milk examination was later improved by Bergey (7), Stewart (8) and Slack (9). The Stokes method, with its various improvements, is known as the "smeared-sediment" method of milk examination. This method is used in a number of American laboratories and is recommended by

the Committee on Standard Methods of Bacterial Milk Analysis (10) appointed by the American Public Health Association.

During the time that improvements were being made on "Stoke's method" Doane and Buckley (11) devised a modification of the ordinary method used in counting the red and white blood cells. This modified method was used for counting the cells in milk. Briefly, the method consisted in making a suspension of the sediment of ten cubic centimeters of centrifuged milk; a small amount of this suspension was then transferred to a Thoma-Zeiss haemocytometer and the cells counted. This method gave a higher degree of accuracy than that obtained by the "smeared-sediment".

The Doane-Buckley so-called "volumetric" method was later modified by Russell and Hoffmann (12). They claim that higher and more accurate results can be obtained by heating milk to 70°C. previous to examination.

A number of American investigators have used one or both of the methods to determine the number of cells in milk and their sanitary significance. Among these investigators are Ward, Henderson and Haring (13), Harris (14), Russell and Hoffmann (15), Bergey (16), Kendall (17), Pennington and Roberts (18), Miller (19), Stone and

Sprague (20), Cambell (21), Hastings, Hammer and Hoffmann (22), Jordan (23), Ross (24) and others.

Prescott and Breed (25) in 1910 suggested a method of counting cells (leucocytes, etc.) in the whole milk directly without the use of the centrifuge. Their method consisted in spreading 0.01 cubic centimeter of milk over an area of 1 sq. cm., drying in air, staining with aqueous methylene blue, and then counting the cells by means of a microscope. This "direct method" showed that the number of leucocytes, or body cells, was much greater than had formerly been supposed.

Savage (26), 1906, worked out a method of counting cells similar to the Doane-Buckley method. His method has been somewhat modified by Hewlett, Villar and Revis (27) who have made extensive studies concerning the nature of cells in milk.

After studying histologically sections from the udders of five cows and one goat, Hewlett, Villar and Revis (28) came to the following conclusion: "This examination of these several udders has shown the paucity of polymorphonuclear leucocytes in the inter-alveolar tissue, and, so far as can be seen, their complete absence in the lumina of the alveoli. Within the lumina of the alveoli cells of the (1) large uni-nuclear, (2) small uni-nuclear,

(3) multi-nuclear, and (4) vacuolated types have been found in some of the specimens, and a study of their appearance confirms our previous views on the nature of these cells, viz., that the large uni-nuclears (generally) and the multi-nuclears are cells of the "germinal" layer. None of the eosinophile type has been detected and their origin therefore remains doubtful.

The results of this examination confirm the opinion we have already expressed that the cellular elements found in milk, either normally or in ordinary catarrhal or non-suppurative mastitis, are tissue cells, and that "pus" cells, in the ordinary acceptance of the term, do not appear under these conditions.

Winkler (29) regards the majority of the cells in the milk as being epithelial cells detached from the glandular layer of the alveoli. He is very emphatic in his statement that leucocytes are practically never found in milk under normal conditions, and that they are never found in the lumina of the alveoli of the glandular tissue of the udder. Michaelis expresses a similar view as to leucocytes being found in normal milk and in the alveoli of udders.

The investigational work of Breed (30) shows that two entirely different kinds of cells are discharged into

the milk of cows. The greater part consists of leucocytes or white blood corpuscles and the rest are epithelial cells, nuclei or fragments of these cells.

Storch (31) was probably the first worker to show that milk from infected udders was abnormal in chemical composition and less acid in reaction than normal milk. Rullmann (32) showed that high leucocytic counts gave a low titratable acidity. He tried to account for this lowered acidity on the ground that the alkali-forming bacteria more than counteracted the effect of the acid-forming streptococci, but could find nothing to confirm this view. Fetzer (33) comes to the same conclusion as Storch.

Höyberg (34) using the Rosolic-acid-alcohol test for determining the reaction of milk, concludes that an excessive number of leucocytes in milk is followed by a lessened acidity. He believes, as does Storch, that this is caused by the passage of blood serum into the milk without change. Baehr (35) has used Höyberg's test (rosolic-acid-alcohol) and reports satisfactory results. Baker and Breed (36) come to the conclusion that Höyberg's reagent is not as satisfactory or as good for general use in detecting the reaction of milk as the brom-cresol-purple test.

VanSlyke and Baker (37) found a relationship between the percent of carbon-dioxide volume and hydrogen ion concentration in milk. VanSlyke and Baker state, "With increase

of P_H value the general tendency of the carbon-dioxide is to increase, while the degree of acidity, as measured by titration, decreases".

In 1874 Robert Hall expressed the belief that milk drawn from the udder is germ-free. Grotenfeldt (38) supported the view of Hall. Grotenfeldt believed that the original sterility of normal milk is due to the fact that the bacteria cannot gain access to the milk glands from without as long as the udder is not injured in any way.

Freudenreich (39) held a similar view: "In the udder milk is germ-free except when the milk glands are diseased, as with tuberculosis or mastitis. In such cases the tubercle bacilli or those organisms causing the inflammation are present in the milk when drawn". He further states that immediately after being drawn it always contains bacteria due to the contaminated surroundings.

Lister's (40) observations on the bacterial content of milk from the udder and the results of his determinations on two small samples, led to the conclusion that milk within the udder is germ-free. This view of milk being germ-free was held as late as 1895 by Conn (41) and others.

The first quantitative study of bacteria in the udder was made under the direction of Lehmann. Schultz (42), a student of his, found that the first milk (fore milk) contained large numbers of bacteria, while samples drawn

midway (middle milk) and the last milkings, or strippings, contained a smaller number of organisms.

Schultz's work was confirmed by Moore (43) who came to the following conclusion: "Freshly drawn fore milk contains a variable number of bacteria, varying in number from a few individuals to many thousand per cubic centimeter. These are distributed among several species. The last milk drawn as the regular milking contains, as compared with the fore milk, very few micro-organisms. It is the exception, however, to find a sample of milk that is free from micro-organisms unless it is taken during the latter milking process from a single quarter of the cow."

Moore suggested the examination of excised tissue from the functioning udders of freshly slaughtered milk cows as a means of settling the question as to whether bacteria enter or are present in the finer ramifications (upper portions) of the udder. Moore (44) later reported the results he obtained by investigating this problem. Ward (45), one of his students, continued this work and isolated bacteria from the finer ramifications of the milk canal as well as from other portions of the udder.

Henderson (46) dissected udders, under aseptic conditions, and found typical udder bacteria and streptococci present.

Harding and Wilson (47), from the results of three

hundred sixty samples, corroborated the results of Stocking (48) in which the middle milk contains a smaller number of germs than those taken from either the fore milk or the strippings.

Bolley and Hall (49), Cohn and Neumann (50), Hastings and Hoffman (51), Harding and Wilson (52), Burri and Hohl (53) and others have shown that the flora of milk taken aseptically from single udders, and even from a single quarter of an udder, may remain constant for many months.

A number of medical workers, such as Escherich (54), Bumm (55), Cohn and Neumann (56), Palleske (57), Honnigman (58), Ringel (59) and Knochenstjerna (60), published work between the years of 1886 and 1893 on the milk of women, taken under aseptic conditions. They found the dominating organisms to be Staphylococcus pyogenes albus and Staphylococcus pyogenes aureus.

Various investigators have made studies of the normal flora of the udders of cows. The results obtained will be only briefly mentioned. Arloing (61) cites cases in which LeBlanc found B. coli communis present in normal udders. Harrison (62) made an examination of twenty-five cows twice and in each examination found members of the B. coli aerogenes group in the milk of two cows. In normal,

fresh milk, Lux (63) found and identified various types of microorganisms, the most prevalent of which were the pyogenic staphylococci which he designates as Staphylococcus mastitis aureus and Staphylococcus mastitis albus. Conn, Esting and Stocking (64) regarded Micrococcus lactis varians as identical with Staphylococcus pyogenes aureus.

A large number of cows' udders infected with streptococci were found to be apparently normal in every other way by Rullmann and Trommsdorf (65). Tartler (66) in his investigation found streptococci present in nine out of twenty-three samples. Puppel (67) withdrawing milk from twelve cows under as sterile conditions as possible found, besides staphylococci and typical udder bacteria, that streptococci were very prevalent. The results of Evans (68) show that of the one hundred and ninety-two samples of milk examined one hundred and thirteen, or fifty-eight and eight-tenths percent, were micrococci belonging to the Staphylococcus pyogenes group. Harding and Wilson (69), in the extensive investigation of nine hundred samples examined for the various types of udder bacteria, showed that seventy-five percent of the microorganisms were micrococci. They further showed that ninety-six percent of the micrococci were gram positive. Justo (70) studied thirty-seven cultures, taken as representative of fifty-

five samples examined. He concluded that the normal flora of the udder consists of two types of micrococci. Steck (71) in his investigations found Staphylococcus pyogenes albus most frequent, then Streptococcus mastitis and Streptococcus lacticus; in addition Bact. abortus (Bang) was common in cows affected with contagious abortion.

Twenty-one of twenty-two samples obtained from five herds by Fleischner and Meyer (72) showed Bact. abortus present. This showed that over sixty-five percent were infected with Bact. abortus. The results of other investigators show that Bact. abortus is quite prevalent in udders of cows. Schroeder and Cotton (73) state, "A favorite habitat for the abortion bacillus is the udder, and the udder is seemingly its only habitat in the bodies of non-pregnant cows.

Bergey (74) states that the presence of streptococci in the udder may cause mammitis. Cooledge (75) says that "milk from actively Bact. abortus infected udders is found to have an average cellular count over five times as high as the apparently normal average"

Zwick and Krage (76) isolated Bact. abortus externally from the milk of a cow which had aborted thirteen months previously. This infected milk showed no macroscopic changes. Even upon clinical examination of the udder and its associated

lymph glands no abnormalities could be noticed. The authors therefore conclude that either the organism is able to pass through the udder without producing lesions at all, or that the lesions are so small as to be unrecognizable clinically. They further injected one goat subcutaneously with 10 cubic centimeters of an emulsion of this organism, and another intravenously with 10 cubic centimeters of an emulsion having half the density of the former. Bact. abortus was isolated for a period of eight weeks from the milk of both of these animals. At the end of this period the two goats were killed. Previous to killing, no macroscopic changes could be noticed in the milk by using "Trommsdorf's method" of examining leucocytes. Clinical examination showed no noticeable alterations from the normal in the udder and its associated lymph glands.

METHODS OF INVESTIGATION.

The literature reviewed has not revealed any definite data as to whether Bact. abortus causes any histo-pathological changes such as macroscopic and microscopic lesions, catarrhal conditions, areas of induration, abscess formation, cell infiltration or any other abnormalities in the udders of cows. No data could be obtained on histological examination of udders infected with Bact. abortus.

The literature has shown some inconsistencies in the origin of the cellular contents of milk. It will be shown in the discussion of one phase of this problem that the majority of the cells originate in the blood stream and a few are derived from the epithelium of the udder, which is a view opposite to that held by some investigators. Not many data have been found where differential blood stains and differential counts have been tried and found successful with milk. It is further hoped that some light may be thrown on other phases of this problem.

With this in mind the experiments were outlined in the following manner:

(1)- The effect of leucocytes in milk on the hydrogen ion concentration and the percent, by volume, of carbon dioxide.

The leucocytes were stained and counted by the Prescott and Breed method (77). The hydrogen ion concentration was determined by the Electrometric Gas Chain Method and Van Slyke's (78) method for the percent by volume of carbon dioxide in milk.

(2)- The relation between the hydrogen ion concentration and the percent by volume of carbon dioxide in milk.

(3)- The comparison of the leucocytes in the fore milk, middle milk and strippings. The Prescott and Breed method was also used for determining the leucocytes in this experiment.

(4)- Staining of milk cells and their origin. A definite amount of whole milk was smeared over a certain area on a slide and dried; the slide was then fixed and stained with Wright's, Jenner's and Giemsa's, and differential counts were attempted. Stained histological slides of two udders were examined and photographs were made to ascertain the origin of these cells in the lumina of the alveoli. The methods of staining will be mentioned on page 47.

(5)- The comparison of the bacteria in the fore milk, middle milk and strippings. Standard Methods of Milk Analysis of the A. P. H. A. of 1928 were used for culturing and counting the bacteria.

(6)- Types of microorganisms most prevalent in milk. Standard Methods of Milk Analysis of the A. P. H. A. were employed. Typical colonies were then fished off on lactose agar slants and microscopical examinations were made of twenty-four-hour-old cultures. The Breed (79) method was used for whole milk. Stained histological slides were examined to see if any organisms could be found which would indicate what might be present in the milk samples.

(7)- The histo-pathological changes caused by Bact. abortus. in an infected udder. Two animals were used for comparison, one with an apparently normal, non-infected udder and one with a naturally infected udder. The apparently

normal, non-infected condition was determined by the agglutination and complement fixation tests on the blood and milk serum. A bacteriological examination was made. This consisted in determining the number and types of bacteria as described above. The purpose of the bacteriological examination was to determine whether an excessively large number of bacteria was present in the various quarters, and the types of organisms which might indicate inflammatory conditions. Leucocytic counts of the three milkings of the various quarters were determined by the Prescott and Breed method. The animal selected was 997 B.

The naturally infected udder was determined in exactly the same manner as that just described for the normal, non-infected udder, with three exceptions. Tests were made of the hydrogen ion concentration and the percent by volume of carbon dioxide in milk from various quarters to see if any abnormalities existed.

Direct-isolation methods as well as guinea-pig inoculations were made to determine whether the udder of the cow, which had been infected for years, was infected before being killed. It was also necessary to determine whether any other organisms were present in this udder giving rise to pathological conditions. Cow 805 was the animal selected for a naturally infected udder.

The two animals were then killed and macroscopic changes sought in the udders. Blocks were then taken from

each udder and fixed in Zenker's fluid, washed in water, run through various percents of alcohol and blocked in paraffin, sectioned and stained by Mallory's eosin and methylene blue method.

The exact details of the methods used in the various phases of this problem will be found between pages 17 and 67.

Method of Determining the Hydrogen Ion Concentration.

All determinations of P_H were made electrometrically, the general technic and apparatus described by Clark being used. The hydrogen was obtained from a tank and was purified by being passed through solutions of mercuric chlorid, alkaline potassium, permanganate and dilute sulphuric acid. The oxygen was burned out. Determinations were made in an air bath to protect the apparatus from drafts. No attempt was made to regulate the temperature around the apparatus, but appropriate corrections were made for this factor. Saturated calomel filaments were used, and frequently checked against each other. Gold plated platinum or solid gold electrodes were used.

Milk samples collected for the determination of hydrogen ion concentration and the percent by volume of carbon dioxide were the same.

Into large test tubes of thirty cubic centimeter capacity 3 to 5 cubic centimeters of paraffin oil was

poured. The tubes were then ready for the collection of milk samples. Just previous to collecting the sample a two-hole rubber stopper containing a thistle tube was fitted into the mouth of the test tube. One end of the thistle tube was placed in the oil. The milk from the particular quarter was then milked into the thistle tube with the least possible exposure to the air. After the required amount of milk was obtained the rubber-stopper thistle tube apparatus was removed and made ready for another tube. This apparatus should be rinsed either with distilled water or by shooting a few streams of milk from the quarter from which the sample is to be collected into the thistle tube before placing the apparatus into another test tube.

Method of Determining Percent by Volume
of Carbon Dioxide.

Baker's modification of Van Slyke's method (80) was used in measuring the percent by volume of carbon dioxide in milk. One and one-half cubic centimeters were used instead of two cubic centimeters as employed by Van Slyke, and twenty percent lactic acid instead of five percent of sulphuric acid. Sulphuric acid coagulum sometimes clogs the apparatus and interferes in the complete removal of carbon dioxide.

One and one-half cubic centimeters of milk was run into the carbon dioxide apparatus. The casein of the milk was dissolved with an excess of twenty percent lactic acid. The dissolving of casein by lactic acid liberated the carbon dioxide which was then measured and corrections made for the inert gases, or absorbed by an excess of a five percent solution of sodium hydroxide.

Leucocytic Counts.

Smears were made the same for the three milkings (fore, middle and strippings) by placing 0.01 cubic centimeter in duplicate on a plain slide. Each amount on the slide was then spread evenly over an area of one square centimeter and placed on a perfectly level stand where it remained until the smears were dry. These smears were stained either immediately or soon.

The staining technic was as follows: The slides with the dried smears were placed in a glass staining jar containing xylol for three minutes to remove the fat, removed and placed in absolute or ninety-five percent alcohol for three minutes to fix, transferred to aqueous alcoholic methylene blue (1:9 dil.) for one to two minutes, then washed in water and air-dried. Counting was always done with an oil immersion objective whose factor was known.

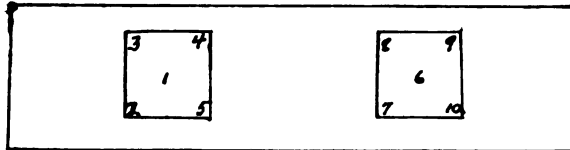
This factor was obtained by using an ocular and drawing the tube out to such length that the diameter of the field measured .16 millimeters. This diameter was determined by a stage micrometer.

$$\text{Area of field} = \pi r^2 \text{ or } 3.1416 \times (.08)^2 = .0201 \text{ sq.m.m.}$$

$$.0201 \text{ sq.m.m.} = 0.0002 \text{ sq.cm.}$$

area of field or factor is 0.0002 or 1/5000
of a square centimeter.

Ten fields were counted on each slide of the duplicate smears. The manner of counting is illustrated below.



The sum of the ten fields multiplied by 50,000 gave the number of cells per cubic centimeter.

Microscopic Method of Examining Milk for Bacteria.

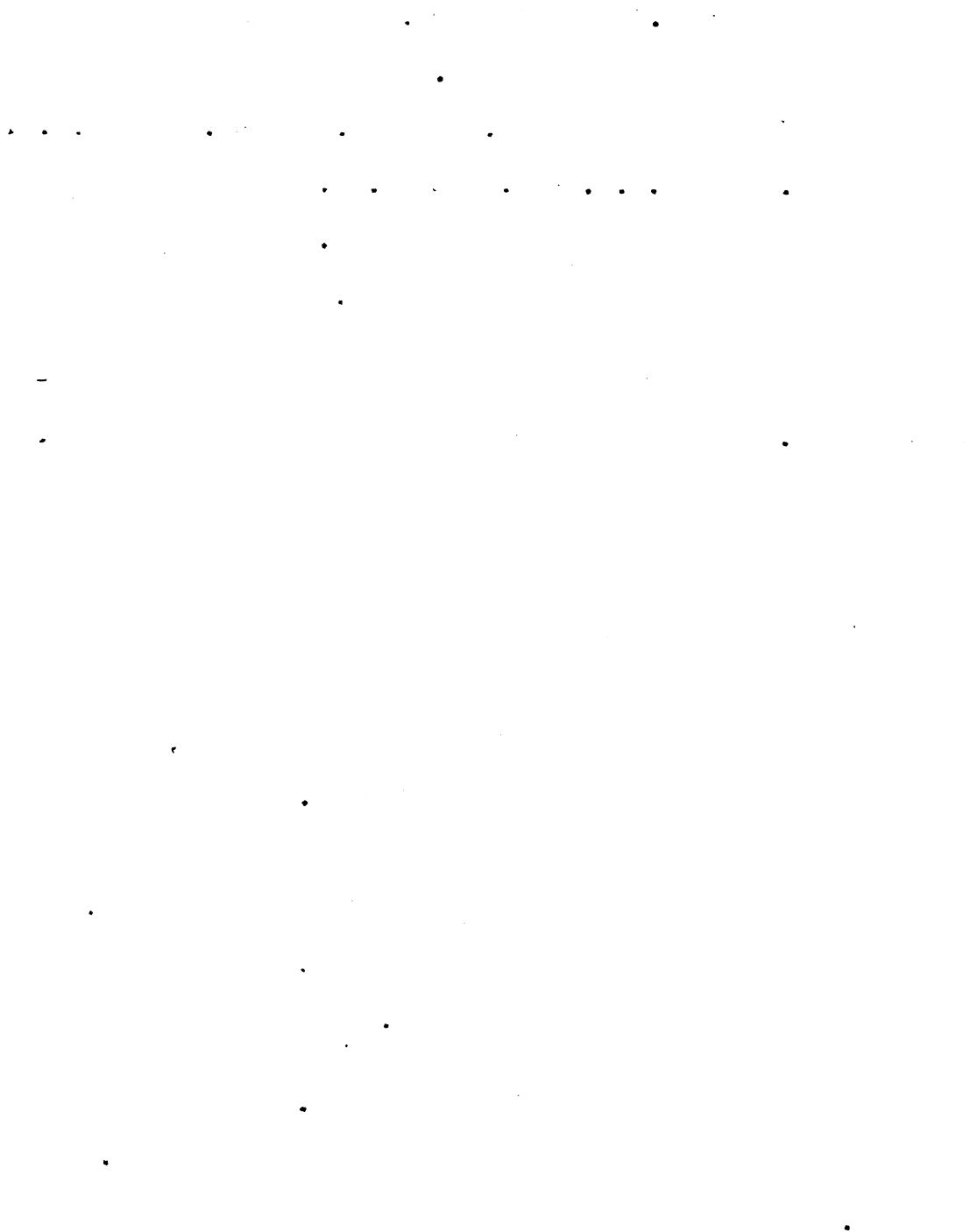
The Breed (81) method was used.

Preparation of Media.

Preparation of liver infusion agar.

This medium was used for the isolation of Bact.
abortus.

To five pounds of ground liver (beef or pork) five



liters of either distilled or tap water were added. The mixture was then placed in flowing steam from thirty to sixty minutes, the length depending on the time it takes to coagulate the liver. This cooking coagulates the albumins and the liquid will contain the soluble albumins, soluble salts, extractions and coloring matter, chiefly hemoglobin.

The mixture was next strained through cheese cloth. To the filtered liquid portion twenty-five grams of sodium chloride (C.P. preferable), fifty grams of Difco or Witte's peptone and one hundred grams of well washed agar were added. The components were then heated over a free flame and stirred constantly. When the salt and peptone were in solution heating was continued until the contents were close to the boiling point. This mixture was then placed in an autoclave and autoclaved for twenty minutes at fifteen-pound pressure.

The solution was then cooled to 50° C. and fifty grams of egg albumin, previously dissolved in water, was slowly added. The egg albumin was added as a clearing agent. After this process was finished, the mixture was placed in an autoclave for one hour at fifteen pounds pressure. It was then filtered through cheese cloth and cotton. After this the medium was corrected to its proper P_H value, which was between P_H 6.6 - 6.8. Should any sodium hydroxide

have been added it was then found necessary to heat the medium over a free flame or asbestos board for fifteen minutes so as to throw down any precipitates that may have been formed and to facilitate filtering. The liver infusion agar, after being boiled for fifteen minutes was next filtered through cotton or glass wool, placed in the proper containers and plugged. The containers with the media were then sterilized for twenty minutes at fifteen pounds pressure.

Although Huntoon (82) claims that about half of the hormone of the media is removed by overheating, employing cotton, cloth or paper in filtration, still the writer obtained excellent results in using cotton for filtration purposes. The proper P_H of 6.6 - 6.8 is absolutely essential for growing Bact. abortus. Any amount of medium, larger or smaller proportionately as one sees fit, may be prepared, but amounts proportional to those just outlined should be employed.

Preparation of Lactose Agar.

Lactose agar was used in all milk-dilution work and in studying the flora of the udder.

To one liter of boiling, distilled water, fifteen grams of well washed agar (market) 5 grams of beef extract and 10 grams of peptone were added. The mixture was then

boiled and stirred until all components were in solution. Distilled water was added if any loss occurred, the medium corrected to the proper P_H value, which should be P_H 6.6 - 6.8 and ten grams of lactose added. The medium was then filtered through cotton. If any sodium hydroxide has been added the mixture should be further heated for fifteen minutes so as to throw down any precipitate that may have been formed, and to facilitate filtering. After this period of boiling, the lactose agar is filtered, placed in proper containers, plugged and the media in the containers sterilized by autoclaving at fifteen pounds pressure for fifteen minutes.

Preparation of Plain Broth.

To one liter of distilled water, ten grams of peptone (either Difco or Witte's), five grams of sodium chloride and two cubes of bouillon were added, or five grams of Liebig's Meat Extract. The contents were then placed over a free flame or asbestos board and well stirred so as to bring all the components into solution. The mixture was boiled fifteen minutes. After being boiled for fifteen minutes the loss of water was corrected and the proper P_H value determined, which should be between P_H 6.6 - 6.8., if no sodium hydroxide had been added. The broth can then be filtered through filter paper, collected in proper containers, plugged and sterilized for

twenty minutes at fifteen pounds pressure. This broth was used for growing Bact. abortus for inoculating Kolle flasks for antigen used in the agglutination and complement fixation tests.

Direct Isolation of Bact. abortus from Milk.

In order to determine whether an udder is infected with Bact. abortus it is very essential that the organism be isolated from the milk. The methods of various investigators in studying the presence of this organism in milk have, as a rule, certain difficulties, and have not been entirely successful. There are two methods of isolation, (1) guinea-pig inoculation and (2) direct cultural. A brief review of the literature on both methods will be given before describing each method that the writer used.

Fabyan (83) and Schroeder (84), working independently, demonstrated the presence of Bact. abortus in milk by means of guinea-pig inoculation. This method is reliable, but it takes from four to twelve weeks for the typical lesions to develop in the spleen and necrotic areas in the liver. Smillie (85) has improved the method of Fabyan and Schroeder in that three to four weeks after inoculation, cultures made from the spleen have been successful. The organism is present in great numbers at the end of this period.

It can very readily be seen that if a large number of samples are examined for Bact. abortus by guinea-pig inoculation, a large expense would be the result and a great deal of time would have to be spent in order to determine the presence of Bact. abortus in milk. The guinea-pig-inoculation method, however, is of great value when direct cultural methods fail, and it is also more reliable.

To isolate Bact. abortus culturally a proper medium is of first importance. Giltner (86) called attention to the use of media made from the uterine wall, fetal membranes, fetus and amniotic fluid. The incorporation of amniotic fluid in agar or gelatin grew this organism successfully. Huddleson (87), experimenting with blood-clot agar, ascitic agar, amniotic agar, fetal agar, glycerin and plain agar, found that all media except glycerin and plain agar grew Bact. abortus well under anaerobic conditions. Stafseth (88), employing liver and spleen infusion agar, has found it far superior to any other media.

Liver agar, with a proper P_H value, has been found to be the best medium for growing Bact. abortus. Huddleson (89) stated that "the H-ion concentration of the medium is the most important factor in the cultivation

of this organism. The H-ion concentration for obtaining the optimum growth lies between 6.6 and 6.4. The limiting H-ion concentration in which growth may be obtained is between 6 and 7.6. The organism grows very slowly at the extreme limits of this range."

A bacteriocidal agent should be employed to eliminate other organisms. Churchman (90) found that gentian violet had a selective bacteriocidal power which inhibited the growth of a large percent of gram positive and a small percent of the gram negative organism. Gentian violet incorporated into liver infusion agar in a final dilution of 1:10,000 does not in the least affect the growth of Bact. abortus.

A proper atmospheric condition is essential for obtaining successful growths from naturally infected material. Bang (91) concluded that the growth depends upon either an atmosphere of nearly pure oxygen, or an atmosphere whose oxygen was considerably less than that of air. Nowak(92) obtained good growths by placing inoculated media cultures of B. subtilis in closed jars. His impression was that its growth caused the proper reduction of oxygen tension so that Bact. abortus could grow readily. Preis (93) using alkaline pyrogallol or acetylene gas for reducing the oxygen tension, claims successful isolation. Holth (94) sealing inoculated

agar slants with paraffin thought the proper oxygen tension was attained as a result of organisms using the air over and over again. Fabyan (95) found that the growth of Bact. abortus was governed by the amount of B. subtilis culture when grown in symbiosis. Attempts by him to grow the organism by means of alkaline pyrogallol were unsuccessful in every case. Huddleson (96) found by connecting an agar slant, which had previously been planted with Bact. abortus to a buffer solution of P_H that a good growth of the organism was obtained in forty-eight hours, and the indicator solution gave a reading of P_H 6.6. By placing tubes of buffer solution, P_H 7, in a number of jars and replacing the air in these with certain percentages of carbon-dioxide gas, after standing several hours the jar containing the ten percent carbon-dioxide gas changed the indicator to P_H 6.6. This was identical with the change produced by Bact. abortus.

Technic of Isolation.

Huddleson's method of isolation of Bact. abortus will be discussed very briefly. In order to isolate this organism milk samples should be centrifuged for two hours at 2500 revolutions per minute. After a two-hour centrifuging the sample is removed from the centrifuge. All but the sediment is removed, which is then smeared on

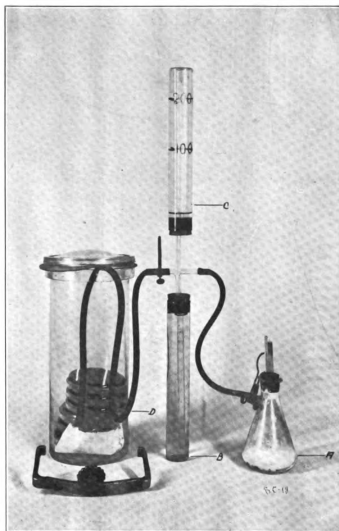
plates of gentian violet liver agar.

This method of isolating Bact. abortus from the spleens and livers of suspected or infected guinea pigs has been found to be of considerable importance. The method of autopsying, removing the organs and the smearing of portions of the organs on gentian violet plates will not be discussed. However, aseptic conditions were maintained at all times.

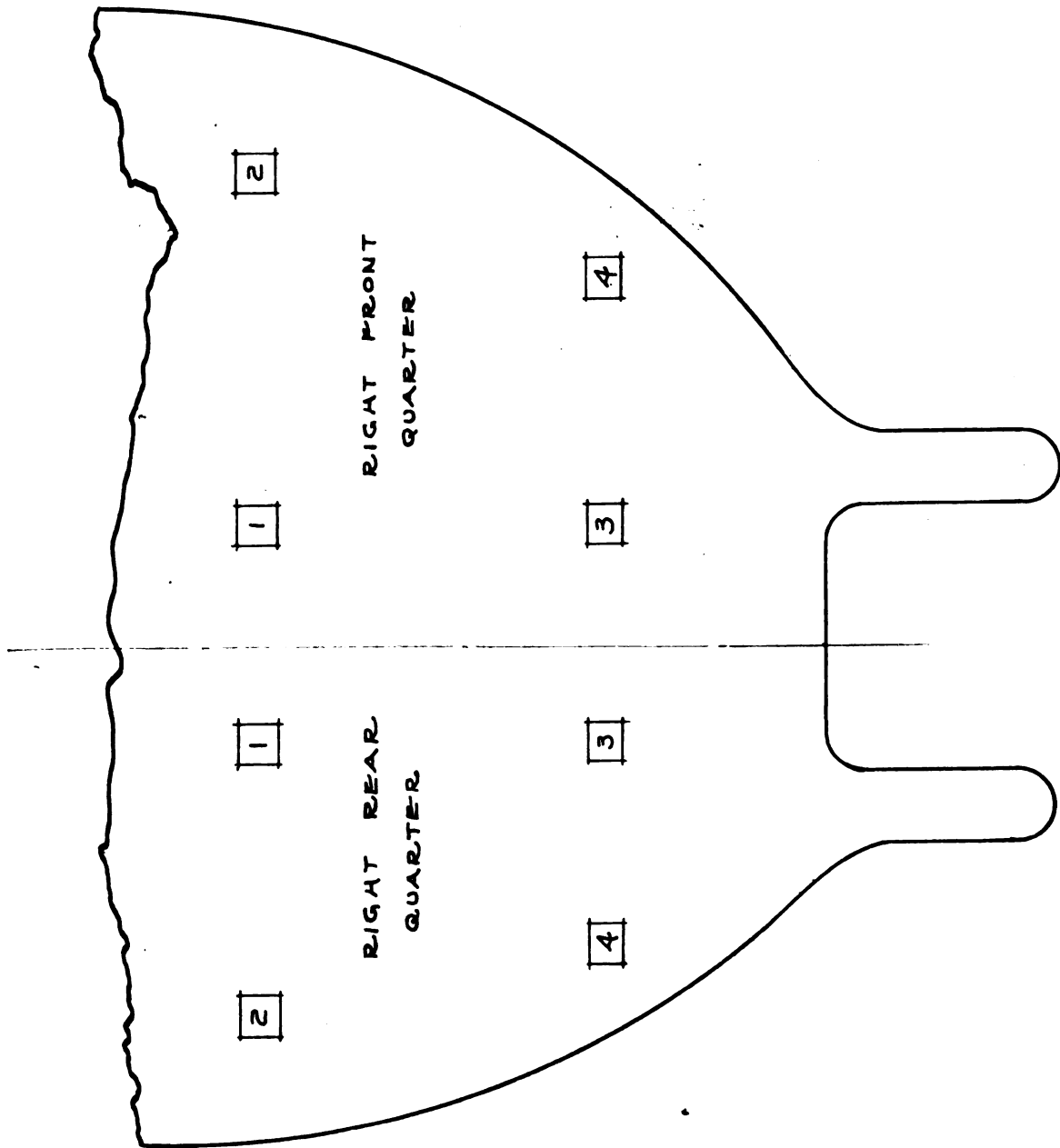
On page 30 is shown the apparatus used, and terms will be designated by the letters of the various articles of the apparatus.

Upon smearing the gentian violet agar plates they were placed in a specimen jar whose volume was known. Calcium carbonate was then placed in flask A and hydrochloric acid added and the cork quickly placed over the top. The carbon-dioxide gas forces the water from cylinder B into the graduated cylinder C. After the desired amount of gas had been obtained in C the rubber tubing at A was closed by the pinch clamp. Then the pinch clamp leading from jar B to jar D was opened and the gas from B was forced into D by the water displacing the volume of CO₂ gas in B. This process can be repeated until the desired amount of CO₂ gas is obtained in the specimen jar D. The amount of CO₂ gas in jar D should be ten percent in isolating Bact. abortus.

By employing carbon-dioxide gas in a similar way excellent results have been obtained by Wherry and Irvin (97) in cultivating tubercle bacilli; by Chapin (98) in growing gonococcus; and by Kohman (99) in isolating and cultivating meningococcus.



- A. Flask for receiving calcium carbonate and hydrochloric acid.
B. Reservoir containing water over which the CO₂ gas is collected.
C. Reservoir for receiving and measuring water displaced by CO₂ gas.
D. Specimen jar for growing cultures under increased CO₂ tension.



SKETCH INDICATING PARTS OF THE UDDER
FROM WHICH BLOCKS WERE TAKEN

Preparation of Udder Tissue for Sectioning.

On page 31 will be noticed the manner in which pieces of tissues or blocks were taken from the various quarters of the udder. Each block (tissue) was from one to two inches long, and from one-eighth to one-fourth of an inch wide. Four blocks were taken from each quarter, the total for each udder being sixteen blocks. The blocks of the various quarters of each udder were taken from correspondingly similar places. After blocks were taken from each udder they were run through the following process:

Fixation.

Fixation is the first step in the preparation of a specimen for sectioning. A good fixative is a reagent that penetrates and kills tissues quickly, preserves the tissue elements - particularly the nuclei - in the condition in which they are at the moment when the reagent acts on them, and hardens them or so effects them that the specimens or tissues will not be altered by various after-steps such as washing, dehydrating, cleaning and sectioning.

Most fixatives are mixtures of different reagents so combined that all the desirable properties may be present in as large a degree as possible. The choice of

a fixative is very difficult in some cases, and a fixative must be employed for the purpose for which the tissue is preserved. The fixative best suited for udder tissue was found to be Zenker's fluid, which is a chemical fixative. This fluid is especially suited for the preservation of nuclear figures, bacteria and fibrils.

Zenker's Fluid.

Bichromate of potassium	2.5 grams
Corrosive sublimate	5 to 8 grams
Water	add 100 c.c.
Glacial acetic acid	5 c.c.

The corrosive sublimate and bichromate of potassium were readily dissolved in water with the aid of heat. Acetic acid should never be added until tissue is to be hardened in the solution, because this acid evaporates readily and also produces changes in chrome salts when present for some time.

The objection to Zenker's fluid is that it forms gritty precipitates in the tissues. These, however, are removed later by alcohol and iodine solution. Zenker's also causes a shrinking of the tissues, but not to such an extent that it is detrimental.

After being removed from the udder, the specimen or block was cut to the proportions previously mentioned

and was placed in one hundred fifty cubic centimeters of Zenker's solution. They were left in this solution for twenty-four hours after which they were removed and washed.

Washing.

The purpose of washing was to remove the excess of the fixative and to wash away any precipitates on the surface of the fixed and hardened tissue.

After being taken out of the Zenker's fluid they were then placed separately in white muslin cloth four inches in diameter. The number of the block and the quarter written with india ink on a slip of paper was then placed on the muslin cloth after which it was tied into a sack. After the blocks with the slips of paper were all tied up into sacks they were then placed in a two-liter, wide-mouthed bottle, one end of a rubber hose being placed in the bottom of the bottle, the other end being attached to a faucet. Enough water was then turned on to keep up a slight circulation. After being washed in water for twenty-four hours they were removed from the bottle and were ready for dehydrating.

Dehydrating.

Dehydrating was to remove the water from the tissues, which made them harder and thereby facilitated sectioning. A soft tissue, no matter how well infiltrated or imbedded

with paraffin, will tear instead of being cut. Alcohol extracts the chrome salts from Zenker's fluid, but the chrome salts are precipitated in light and it is desirable, although by no means necessary, to keep all such specimens in the dark. A tissue should approach as nearly as possible the hardness of the paraffin in which it is embedded. This is accomplished by passing the tissue through various percentages of ethyl alcohol, thereby removing the water, as has been previously stated, and hardening the tissue..

Tissues were always placed first in low percentages of alcohol which were gradually increased to absolute alcohol. It is quite apparent that if high percentages were used first, water would be extracted from the cells and finer structures so rapidly that bursting of the cells as well as distortion of the tissue elements would take place.

Wide-mouthed bottles of five hundred cubic centimeter capacity were arranged as follows, filled with a certain percentage of alcohol, labeled with the quarter and number of the block of that certain quarter. A volume of alcohol from thirty to fifty times greater than the volume of the immersed tissue was introduced into each flask.

Block	RR	RF	LR	LF
1	0	0	0	0
2	●	0	0	0
3	0	0	0	0
4	0	0	0	0

RR 1 = right rear quarter, number
1 block, etc.

Each sack was then opened and the tissues transferred to their respective bottles. Eighty percent alcohol was then added to each bottle, and the bottle tightly stoppered. Tissues were left in eighty percent for twenty-four hours. It was found best to pare the tissues down to their proper size at this time. With the greater percent of water removed the tissues become harder and neater work is the result. Each tissue was pared down to approximately three-fourths of an inch long, one-half inch wide and one-eighth of an inch thick. The tissue was then returned directly to the container from which it was taken.

The alcohol was now poured off and as much removed from each bottle as possible, but the tissues were not removed. Then ninety-five percent alcohol was added and the tissues remained in this for twenty-four hours. The same process was followed in pouring off the alcohol as was described above, and absolute alcohol added. This

was left for twelve hours, at the end of which the specimens were ready for the clearing process.

In changing from eight percent to ninety-five percent and from ninety-five percent to one hundred percent alcohol one should always remove as much as possible of the preceding percentage of alcohol. This is especially important when adding the absolute alcohol as a small amount of the ninety-five percent will dilute the absolute, seriously inhibiting its full action. All bottles were tightly stoppered to prevent evaporation. In no case were the tissues allowed to become dry as this would have resulted in their becoming very hard.

Tissues can be stored in eighty percent alcohol for an indefinite period without in any way impairing the specimens. Should one have such a large number of specimens that it is impossible to use them immediately they can be carried on from this point later. A dark, cool place should be used whenever possible in storing specimens in eighty percent alcohol, and the bottle or vial should be tightly stoppered.

Clearing.

Clearing was mainly for the purpose of rendering the tissue more transparent. Clearing permits a better penetration of light through the tissue, aiding materially

in bringing out greater detail in microscopic examinations.

Cedar oil was the reagent used in clearing the tissues. Its clearing property is due to its infiltrating power. The volume used was ten times greater than that of the tissue immersed in it. Chemically pure oil was used by the writer. The tissue when first placed in the oil floated, but the alcohol was soon replaced by the oil, whereupon the tissue sank, becoming a dark brown.

The writer followed the same system in arranging and labeling the vials for clearing as was just described in the dehydrating process. Two changes of the cedar oil were made, the first being at the end of six hours, and the second at the end of seventeen hours. The blocks were then ready for infiltration.

Infiltration.

Infiltration is the process of filling the interstices of the tissues with a solid medium, paraffin. Infiltration must be done with a substance which will readily infiltrate when in fluid, and later, when congealing, will hold the tissue elements in their places. It must also be a substance which can be thinly sectioned, with sections neither too soft nor too brittle. The two methods commonly used are paraffin and celloidin.

For the general laboratory work, the cheapness and ease of handling make the paraffin method far superior. The paraffin used for infiltration was called "Best White". It had a melting point between 59° C. and 62° C.

The following method was used for infiltrating the udder specimens: Paraffin was placed in vials which had been labeled for them. The amount of paraffin was about five times the volume of the immersed tissues. After this the vials were placed in an oven which was kept at 62° - 63° C. As soon as the paraffin was melted the specimens were placed in their proper vials. The changes of paraffin were made at the end of the second, fourth and sixth hours. After this process the tissues were ready for blocking.

As oil of cedar is a solvent for paraffin it was necessary to make a number of changes of paraffin so as to get rid of all the oil of cedar. This was absolutely necessary as the presence of any oil lowered the density, thus causing the paraffin within the specimen to be considerably softer than the surrounding tissue. It will be readily seen what the result of this would be when attempting to section the tissues later on.

Specimens were never left in a paraffin bath longer than was absolutely necessary. The limit was found to be about eight hours; if left in a longer time the tissues were excessively hardened.

Blocking.

Blocking is the process by which paraffin is formed into a solid block around the infiltrated tissue. This process is most difficult to master and requires skill and considerable experience.

A flat, steel plate with two L-shaped bars of lead was found to be very unsatisfactory. A small paper box one inch long and three-quarters of an inch wide was used. Pieces of paper (ordinary white stationery) three inches by four inches were prepared. They were then put over a square stick (dimensions, one inch by three-quarters of an inch) and the two opposite sides creased in such a manner that when the paper was removed it was in the shape of a box. After the required number of paper boxes were made they were labeled with the specimens to be placed in them.

The technic consisted in heating the paraffin in the vials with the specimens to 64° C. in an oven. It was then ready for use. Each specimen was taken out of the vial in which it had been infiltrated with paraffin, placed in a paper box and its edges squared to the sides of the box. Melted paraffin was then poured over the tissue until the box was completely filled. Air was blown over the surface of the melted paraffin until a scum was formed. The whole was then completely im-

mersed in cold water having a temperature between 8° C. and 12° C. It remained in the cold water until the whole mass was thoroughly hardened, after which it was taken out and the paper removed from the paraffin block as soon as it had dried. The block was then placed in a properly labeled vial ready for sectioning.

Such difficulties as cavity-forming during the cooling process were met in but few cases. Whenever this happened it was found to be due to not having the water cold enough and not having a sufficient scum on top. This, however, can be corrected very easily, and in no case was the cavity of such proportion as to include the embedded tissue. Paper boxes are far superior to metal plate blocks in that they are easily made, inexpensive and offer none of the difficulties of crystallizing and cavity formation so often experienced with metal plates. There is no possibility of confusion as the boxes are labeled, and blocking can be done very quickly as there is no loss of time in waiting for the blocks to harden as is the case with metal which requires the hardening of the block before they can be removed. Paraffin blocks will keep indefinitely if kept in a dark, cool place.

Sectioning.

Sectioning is the process of cutting thin sections from the tissue embedded in the paraffin block.

The present method of obtaining sections is by the use of a machine called the microtome. A Spencer Horizontal Microtome was used.

Preparatory to sectioning, the paraffin was trimmed with a sharp knife or razor, the edges being trimmed parallel to the edges of the embedded tissue leaving only enough about the edges to hold it in shape properly while being sectioned. The paraffin block was then attached to a steel disk with corrugated surface. This was done by heating the disk sufficiently to melt the paraffin. The block was then pressed against the disk and the two cooled immediately in cold water. The steel disk was then clamped in the microtome with the lower edge of the block parallel to the edge of the knife. The block was then ready for sectioning.

The microtome was then adjusted to the thickness to which the tissue was to be sectioned. The sections were cut in long ribbons of twelve inches each and placed on white paper, as many being cut as were desired. These sections were then ready to be fixed to the slides.

Mounting on Slides.

The mixture used for attaching paraffin sections to slides is Mayer's Glycerin-Albumin mixture.

Mayer's Mixture: Equal parts of white of egg and of glycerin were taken. The mixture was then thoroughly beaten and filtered, or after standing for some time it was found possible to decant. Enough sodium salicylate was then added to make the solution one percent. The sodium salicylate acted as a preservative agent. Egg albumin is dissolved by acids and alkalies; accordingly when such reagents were used some other mounting agent was resorted to.

The mounting of sections was done as follows: The sections in the ribbons were first cut apart. After this procedure a thin film of Mayer's mixture was very evenly spread over the surface of a slide with the forefinger. A section (or a number of sections) was then transferred to the slide. Just enough water was added to float the section, and the slide was then placed over a small flame for a few seconds. The heat softened the paraffin and the surface tension flattened out the section removing all the wrinkles. The heating of the slides was found to be a most delicate process, but one in which proficiency can be acquired after a short time. After the section

was properly flattened the slide was removed from the flame and the water removed by slightly tilting the slide and by rubbing the finger along its entire length. Most of the water was removed in this manner. The slide with the attached section was then placed in a slide box. The box was then placed in a 37° C incubator for three days, or left at room temperature for about a week. The sections were then ready for the staining process. The slides may be stored for an indefinite period of time at this point in the procedure.

Staining.

Staining is the process of coloring artificially the various tissue strata for the purpose of microscopic study. Various tissue elements seem to have a special affinity for certain dyes which makes it possible to differentiate the various structures of a particular tissue. For differentiating purposes a stain with a counter stain is used.

Mallory's eosin and methylene blue stain is the best general stain yet devised to use on paraffin sections of tissues fixed in Zenker's fluid (100). It was especially good as a nuclear stain, and at the same time brought out with a great deal of differentiation all the various structures in the different tissues. This stain was found to be excellent for photomicrography when

Panchromatic Wratten M plates were used. It was found to be far superior to Eosin and Haematoxylin stain for microscopic examination and for photomicrographin work.

Before describing the technic of staining it is highly important to consider several factors. All stains and other reagents used were placed in glass staining jars which contained grooves on two sides for holding slides in place. By backing two slides against each other it was possible to run ten slides through at one time, and after one has some experience in staining it is possible to keep two or three sets of ten slides going at a time.

Method and Time Employed.

Jar 1 - Xylol	3 - 5 minutes
" 2 - "	3 - 5 "
" 3 - 95% Alcohol	5 "
" 4 - Iodine	5 - 8 "
" 5 - 95% Alcohol	5 "
" 6 - Eosin	15 - 20 "

Wash in four changes of water.

" 7 - Methylene blue 10	15 minutes
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Wash in four changes of water.

" 7 - Decolorize in 95% colophonium alcohol.	
" 8 - Complete decolorization with absolute alcohol.	
" 9 - Xylol	5 minutes

" 10 - Xylol - leave slides in jar until ready for mounting with xylol balsam.	
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In jar 7 with ninety-five percent colophonium alcohol the slide must be kept in constant motion so that the decolorisation will be uniform. The decolorisation can be controlled with a microscope, but after one works with a certain tissue one can tell quite readily how far to carry the decolorization process by holding the slide towards a window. Proper decolorisation is attained when the pink color has returned and the nuclei are still dark blue. The decolorization was then completed in jar 8.

A few drops of a ten percent colophonium solution was sufficient when placed in jar 7. Enough saturated alcoholic iodine solution was added to the ninety-five percent alcohol in jar 4 to make it a rich amber color. By a chemical combination the iodine removes any traces of precipitate from the tissues caused by Zenker's fluid.

Mounting.

Previous to mounting it is essential to have clean cover glasses. These were cleaned by leaving in ten percent sodium hydroxide for five minutes, then transferred to distilled water and washed in several changes, and finally transferred to ninety-five percent alcohol and each one dried with a cheesecloth. Every cover glass must be scrupulously clean. The most suitable cover

slip is a square or round cover slip with a diameter of 18 mm. The cover glasses are fixed to the slides by Canada balsam. The balsam was dissolved in xylol until it had the consistency of thin syrup.

After having followed the steps just mentioned the labeled slides with the stained tissues were removed from jar 11 and placed on a flat surface. A drop of xylol was then added to the stained section and a cover glass pressed firmly down on it. Pressing down is essential as the smaller the amount of balsam present under the cover glass the less aberration will be the result when examining microscopically. This clamping is absolutely essential in photomicrography.

After the specimen had been mounted the slide was placed in a horizontal position on a level table for forty-eight hours. The slide was then labeled with a permanent label and put in a slide box in a dark place, as light will fade the color of the stain. The balsam will not be dry until two or three weeks later and great care must be taken in handling it. In no case is it advisable to use an oil immersion lens until the balsam is perfectly dry.

Staining Methods of Milk Cells.

The three stains used were Wright's, Jenner's and Giemsa's.

Slides were cleaned with alcohol and then held over a free flame in order to get rid of all dirt, fatty material and moisture. This is essential as it aids materially in permitting the smearing of a thin film of milk as will be described later.

A clean slide was then placed over a cardboard which had been ruled off in squares of one square centimeter each. One-hundredth of a cubic centimeter of fresh, whole milk was placed on the slide and smeared over an area of three of these squares. After the film had been dried in air it was ready for any of the three stains mentioned above.

Wright's Stain:

1 - Xylol - 3 minutes

2 - Ethyl alcohol - 3 minutes

3 - The smear was then covered over with a noted amount of staining fluid by means of a medicine dropper. After one minute a similar amount of distilled water was added. (The distilled water must be added very quickly to do away with the precipitates which form if the water is added slowly). This mixture was allowed to remain from two to three minutes according to the intensity desired.

4 - Wash in distilled water for thirty seconds, or until a pink color appears.

5 - Dry in air and examine.

Jenner's Stain: Identical to the methods used
in Wright's.

Giemsa's Stain:

- 1 - Xylol - 3 minutes
- 2 - Absolute methyl alcohol - 3 minutes
- 3 - Flood slide with stain - 12 to 15 minutes
- 4 - Wash the film until it has a slight pink tinge.
- 5 - Dry in air and examine.

The purpose of the xylol was to dissolve any fat particles present in the film. The purpose of the ethyl and methyl alcohol was to fix the film so it will not wash off from the slide later on, and to prepare the cells for the stains to be employed later.

The Complement Fixation Test.

The complement fixation test was discovered by Bordet and Gengou (101) in 1901 and is generally called the Bordet and Gengou phenomenon. It has been used in bacteriological investigations as a method of diagnosis for the determination of specific antibodies in the serum of animals.

Wassermann and his collaborator (102) applied it to the diagnosis of syphilis. In the veterinary world it is best known for the diagnosis of glanders and infectious abortion.(103).

A great many investigators have shown that the complement fixation test is of great value in the diagnosis of infectious abortion. Some of these investigators are Hadley and Beach (104), Surface (105), Wall (106) and Gibbs and Rettger (107).

The complement fixation test requires the following components:

1. Antigen
2. Suspected serum
3. Complement
4. Hemolysin
5. Erythrocytes
6. Physiological salt solution (0.85%)

The preparation and titration of the bacterial antigens has presented great difficulties and this has led to the abandonment of the test for abortion in some laboratories.

The antigen was prepared by growing various strains from forty-eight to seventy-two hours at 37° C. in Kolle flasks on liver agar. The growth was then washed off with a solution containing a 0.5 percent phenol and an 0.85 percent solution of sodium chloride and filtered through a cotton filter. It was found that the best antigenic properties could be obtained with an antigen from three to four days old. In this procedure a

polyvalent antigen was prepared consisting of four different strains of organisms. This mixture was next standardized to turbidity five McFarland's Nephelometer (108). The antigen, if kept at ice-box temperature, will keep for months. Antigen has been used by the writer for a period of eight months and no anti-complementary properties have been noticed.

Preparation of Suspected Serum.

The preparation of the serum depends on whether the sample is milk or blood. To ten cubic centimeters of milk in a test tube four drops of rennet were added. The test tube was next placed in a water bath at 37° C. for thirty minutes, which is sufficient time for the formation of the rennet curd. The rennet-curd tube was then placed in a centrifuge and centrifuged for fifteen minutes at two thousand revolutions. After the designated time of centrifuging a clear serum was obtained. If the serum is cloudy it may be filtered through a filter paper. The work of Lane-Clayton (109) has shown that milk contains only a very small amount of complement, therefore it is not necessary to inactivate milk serum. Two cubic centimeters was the smallest amount in which complement was demonstrated to be present. Should milk serum be retained for any length of time add a sufficient amount of 5 percent phenol to make the solution 0.5 percent as phenol will prevent any microbial growth.

The blood of a cow was obtained for a test in the following manner: A halter was placed on the cow's head, the rope of which was drawn over the top of the stanchion until the head was at an angle of forty-five to seventy degrees. The jugular vein was then dilated by pressing with the thumb against the vein. A quick puncture was then made with a large hypodermic needle. The blood was collected in a sterile test tube and allowed to clot. The serum was next centrifuged and the clear fluid pipetted off and inactivated by heating the blood serum at 56° C. for thirty minutes. A concentration of 0.5 percent phenol in the blood serum will preserve it. A very sharp needle is one of the prerequisites for successful bleeding. The next is a needle of proper size. The size most suitable for bleeding cattle is gauge 12.

Preparation of Complement.

For complement an animal should have the following requirements: One whose complement is potent and uniform for that species, and which can be kept and handled easily. The guinea pig was found to be the best animal for this purpose.

There are various methods of bleeding guinea pigs. In some laboratories cutting the jugular vein is resorted to. This method is rather expensive as a pig is killed each time. A second method is Wenner's (110), and

a third is that of Gibbs and Rettger (111) who claim to have refined Wenner's method. The last and most desirable is bleeding from the heart.

Bleeding was done in the following manner: The guinea pig was firmly fastened on its back to an animal board. A small space over the left side of the thorax on a midline between the two fore limbs was clipped free from hair and the area disinfected with fifty percent alcohol. The animal was next etherized until the beat of the heart slowed up slightly. (It is not absolutely necessary to etherize but this facilitates bleeding in that the animal is quiet.)

After etherizing, a 22 gauge needle, with or without a syringe attached was inserted between the fourth and fifth ribs in a diagonal position toward the opposite side of the body. It is essential that the needle enter the left side of the heart as blood will flow more freely from there than from the right side. A syringe (Luer type) attached to the needle is preferable because blood can be withdrawn very rapidly and at the same time a known quantity can be secured. About seven cubic centimeters was withdrawn from each pig without any injury. Several guinea pigs were bled each time for complement for the complement fixation test proper. The blood was then pooled and placed in a centrifuge tube, defibrinated and centrifuged. The serum was then pipetted

off, placed in a sterile test tube and plugged. This was used immediately, or after twenty-four or forty-eight hours.

A more potent complement was obtained by placing uncentrifuged, clotted blood in an ice-box for twenty-four hours. Complement kept at room temperature deteriorated very rapidly.

Preparation of Hemolysin.

There are two methods for building up hemolysin, the slow and the quick method.

Examples of the slow method are those of Stitt (113) and Huddleson (114). Gibbs and Rettger (115) and Coca (116) use the quick method.

The most satisfactory method found by the writer in building up hemolysin in rabbits was a quick method devised by himself. One and five-tenths cubic centimeters of well washed, concentrated sheep cells (in sterile saline solution) was injected intravenously into the marginal vein of the rabbit. A second injection of one and five-tenths cubic centimeters of fresh, well washed cells (seven times) of the same concentration as before was injected two days later. A final injection of two cubic centimeters, same strength as before, was made four days after the first injection. Three to four days after the last injection the rabbit was bled from the marginal

vein of the ear, or from the heart, and the blood serum tested for its hemolytic properties. Should one-thousandth of a cubic centimeter of hemolysin in a definite amount of complement hemolyze five -tenths of a cubic centimeter of a two and five-tenths percent suspension of sheep erythrocytes in thirty minutes the serum was considered satisfactory for the test.

The rabbit was then partly anesthetized and bled from the heart. Fifty cubic centimeters of blood were collected either in a sterile, dry culture dish or in a flask, and the blood allowed to clot. This allows the serum to separate from the clot.

The serum was next poured into a sterile centrifuge tube and centrifuged. The clear, supernatant liquid was next drawn off, placed in sterile test tubes and inactivated so that the complement (thermolabile substances) will be destroyed, heating at 56° C. for thirty minutes. The hemolytic antibody (thermostable substance) will not, however, be destroyed. Enough phenol was added so that the serum contained five-tenths percent phenol, which acted as a preservative. A second bleeding of similar amount was made four days after the first, and the same technic was employed. The hemolytic serum was then placed in small vials, the stoppers sealed with paraffin and placed in a cool, dark place. Here the serum will keep for several years.

Preparation of Erythrocytes.

The blood was withdrawn from the jugular vein of the sheep by means of a large hypodermic needle and collected in a sterile flask containing glass beads. After collecting the desired amount of blood, the flask was moved in a circular motion for five minutes in order to defibrinate the blood. The blood was then strained through a sterile gauze into the required number of centrifuge tubes. The gauze removes any fibrin or small pieces of glass present in the blood. It was then centrifuged until the cells were thrown down after which the supernatant liquid was drawn off and physiological salt solution added in order to wash the cell. This is repeated about five times in order to wash out all remaining serum from the blood erythrocytes.

A very sharp, sixteen-gauge needle should be used. A two and five-tenths percent solution of corpuscles was used in all titration.

Erythrocytes, even if washed every day, were found unsatisfactory if used more than four days after they had been withdrawn from the sheep.

Titration of Components.

The complement was the first reagent titrated. It was diluted one to four with physiological salt solution

(0.85 percent). The mixture was then titrated to find the smallest amount of complement that will hemolyze five-tenths of a cubic centimeter of a two and five-tenths percent solution of sheep corpuscles mixed with a constant amount of hemolysin kept in a water bath at 37° C. for thirty minutes. The amount of complement that will cause complete hemolysis may vary for different guinea pigs; therefore, before running the complement fixation test the complement must be titrated to find the amount required.

Four one-hundredths of a cubic centimeter of a one to four dilution usually resulted in complete hemolysis. Two times the titer was used because antigen absorbs a slight amount of complement. This in itself will lead to an error; further, it is absolutely essential to have more complement than the amount required for the test.

Table I

Shows Titration of the Complement.

Tube No.	'Complement' Dil. 1:4	'Hemolysin' 1 %	'NaCl' .85%	'Sheep' Corp.	'bath' Incubate in 37° C.	'Results'
1	.02	2 x t (a)	1.5cc	.5cc	30 min	No hemolysis
2	.04	2 x t	1.5cc	.5cc	30 min	Hemolysis
3	.06	2 x t	1.5cc	.5cc	30 min	"
4	.08	2 x t	1.5cc	.5cc	30 min	"
5	.1	2 x t	1.5cc	.5cc	30 min	"
6	0	2 x t	1.5cc	.5cc	30 min	No hemolysis
7	.1	0	1.5cc	.5cc	30 min	"

(a) t = the titer found on a previous titration.

Table I shows that four one-hundredths of a cubic centimeter was the highest dilution which caused complete hemolysis. Two times this amount (four one-hundredths) is taken in the final test, which is eight one-hundredths of a cubic centimeter.

Tube six was a control to show that hemolysin will not cause hemolysis. Tube seven was a control to show that complement does not exhibit hemolytic properties. Controls are absolutely essential to act as checks; this prevents errors in later results.

Titration of Hemolysin.

In running the complement fixation test it is absolutely necessary to know the quantity of hemolysin that will completely hemolyze five-tenths of a cubic centimeter of a two and five-tenths percent solution of sheep erythrocytes mixed with a complement (two times titer) of known strength of titer.

The preparation of hemolysin has been previously described. A one-percent solution of hemolysin is made for titration.

In Table II it will be seen that 0.02 of a cubic centimeter is the hemolytic titer and 0.02 is the unit to be used. Tubes nine and ten were control tubes. Had tube nine shown hemolysis it would have shown that the complement possessed hemolytic properties.

Tube ten contained a large amount of hemolysin to show whether hemolysin in the absence of complement would show complementary properties. Two times the titer was always taken, but for the sake of convenience hemolysin was diluted to a point where one-tenth of a cubic centimeter would contain twice the titer. The one percent hemolysin was diluted one to five so that one-tenth of a cubic centimeter could be employed in the final test.

Table II
Shows Titration of hemolysin.

Tube No.	'NaCl ' .85%	'Complement ' 1:4	'Hemolysin ' 1%	'Sheep ' Corp. ' 2.5%	Incubate in ' H ₂ O Bath ' 30" at 37° C.	Results
1	'1.5cc	' 2 x t(a)	' .005 cc	' .5 cc		' No hemolysis
2	'1.5cc	' 2 x t	' .01 cc	' .5 cc		' Hemolysis
3	'1.5cc	' 2 x t	' .02 cc	' .5 cc		' "
4	'1.5cc	' 2 x t	' .04 cc	' .5 cc		' "
5	'1.5cc	' 2 x t	' .06 cc	' .5cc		' "
6	'1.5cc	' 2 x t	' .1 cc	' .5 cc		' "
7	'1.5cc	' 2 x t	' .15 cc	' .5 cc		' "
8	'1.5cc	' 2 x t	' .2 cc	' .5 cc		' "
9	'1.5cc	' 2 x t	' 0 cc	' .5 cc		' No hemolysis
10	'1.5cc	' 2 x t	' .2 cc	' .5 cc		' " "

(a) T = titer found on titration.

Titration of Antigen.

It is quite necessary to know the smallest amount of antigen that will fix complement in the presence of a known, specific antibody. Antigen has the power of absorbing complement in large quantities, therefore it is necessary to know the smallest amount of antigen that will absorb complement in the absence of a specific antibody.

1. The first part of the document is a list of names and addresses, which are arranged in three columns. The names are written in a cursive script, and the addresses are written in a more formal, printed style. The list appears to be a directory or a roster of some kind.

2. The second part of the document is a series of short, handwritten notes or entries. These are arranged in three columns, corresponding to the columns of the list above. The notes are written in a cursive script and appear to be brief descriptions or comments related to the names and addresses listed above.

3. The third part of the document is a series of short, handwritten notes or entries. These are arranged in three columns, corresponding to the columns of the list above. The notes are written in a cursive script and appear to be brief descriptions or comments related to the names and addresses listed above.

A positive milk serum of Bact. abortus was used for the specific immune serum. This serum was positive in 0.05 c.c. used in previous tests.

Table III
Titration of Antigen.

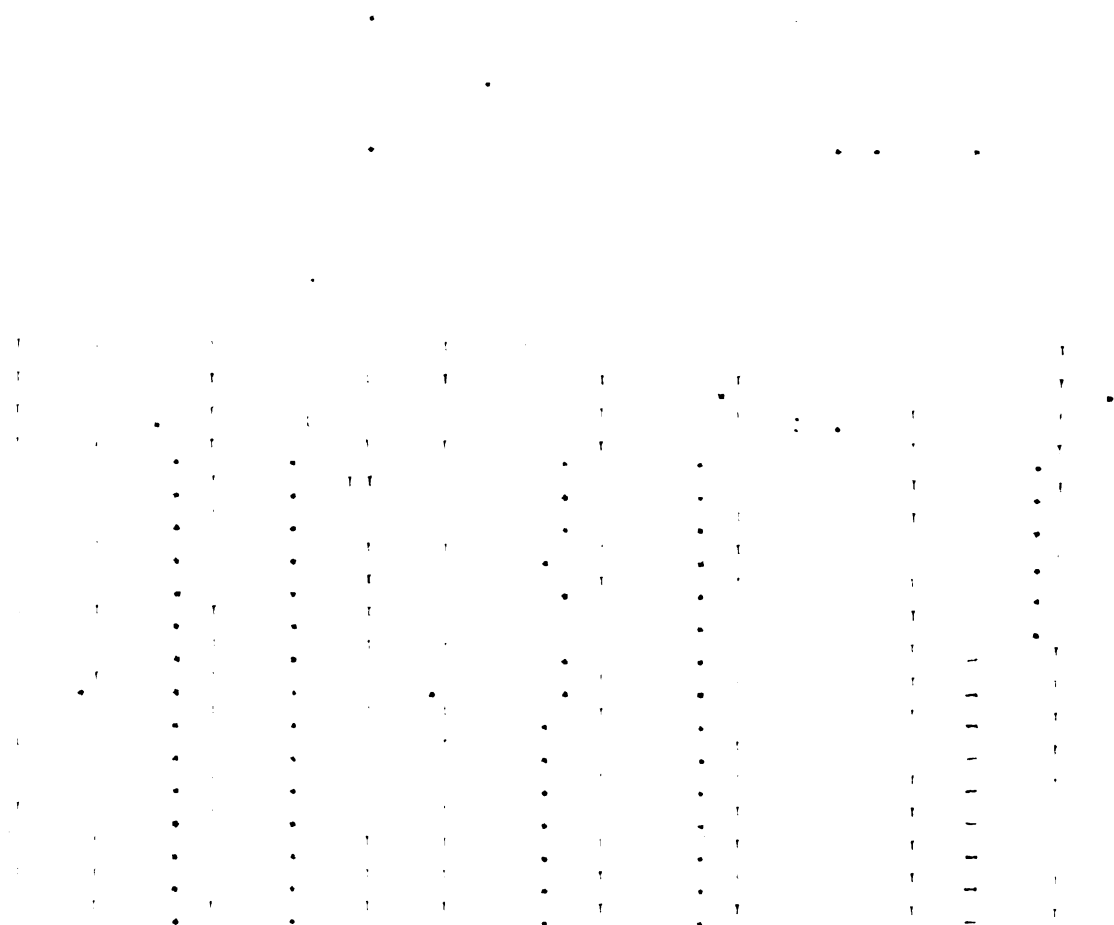
Tube No.	'Immune' Serum	'Comple-ment' Dil. 1:4	'NaCl' .85 %	'Antigen'	'Hemolytic Am-'boceptor'	'Sheep' Cells' 2.5%	'Results'
1	.02 cc	2 x t(a)	1.5 cc	.01 cc	0.1cc	.5cc	P
2	.02 cc	2 x t	1.5 cc	.02 cc	0.1cc	.5cc	No hemolysis
3	.02 cc	2 x t	1.5 cc	.05 cc	0.1cc	.5cc	" "
4	.02 cc	2 x t	1.5 cc	0.1 cc	0.1cc	.5cc	" "
5	.02 cc	2 x t	1.5 cc	.2 cc	0.1cc	.5cc	" "
6	.02 cc	2 x t	1.5 cc	0 cc	0.1cc	.5cc	Hemolysis
7	-	2 x t	1.5 cc	.02 cc	0.1cc	.5cc	" "
8	-	2 x t	1.5 cc	.04 cc	0.1cc	.5cc	" "
9	-	2 x t	1.5 cc	0.1 cc	0.1cc	.5cc	" "
10	-	2 x t	1.5 cc	0.2 cc	0.1cc	.5cc	" "
11	-	2 x t	1.5 cc	0.4 cc	0.1cc	.5cc	No hemolysis
12	-	2 x t	1.5 cc	0.5 cc	0.1cc	.5cc	Hemolysis
13	-	2 x t	1.5 cc	0.6 cc	0.1cc	.5cc	" "
14	-	2 x t	1.5 cc	0.7 cc	0.1cc	.5cc	" "
15	-	2 x t	1.5 cc	0.8 cc	0.1cc	.5cc	" "

(a) t = titer found on titration.

(b) t = .02 but made up as described previously.

P = Partial hemolysis.

Table III shows that .02 c.c. is the smallest quantity that will fix complement. Four one-hundredths of a cubic centimeter was the smallest amount of antigen that would absorb complement without the presence of immune serum as was shown by tube eleven. In the final test it was found best to employ four times the titer which is eight one-hundredths of a cubic centimeter, but since this was inconvenient in pipetting,



one-tenth of a cubic centimeter was the amount used. Since four-tenths of a cubic centimeter of antigen is anticomplementary we are safe in employing one-tenth of a cubic centimeter of antigen in the final test.

Tube six was the control to show whether the immune serum had any antigenic properties. Some immune serums have the antigenic properties of combining the complement and throwing off the results. Tubes seven to fifteen did not contain immune serum.

The Complement Fixation Test Proper.

The purpose of the complement fixation was to test for the specific antibody present in the serum, and for the concentration in which it was found. A positive complement fixation test may also mean either that the animal is infected at the time of testing, or has been infected at some time in the past.

The following outline was used in running the final test for infectious abortion on the blood and milk serum of cows, and the blood serum of guinea pigs.

Table IV.

Shows the Test as it Was Used.

Tube No.	NaCl Solution	Specific Serum	Antigen	Complement	Incubate at	Hemolytic Amboceptor	Sheep Corp.	Results
				1:4	30° C.		2.5%	
1	1.5 cc	1.00	2 x t cc	2 x t (b)	30° C.	2 x t cc	.5 cc	No hemolysis
2	1.5 cc	.04 cc	2 x t cc	2 x t	30° C.	2 x t cc	.5 cc	"
3	1.5 cc	.02 cc	2 x t cc	2 x t	30° C.	2 x t cc	.5 cc	"
4	1.5 cc	.005 cc	2 x t cc	2 x t	37° C.	2 x t cc	.5 cc	Hemolysis
5	1.5 cc	.1 cc	2 x t cc	2 x t	37° C.	2 x t cc	.5 cc	"
6	1.5 cc	.1 cc	0 cc	2 x t	37° C.	0 cc	.5 cc	No hemolysis
Control I	1.5 cc	0 cc	0 cc	0	30° C.	0 cc	.5 cc	"
Control II	1.5 cc	0 cc	0 cc	2 x t	30° C.	2 x t cc	.5 cc	Hemolysis
Control III	1.5 cc	0 cc	2 x t cc	2 x t	30° C.	2 x t cc	.5 cc	"
Control IV	1.5 cc	0 cc	2 x t cc	0	30° C.	2 x t cc	.5 cc	No hemolysis

(b) t = titer found on titration
X t = .05 the titer.

— *Journal of the American Medical Association*, 1997

Figure 1. The effect of the concentration of the solution on the adsorption of the dye. The concentration of the solution was 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 15.0, 20.0, 30.0, 40.0, 50.0, 60.0, 70.0, 80.0, 90.0, 100.0, 150.0, 200.0, 300.0, 400.0, 500.0, 600.0, 700.0, 800.0, 900.0, 1000.0, 1500.0, 2000.0, 3000.0, 4000.0, 5000.0, 6000.0, 7000.0, 8000.0, 9000.0, 10000.0, 15000.0, 20000.0, 30000.0, 40000.0, 50000.0, 60000.0, 70000.0, 80000.0, 90000.0, 100000.0, 150000.0, 200000.0, 300000.0, 400000.0, 500000.0, 600000.0, 700000.0, 800000.0, 900000.0, 1000000.0, 1500000.0, 2000000.0, 3000000.0, 4000000.0, 5000000.0, 6000000.0, 7000000.0, 8000000.0, 9000000.0, 10000000.0, 15000000.0, 20000000.0, 30000000.0, 40000000.0, 50000000.0, 60000000.0, 70000000.0, 80000000.0, 90000000.0, 100000000.0, 150000000.0, 200000000.0, 300000000.0, 400000000.0, 500000000.0, 600000000.0, 700000000.0, 800000000.0, 900000000.0, 1000000000.0, 1500000000.0, 2000000000.0, 3000000000.0, 4000000000.0, 5000000000.0, 6000000000.0, 7000000000.0, 8000000000.0, 9000000000.0, 10000000000.0, 15000000000.0, 20000000000.0, 30000000000.0, 40000000000.0, 50000000000.0, 60000000000.0, 70000000000.0, 80000000000.0, 90000000000.0, 100000000000.0, 150000000000.0, 200000000000.0, 300000000000.0, 400000000000.0, 500000000000.0, 600000000000.0, 700000000000.0, 800000000000.0, 900000000000.0, 1000000000000.0, 1500000000000.0, 2000000000000.0, 3000000000000.0, 4000000000000.0, 5000000000000.0, 6000000000000.0, 7000000000000.0, 8000000000000.0, 9000000000000.0, 10000000000000.0, 15000000000000.0, 20000000000000.0, 30000000000000.0, 40000000000000.0, 50000000000000.0, 60000000000000.0, 70000000000000.0, 80000000000000.0, 90000000000000.0, 100000000000000.0, 150000000000000.0, 200000000000000.0, 300000000000000.0, 400000000000000.0, 500000000000000.0, 600000000000000.0, 700000000000000.0, 800000000000000.0, 900000000000000.0, 1000000000000000.0, 1500000000000000.0, 2000000000000000.0, 3000000000000000.0, 4000000000000000.0, 5000000000000000.0, 6000000000000000.0, 7000000000000000.0, 8000000000000000.0, 9000000000000000.0, 10000000000000000.0, 15000000000000000.0, 20000000000000000.0, 30000000000000000.0, 40000000000000000.0, 50000000000000000.0, 60000000000000000.0, 70000000000000000.0, 80000000000000000.0, 90000000000000000.0, 100000000000000000.0, 150000000000000000.0, 200000000000000000.0, 300000000000000000.0, 400000000000000000.0, 500000000000000000.0, 600000000000000000.0, 700000000000000000.0, 800000000000000000.0, 900000000000000000.0, 1000000000000000000.0, 1500000000000000000.0, 2000000000000000000.0, 3000000000000000000.0, 4000000000000000000.0, 5000000000000000000.0, 6000000000000000000.0, 7000000000000000000.0, 8000000000000000000.0, 9000000000000000000.0, 10000000000000000000.0, 15000000000000000000.0, 20000000000000000000.0, 30000000000000000000.0, 40000000000000000000.0, 50000000000000000000.0, 60000000000000000000.0, 70000000000000000000.0, 80000000000000000000.0, 90000000000000000000.0, 100000000000000000000.0, 150000000000000000000.0, 200000000000000000000.0, 300000000000000000000.0, 400000000000000000000.0, 500000000000000000000.0, 600000000000000000000.0, 700000000000000000000.0, 800000000000000000000.0, 900000000000000000000.0, 1000000000000000000000.0, 1500000000000000000000.0, 2000000000000000000000.0, 3000000000000000000000.0, 4000000000000000000000.0, 5000000000000000000000.0, 6000000000000000000000.0, 7000000000000000000000.0, 8000000000000000000000.0, 9000000000000000000000.0, 10000000000000000000000.0, 15000000000000000000000.0, 20000000000000000000000.0, 30000000000000000000000.0, 40000000000000000000000.0, 50000000000000000000000.0, 60000000000000000000000.0, 70000000000000000000000.0, 80000000000000000000000.0, 90000000000000000000000.0, 100000000000000000000000.0, 150000000000000000000000.0, 200000000000000000000000.0, 300000000000000000000000.0, 400000000000000000000000.0, 500000000000000000000000.0, 600000000000000000000000.0, 700000000000000000000000.0, 800000000000000000000000.0, 900000000000000000000000.0, 10000000

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100% 90% 80% 70% 60% 50% 40% 30% 20% 10% 0%

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[illegible]

The serum in question is called a three-plus serum as the first three dilutions showed no hemolysis of the sheep corpuscles. Two one-hundredths of a cubic centimeter is the smallest amount of serum that has fixed or combined with complement. Should tubes one to four show hemolysis the milk serum is negative. The theory of the complement fixation test is based on the fixation of complement. If the serum is a positive one (immune bodies present) the bacterial cell, specific antibody and complement will unite, and, on addition of erythrocytes and hemolysin no hemolysis will result. Ordinarily no two can combine without the third, as antigen and complement or immune serum and complement. This will be true, however, only in cases where the antigen is present in large enough amounts to absorb the complement, or when enough antigen is contained in the immune serum so that the complement will be bound. Should the serum be negative, the complement will not be bound, and on addition of hemolysin and erythrocyte, complement, hemolysin and erythrocyte combine resulting in a lysis of the erythrocytes.

Tubes five and six were the controls for the milk serum. If tube five shows no hemolysis the milk serum has the power of combining with complement, or is

anticomplementary. If tube six shows hemolysis it is an indication that the serum possesses hemolytic properties. Should some of the tubes from one to four show no hemolysis and there is no hemolysis present in tube five then it is concluded that the complement of the guinea pig contains immune bodies (abortion antibodies) and that enough antigen is present in the complement (inactivated blood serum) to fix complement and that the guinea pig shows Bact. abortus infection. The writer has detected positive guinea pigs by getting opposite results in tube five and Control III, and in some cases in Control II.

Control I shows whether physiological salt solution or sheep erythrocytes have any other properties, such as complementary and hemolytic. Control II indicates whether the complement shows antigenic or serum properties. No hemolysis may indicate a positive pig. Should Control III show no hemolysis it would mean either a positive guinea pig or that the antigen has the power of absorbing the complement. If tube five and Control II show no changes then the antigen would be anticomplementary. Should tubes five, Control II and Control III show opposite results one might investigate to see whether the complement contains specific antibodies (abortion). Invariably on retesting the guinea-pig blood serum (inactivated) it indicated a positive pig.

The Agglutination Test.

The agglutination or clumping of the bacteria was noticed as early as 1891. From the year 1891 to 1895 Metchnikof (117), Charrin and Roger (118), Isaef and Ivanoff (119), Washbourn (120) and several workers made this observation that when bacteria were added to homologous immune serum clumping would result.

In 1896 Gruber and Durham (121) in Vienna notices the specificity of agglutination when colon bacilli and cholera spirilla were added to homologous immune serum. A few months after Gruber and Durham published their work, Widal and also Grünbaum (apparently independent of Widal) (122) applied the reaction to the diagnosis of infectious disease.

The agglutination reaction may be considered in one sense a truly colloidal reaction. Bechtold (123), Neisser and Friedemann (124) and Sears and Jamieson (125) have shown that bacteria in suspension may be compared very closely with true colloidal suspension in that the bacteria carry a definite and uniform electrical charge.

Bacteria, not agglutinated, in a weak salt solution upon the addition of agglutinin are agglutinated. Two explanations are plausible: that the agglutinin

attacks the membrane or outer coating protoplasm, or that the absorption of agglutinin has "sensitized" the bacteria to the action of the electrolyte.

The agglutination test has been successfully applied in the following diseases: typhoid (Gruber-Widal reaction), paratyphoid, pneumonia, glanders, bacillary white diarrhea of chickens and in abortion. The agglutination test employed by the writer acted as a check on the complement fixation test. The relationship between lytic and agglutination bodies was another, and the concentration of the agglutinating antibodies present in either the blood or milk serum, another.

Preparation of Antigen.

Antigen was prepared in the same manner as in the complement fixation test, but instead of using turbidity 5, turbidity 3 McFarland's nephelometer was employed.

Preparation of Serum.

The milk serum was prepared in the same manner as has been previously described.

The blood serum was also prepared in the same manner as has been previously described with the exception that heating to 56° C. for thirty minutes was not necessary.

Table V

Shows Test as it Was Used.

Tube No.	Antigen	Serum (a)		Results
1	2 cc	0.1 cc	INCUBATE IN H ₂ O BATH FOR 2 HOURS AT 56°C.	+
2	2 cc	0.05 cc		+
3	2 cc	0.025cc		+
4	2 cc	0.01 cc		P
5	2 cc	0.005cc		-
6	2 cc	0		-

(a) Serum in question, whether positive or negative

+ = Positive

- = Negative

p = Weak positive

The serum in tubes 1, 2, 3, 4 and 5 were diluted one to twenty, one to forty, one to eighty, one to two hundred and one to four hundred.

The serum is a three-plus positive serum, showing partial agglutination in tube four. Control tube six should show no settling out or flocculation. If it should show either of these the test is of no value.

Histories of Cows Used in Experiments.

The histories of the cows used in these experiments will be outlined very briefly. At this time only brief mention will be made of the work done with cows. This will be taken up in detail on page

Cow 997 B.

Cow 997 B was a Jersey, born December 12, 1916, at the Michigan Agricultural College abortion barn. At the time of birth her blood was negative. While a calf she was used for a milk-feeding experiment, being fed with naturally infected milk. As a result of this feeding she developed a weak positive reaction in the blood which lasted four weeks. This might be accounted for in that abortion antibodies must have been taken into the intestinal tract.

January 1st, 1918, an effort was made to infect her through the vagina, with negative results. One June 6th, 1918, thirty cubic centimeters of living abortion bacilli were introduced into the vagina, with negative results. 997 B always gave birth to normal calves during her whole history. Calves were born in 1918, 1919, 1920 and 1921. After the last calf she developed an endometritis which became chronic, but which was completely healed at the time the animal was killed.

From April 14th to June 30th, 1921, various tests such as the agglutination, complement fixation, bacterial and leucocytic counts and bacteriological examinations, as well as differential staining methods were run on the milk, taken under aseptic conditions, to determine if the udder was normal in every respect.

The agglutination and complement fixation tests showed that the blood and milk were negative. The bacterial, leucocytic and differential staining method demonstrated that no abnormalities could be detected in the milk. 997 B was always negative to the agglutination and complement fixation tests, both of the milk and the blood serum, throughout her history. At no time was Bact. abortus isolated from the udder.

This animal was killed June 30th, 1921, and blocks taken from the udder for histo-pathological work. Clinical examination showed the dissected udder to be normal in every respect. The sections obtained from this udder for microscopic work were taken as representative of an apparently normal udder.

Cow 805.

Cow 805, a Holstein, was born in December, 1916. She was bought by the Bacteriology Department of the Michigan Agricultural College September 7th, 1917. On

May 29th, 1918, June 7th, 1918 and June 14th, 1918, she was injected subcutaneously and intravenously with suspensions of living Bact. abortus cultures to determine whether she could be infected. She has given a positive reaction since June 14th, 1921.

Bred October 1st, 1918, she aborted her first calf on May 31st, 1919. Since that time she has given birth to two normal calves. She was treated with killed vaccines in November and living cultures, or vaccines of abortion bacilli, November 9th, 1920. The purpose of these treatments was to determine the effect of the vaccines on a positive udder. At first the udder was negative to the complement fixation and agglutination tests, but after a month or so became positive and continued so until the animal was killed. She gave birth to a live, normal calf October 13th, 1921.

From July 22nd to November 2nd, 1921, various tests such as the agglutination, bacterial and leucocytic counts, hydrogen ion concentration and carbon dioxide volume determinations, as well as differential staining methods, were done on the milk of the udder, taken under aseptic conditions, to determine whether the milk was abnormal or normal in every respect.

The agglutination and complement fixation tests showed that the blood and the milk were positive. Guinea-pig inoculation showed that the udder was infected with Bact. abortus.

805 was killed November 2nd, 1921, and blocks taken for histo-pathological work. Clinical examination revealed no macroscopic lesions or areas of induration in the dissected udder. The sections obtained from this udder for microscopic work were studied as typical of a Bact. abortus infected udder.

Cow 104.

Cow 104, a Jersey, was purchased in June, 1921, by the Bacteriology Department of the Michigan Agricultural College. She was four years old at the time. She had aborted once in 1920, but was sterile when the Department purchased her. The blood of 104 was always strongly positive while the milk was negative.

On July 20, 1922, four different strains of Bact. abortus were injected into the udder. One strain was injected to a quarter. The purpose of these injections was to determine the effect of the presence of Bact. abortus in the udder in its relation to the increase of leucocytes, the decrease in hydrogen ion concentration and the increase in percent by volume of carbon dioxide in milk, also for a comparison of the hydrogen

ion concentration and the percent by volume of the carbon dioxide.

The milk was positive for a short time and then became negative. Milk-staining methods were also employed to study the nature and origin of the cells. This animal is still in the Department herd.

Experimental Work.

1. The Effect of Leucocytes in milk on the Hydrogen ion Concentration and the Percent by Volume of Carbon Dioxide.

This work was undertaken with a view to studying a naturally infected udder, and, if possible, an artificially infected cow's udder. The two cows used were 805 and 104, whose histories have already been given.

Four different strains of Bact. abortus were introduced through the ducts of the teats into the different quarters of the udder of cow 104 to see whether it would be possible to infect them. Only one strain of a forty-eight-hour culture was introduced into each quarter. The reason for using a culture of this age was to insure the introduction of a large number of organisms and thus make the infection of the udder more certain.

The technic employed in this phase of the problem has already been outlined in Methods of Investigation.

Table VI

Shows the Relationship of the Leucocytes to the P_H Concentration and the Percent by Volume of CO_2 in an Artificially Infected Udder - COW 104.

100

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I	I	I	I	I	I	I	I	I	I
I	I	I	I	I	I	I	I	I	I
I	I	I	I	I	I	I	I	I	I
I	I	I	I	I	I	I	I	I	I
I	I	I	I	I	I	I	I	I	I

I	I	I	I	I	I	I	I	I	I
I	I	I	I	I	I	I	I	I	I
I	I	I	I	I	I	I	I	I	I
I	I	I	I	I	I	I	I	I	I

Table VI shows the effects of the leucocytes on the P_H concentration and the percent by volume of carbon dioxide in an artificially infected cow's udder. Serum reactions were employed to show whether the animal was infected. The serum tests showed that she was a reactor, reacting to both the complement fixation and agglutination tests throughout the experiment.

The term leucocytes in milk in this problem will mean the cellular elements which resemble, or are the same as the leucocytes found in the blood stream unless otherwise designated.

Table VII shows the effects of the leucocytes on the P_H concentration and the percent by volume of carbon dioxide in a naturally infected udder, that of cow 805. Serum reactions, direct isolation methods, as shown in Table VII, and guinea-pig inoculations, as shown in Table VIII, were employed to show whether the udder of 805 was infected during the experiment.

The results of Table VI will be discussed in detail. It may be stated that leucocytic counts were made for forty-nine samples, and seventy-six samples were examined for the P_H concentration and the percent by volume of carbon dioxide in milk.

The average of twelve samples of milk examined previous to the introduction of the different strains of Bact. abortus showed a P_H concentration of 6.6, and 9.7 percent of carbon dioxide by volume. From these averages it was concluded that the udder was normal in this respect.

The work of Baker (126) shows that the hydrogen ion concentration of normal milk is from 6.5 to 6.6, and that it contains by volume ten percent carbon dioxide, and that blood serum has a P_H concentration of 7.6 and sixty-five percent by volume of carbon dioxide. Other investigators have shown that P_H values vary from 6.39 to 6.81, the usual range being between 6.5 and 6.6.

It will be noticed that one week after inoculation the highest leucocytic counts as a rule gave the lowest P_H concentration and a greater percent by volume of carbon dioxide. These high leucocytic counts do not represent the phenomenon expected of an artificially infected udder at this time, which is chiefly due to the introduction of foreign materials, such as broth and its components.

Four samples, examined on August 8, October 5, October 9 and December 1, brought out very strikingly that when an increased leucocytic count was obtained there was

invariably a decreased hydrogen ion concentration and a greater percent by volume of carbon dioxide. These are the results of experiments with an artificially infected udder.

It is interesting to note that the leucocytic counts were done by the writer while the P_H concentration and carbon dioxide volume were determined by a member of the Experiment Station staff and not until the end of the nineteenth week were the results of the P_H and carbon dioxide work obtained by the writer.

Table VI shows that in the udder five days after the introduction of the various strains of Bact. abortus no immune bodies had been formed, but on August 8, they were present in the udder in great numbers. A test run at the end of the nineteenth week, December 1, shows that all agglutinating and complement fixation antibodies were present only in small numbers in the milk coming from the udder. However, it may be assumed that cow 104 was infected during the greater portion of the experiment.

Before the introduction of the Bact. abortus strains into the various quarters the hydrogen ion concentration and the percent by volume of carbon dioxide of the various quarters were normal, but after the intro-

duction of these strains and throughout the entire nineteenth week the hydrogen ion concentration was less, and the percent by volume of carbon dioxide greater than before.

The results discussed above should be taken as an indication of the various phenomena taking place in an artificially infected udder.

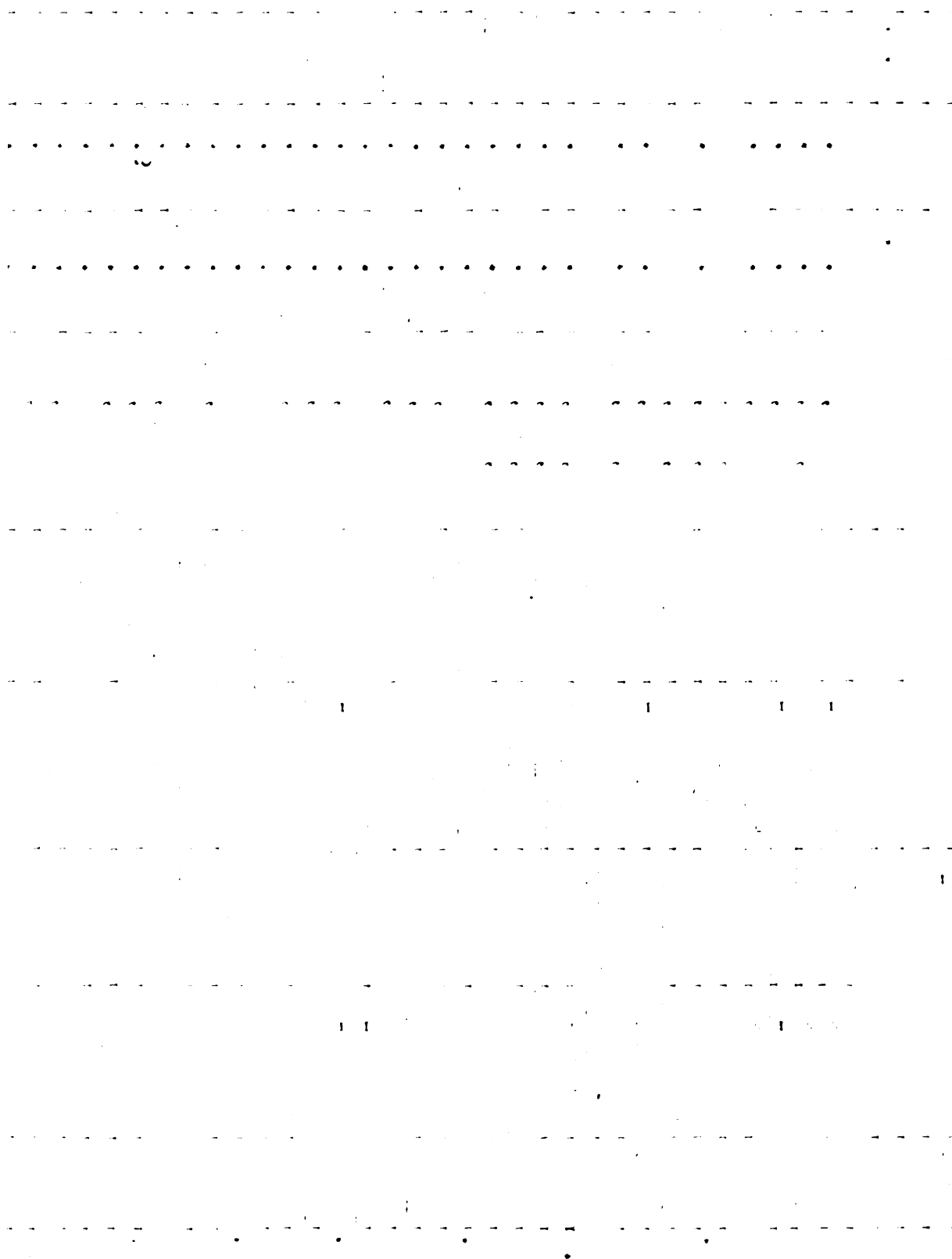
The results of Table VII will be discussed briefly and only the most important matters will be mentioned. Cow 805, an animal whose udder had been infected over a period of a number of years, was taken as the representative of a naturally infected udder. Although Bact. abortus could not be isolated by the "direct method" of isolation from the sediment of the centrifuged milk, still guinea-pig inoculation showed that the udder was infected. Guinea pigs injected with the milk of various quarters were separated from each other so that one could not infect the other. The milk and blood serum tests showed that antibodies were present in large numbers or in low concentration in cow 805.

Forty-three samples showed that the percent by volume of carbon dioxide, the P_H concentration and leucocytic counts were, with but few exceptions, normal throughout the whole experiment.

Table VII

Shows the Relationship of the Leucocytes to the P_H Concentration and the Percent by Volume of CO_2 in a Naturally Infected Udder-COW 805

Date	Quar-	Complement	Fixa-	Agglutination	Test	Leucocytic	P_H	CO_2	Remarks
	ter	tion	tion			Count			
	Milk	Milk	Blood	Milk	Blood		Conc.		D.M.
		Serum	Serum	Serum	Serum				



Very high leucocytic counts were accompanied by a decreased hydrogen ion concentration and an increased percent by volume of carbon dioxide. Especially worthy of note are the results obtained on August 10, with the four samples. The right rear quarter sample with a count of 4,650,000 gave a P_H concentration of 6.90 and a carbon dioxide volume of fourteen percent; the right front, with a count of 2,550,000, gave a P_H concentration of 6.9 and a carbon dioxide volume of thirteen percent; the left rear, with a count of 2,650,000, gave a P_H concentration of 6.90 and a carbon dioxide volume of fifteen percent; and the left front, with a count of 30,550,000, gave a P_H concentration of 7.5 and a carbon dioxide volume of twenty-three percent.

The sample from the left front quarter on August 10 showed the extremely low P_H concentration of 7.5, which is very close to that of blood serum, P_H 7.6, and a carbon dioxide volume of twenty-three percent was obtained, whereas the carbon dioxide of blood serum is sixty-five percent by volume.

It might be added in referring to Table VII that the abnormally high leucocytic count of 30,550,000 should not be taken as representative

as it will be noticed that it was taken from the strippings.

Where the leucocytic count for a sample is small - under one or two million - it would be natural to expect that the P_H concentration and carbon dioxide volume would show a great variation. Table VII brings this fact out very clearly.

Table VIII shows the results obtained by inoculating guinea pigs intraperitoneally with five cubic centimeters of milk. The guinea pigs were killed after a certain period and the spleens examined for lesions and the livers for necrosis. Smears from the spleens were made on gentian violet agar plates and the method used was that for isolating Bact. abortus as described on page 24. Previous to killing, the animal's blood was obtained by bleeding from the heart. The blood was allowed to clot and the usual technic for preparing blood serum was followed and the agglutination and complement fixation tests then employed.

The results obtained in Table VII represent the variation existing between leucocytic counts and the P_H concentration and the percent by volume of

Table VIII

Shows the Results of Guinea Pig Inoculation with Whole Milk - COW 805.

Date of Inoculation ^a	Date Killed	Complement Fixation Test.	Agglutination Test	Lesions	Neurosis	Isolation of	Remarks
		.1 .04 .02 .005	.1-.05-.025-.01-.005	Spleen	Liver	Bact. abortus on G.V. plates	
April	July 21 1921					1 2 3 4	
28	R R			+	+		
	R F			+	+		
1921	L R			+	+		
	L F			+	+		
							No isolation or serum tests run.
August 8	R R	+	+	+	+		
	Nov. 18	+	+	+	+		
1921	R F	+	+	+	+		
(in dup. b)	Nov. 18	+	+	+	+		
	L R	-	-	-	-		
	Sept. 10	+	+	+	+		
	L F	+	+	+	+		
							Isolation not Attempted.

carbon dioxide in milk taken from a naturally infected udder.

2. The Relation between the Hydrogen Ion Concentration and the Percent by Volume of Carbon Dioxide in Milk.

The first part of this study has been a discussion of the results obtained as shown in Tables VI and VII, with the conclusion that an increase in the leucocytic count gives a decrease in the hydrogen ion concentration, and an increase in the percent by volume of carbon dioxide in milk.

The phase of the problem considered here will be undertaken to show that a relationship exists between the hydrogen ion concentration and the percent by volume of carbon dioxide in freshly drawn milk. The factors which cause a decrease in hydrogen ion concentration and an increase in the carbon dioxide volume will be taken up in the discussion of Tables IX and X. The two cows used in this phase of the problem were 805 and 104, whose histories have already been given.

The technic employed in this part of the work has already been outlined on page 17.

Table IX.

Shows the Relationship between the P_H Concentration and the Percent by Volume of Carbon dioxide in an Artificially Infected Udder.

COW - 104.

(Same as Table VI except for the omission of the data on leucocytic counts. See page 73.)

Table IX shows the relation between the P_H concentration and the percent by volume of carbon dioxide in milk from an artificially infected udder. Serum reactions were employed to show whether the animal was infected or not. The serum tests showed that she reacted during the greater part of the nineteen week period and might be regarded as a cow that was artificially infected.

Table X shows the relationship between the hydrogen ion concentration and the percent by volume of carbon dioxide in a naturally infected udder. Serum reactions, direct isolation methods, as shown in Table X, and guinea-pig inoculations as shown in Table XI were employed to show whether the udder of 805 was infected during the experiment.

The results of the data obtained as shown in Table IX will now be discussed.

Twelve samples were examined before cultures of Bact. abortus were introduced into this animal, and sixty-four after the introduction of the organisms, making a total of seventy-six samples.

The average of the twelve samples of milk examined previous to the introduction of different strains of Bact. abortus showed a P_H concentration of 6.56, and 9.7 percent of carbon dioxide volume. This showed that the udder was normal in every respect and served as a basis for the experiment as to whether a decrease in the P_H concentration gave a higher percent by volume of carbon dioxide in milk after the udder was artificially infected.

It will be noticed that on July 21, twenty-four hours after the introduction of Bact. abortus a decreased P_H concentration and an increased percent by volume of carbon dioxide were obtained in the samples examined. This should probably not be regarded as due to the introduction of the organisms but rather to the presence of foreign material in the emulsion. The changes obtained later on, however, should be regarded as due to Bact. abortus.

The results of the samples examined on July 23, and 29, August 8, October 9, and throughout the experiment where there was a decrease in the P_H con-

centration showed an increase in the percent by volume of the carbon dioxide. The results after August 8 may be regarded as those of an artificially infected udder. The reason for this has already been discussed.

The reason for getting a decreased P_H concentration followed by an increased carbon dioxide volume will be taken up very briefly. It is probable, however, that the increased percent by volume of the carbon dioxide content of milk having a lowered acidity of P_H concentration, is due to the entrance of alkaline bicarbonates from the blood. The acidity would be decreased or lessened by increased amounts of basic phosphates, or even albumins. Changes in the secretion of the milk in relation to the substances just mentioned could easily explain the changes in reaction actually noted without assuming the entrance of excessive amounts of unchanged blood serum or lymph. These chemical changes are only partially understood, and, as has been shown, an increased leucocytic count results in a decreased P_H concentration and an increased percent by volume of carbon dioxide in an artificially infected udder and in a naturally infected udder.

A comparison of nineteen samples from each quarter of the udder is as follows: right rear quarter, P_H 6.7 and percent by volume of carbon dioxide 12.9; right front, P_H 6.6 and percent carbon dioxide volume 12.4; left rear, P_H 7.3 and carbon dioxide volume 17.2 percent; left front P_H 6.8 and carbon dioxide volume 13 percent. The averages of these samples show that with a decreased P_H concentration there is an increase in the percent by volume of the carbon dioxide.

The lowest P_H concentration was 7.2, with a corresponding carbon dioxide volume of 41 percent. This was obtained two days after the introduction of the various strains of Bact. abortus. This may be explained by the fact that blood serum from a normal animal has a P_H concentration of 7.6 and 65 percent by volume of carbon dioxide. Inflammation of the udder at this time caused by the introduction of foreign material greatly increases the P_H concentration and the percent by volume of carbon dioxide, due in all probability to the direct passage of the blood serum through the epithelium of the alveoli into the lumina of the alveoli without being changed to any great extent.

The results in general show as specific a relationship between the P_H concentration and the percent by volume of carbon dioxide in samples of fresh milk drawn from an artificially infected udder.

Table X.

Shows the Relationship between the P_H Concentration and the Percent by Volume of Carbon Dioxide in a Naturally Infected Udder - COW 805.

(Same as Table VII except for the omission of the leucocytic counts. See page 78.)

Table XI.

(See Table VIII, page 81).

Before discussing the results recorded in Table X several matters will be taken up very briefly. Cow 805 was the animal used in this experiment and was taken as representative of animals with naturally infected udders. The manner of determining this has already been given.

Table XI refers to Table VIII which confirms the fact that the udder was infected with Bact. abortus during the experiment. The history of the animal has already been given and shows that the udder had been infected for years.

Forty-three samples were examined for the hydrogen ion concentration and the percent by volume of carbon dioxide in milk. Eleven samples from each the right rear, left rear and left front, and ten samples from the right front quarter were examined. Table X shows that in determining the P_H and the carbon dioxide values of a sample of the right front quarter curdling took place and made readings impossible.

In looking over the data in this table one will notice that when a decreased P_H concentration was obtained in this naturally infected udder there was a corresponding increase in the percent by volume of carbon dioxide. Since the hydrogen ion concentration and the percent of carbon dioxide volumes were so close to normal, as one will note in looking over the table, comparisons could not be brought out as well as with the results recorded in Table IX for cow 104.

With a decreased hydrogen ion concentration, however, there was a corresponding increase in the carbon dioxide volume, as was clearly demonstrated in the four samples of August 10.

A comparison of the averages obtained for the eleven samples examined from the right rear, left rear and left front quarters with those of the ten samples from the right front quarter shows the following: the right rear has a P_H concentration of 6.5 and a carbon dioxide volume of 9.1 percent; the right front, a P_H concentration of 6.5 with a carbon dioxide volume of 9.1 percent; the left rear, a P_H concentration of 6.5 with a carbon dioxide volume of 10 percent; and the left front, a P_H concentration of 6.6 with a carbon dioxide volume of 10 percent. The averages of the hydrogen ion concentrations and carbon dioxide volumes are so close that a comparison as to variation is not possible as in the case of cow 104. This fact was brought out, however, that when the P_H concentrations of the various quarters were so close the percents by volume of carbon dioxide were correspondingly close. In this naturally infected udder the P_H concentration and the percent by volume of carbon dioxide were so close to normal all through the experiments that great variations could not be expected nor were they obtained in this udder.

3. The Comparison of the Leucocytes in the Fore Milk, Middle Milk and Strippings.

The first two phases of this thesis discuss somewhat in detail (1) the comparison of the leucocytes with the hydrogen ion concentration and the percent by volume of the carbon dioxide, and (2) the relationship between the hydrogen ion concentration and the percent by volume of carbon dioxide in the milk. The two animals used were 805 and 104 of which considerable mention has been made.

The third phase of this problem will deal with a comparison of the leucocytes in the three portions of the milkings. As very little literature was available in regard to the relationship of the leucocytes of the three milkings of normal cows, and no literature at all on an udder naturally infected with Bact. abortus, it is hoped to give here a comparison of the number of leucocytes in the three milkings both in a normal, non-infected udder and in an udder naturally infected with Bact. abortus. The two cows used in this phase of the problem were 805 and 997 B.

Shows the Comparison of the Leucocytes in Three Portions of the Milkings of an
Apparently Normal Udder - COW 997 B.

Date	'Quarter	'Complement	'Fixa-	'Agglutination	'Leucocytic	Count of Various Portions	Remarks
		'tion	'Test	'Test		of Milk	
		'	'	'	'Fore	Middle	Strippings
Apr.	R R	-	-	-	250,000		100,000
	R F	-	-	-	250,000		750,000
14	L R	-	-	-	50,000		150,000
							400,000

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Table XII compares the number of leucocytes in the three portions of the milkings of an apparently normal udder. Serum reactions on the blood and milk were employed. The leucocytic counts, as will be brought out later, were not abnormal. The animal was found to be apparently normal in every respect.

Table XIII shows the comparison of the number of leucocytes in the three milkings of a naturally infected udder. Serum reactions, direct isolation methods, as shown in the table, and guinea-pig inoculations, as shown in Table XIV, were employed to determine whether the udder of 805 was infected during the experiment.

The results as shown in Table XII will now be discussed.

Leucocytic counts were made of forty samples of fore milk, forty-four samples of strippings and sixteen samples of middle milk. In comparing the fore milk and the strippings between April 14 and May 11 the results show that in most cases the stripping counts ran higher than those of the fore milk. The samples of April 19 and April 28 are especially good examples of this.

It is to be further noticed that from May 11 to June 7 there is no marked difference between fore milk and middle milk, and that on certain dates one of the

Table XIII

Shows the Comparison of the Leucocytes in Three Portions of the Milkings
of a Naturally Infected Udder - COW 805.

Date	Quar-	Complement Fixa-		Agglutination Test	Leucocytic Counts of Various				D.M.	Remarks	
		tion Test			Portions of Milk						
		'Milk	'Serum	'Milk	'Blood	'Serum	'Fore	'Middle	'Strippings	'a	
July	RR	+	+	+	-	+	+	+	+	+	450,000 U
	RR	+	+	+	+	+	+	+	+	+	34,750,000 U
	LR	+	+	+	-	+	+	+	+	+	500,000 U
	LR	+	+	+	+	+	+	+	+	+	1,050,000 U
Aug.	RR	+	+	+	+	+	+	2,200,000	1,800,000	2,100,000	
	RR	+	+	+	+	+	+	30,800,000	32,950,000	31,950,000	
	LR	+	+	+	+	+	+	650,000	1,100,000	1,150,000	
6	LR	+	+	+	+	+	+	1,400,000	2,400,000	2,300,000	
	LR	+	+	+	+	+	+				
Aug.	RR	+	+	+	+	+	+	1,350,000	1,450,000	2,300,000	
	RR	+	+	+	+	+	+	2,100,000	2,050,000	2,350,000	

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milking was greater than the other and on other dates vice versa. The stripping counts were higher than those of the other two milkings in every case but two, namely on May 19 in the milk from the left rear quarter, and in the milk from the right front quarter on June 7.

The averages of the four samples of fore milk, middle milk and strippings collected on various occasions from each quarter as shown in Table XII are as follows: right rear quarter, fore milk, 237,500, middle milk 312,500 and strippings 1,700,000; right front quarter, fore milk 362,000, middle milk 612,500 and strippings 1,425,000; left rear quarter, fore milk 550,000, middle milk 712,500 and strippings 1,662,000; and left front quarter, fore milk 150,000, middle milk 250,000 and strippings 525,000. Greater numbers of leucocytes were obtained in strippings than in the other two milkings, and more in the middle milk than in the fore milk, as is shown by the above averages. The general averages of the different milkings will bear this out as will be shown presently.

An average for sixteen samples, taken from all quarters, of the fore milk, middle milk and strippings

Taken at the same time on certain dates, shows the average of the fore milk to be 353,125, the middle milk, 465,625 and the strippings, 1,337,500. The above shows that a greater number of leucocytes are found in the strippings than in the other two milkings, and even the middle milk gave a slightly higher cellular count than was found in the fore milk. This confirms the results obtained from the various quarters as previously cited.

The fact that higher counts were obtained in the middle milk and strippings was due in all probability to an irritation of the pliable udder tissue during the milking process. As the milking process continues the irritation and pressure of the outer connective tissue which envelops the pliable udder tissue become greater and an increase in leucocytes results, which is greatest in the strippings.

Another plausible explanation is the close relationship between the blood vessels and the epithelium of the alveoli. The irritant with the pressure of this outer connective tissue covering of the pliable udder tissue causes a diapedesis of the leucocytes between the endothelial cells of the blood capillaries into the epithelium of the alveoli. The diapedesis increases as the milking process continues and is

greatest in the last secreted milk of the strippings. Another factor which must not be overlooked is the fact that a larger number of desquamated epithelial cells should be expected in the strippings, thereby causing an increase in the leucocytic count.

Twenty-four samples of fore milk, twenty-four of middle milk and twenty-eight of strippings were examined for leucocytic counts. Only twenty-four counts of the stripping samples will be compared with twenty-four samples of the other two milkings taken at the same time in discussing Table XIII.

The strippings in nearly all cases yield greater counts, as one can see by looking over Table XIII. One interesting factor to be noted is that just before calving the serum reactions were especially positive, showing positive agglutination and positive complement fixations in the highest dilutions of both blood and milk serum. The highest counts were obtained at this period. No explanation can be given unless it is a fact that milk is higher during the end of the lactation period. This, however, cannot be stated with certainty. Some investigators have shown that the counts in the later stages of lactation are higher, and others have shown no increase in

Table XIV.

Same as Table VII - See page 78.

Table XV.

Averages of six samples each of fore milk, middle milk
and strippings, taken from each quarter.

Quarters	Fore Milk	Middle milk	Strippings
Right Rear	625,000	650,000	916,667
Right Front	658,317	705,833	729,167
Left Rear	200,000	705,833	729,167
Left Front	566,667	1,133,333	1,600,000

the leucocytic counts. If this is not the case it might be due to an active infection of the organism causing an inflammatory condition in the udder, which may account for the greatly increased leucocytic count.

The average counts of six samples collected on various occasions from each quarter, of the fore milk, middle milk and strippings are as shown in Table XV.

These averages show that smaller numbers were found in the fore milk, larger in the middle milk, and the largest numbers in the strippings. The general average of the three milkings will bear this out as will be shown presently.

An average of twenty-four samples, from all quarters, of the fore milk, middle milk and strippings taken at the same time on certain dates, shows the average of the fore milk to be 512,496, the middle milk 703,541 and the strippings 909,375. These averages show that the fore milk counts were the least, the middle milk greater and the strippings the greatest. These results were obtained from a naturally infected udder.

The results obtained in the normal animal, 997 B, and the naturally infected animal 805 correspond in every detail. Breed (127) examined the milk of one

hundred twenty-two cows and found an average cellular count of 868,000 per cubic centimeter. Cooledge (128), examining the whole milk of forty-seven apparently normal cows, found an average cellular count of 930,000. The average leucocytic count of the three milkings of 805 was 408,471 and that of 997 B was 718,783. The udders of both these animals may be considered normal as to leucocytic counts. This also shows that a naturally infected udder like that of 805 did not have a higher cellular count than that of a non-infected udder, as that of 997 B.

4. Staining of Milk Cells and their Origin.

Differential counts have been made of milk and employed in a manner similar to that applied to the blood. The basis of these counts has been qualitative, assuming that the cells present were true leucocytes. These attempts have not been made in the interest of science alone, but to bring about a hygienic control of milk supplies. This being the case it is very important to ascertain beyond all doubt whether a differential count can be made on the basis of the cells in the milk being true leucocytes as those found in the blood. Savage has made use of a classification into polymorphonuclear cells, lymphocytes, large leucocytes and doubtful cells. He states that the presence of any particular

form of leucocyte cannot be taken as of diagnostic value.

Winkler and Michaels emphatically state that leucocytes are practically never found in milk under normal conditions, and never found in the lumina of the alveoli of glandular tissue. Hewlett, Villar and Revis express their view that the cellular elements found in milk in normal as well as in ordinary catarrhal or INTERSTITIAL non-suppurative arthrites are tissue cells and that leucocytes do not appear in milk under these conditions. Breed holds the opposite view that the larger percent are leucocytes or white corpuscles, the smaller percent epithelial cells (nuclei or fragments of these cells).

The fourth phase of this problem deals with the staining of milk cells and their origin. The writer hopes to show that a rough differential count can be made of whole milk, showing that the greater percent are true leucocytes, the minority being epithelial cells and fragments of these cells. An attempt will be made to account for the difficulties encountered in staining the leucocytes of the milk with ordinary blood stains. Differential counts were made of only four quarters of 997 B.

By means of photographs cells will be shown the lumina of two alveoli which represent true leucocytes such as those found in the blood stream. A further proof as to whether leucocytes originate in the epithelium or the blood stream will be brought out in the histological examination of stained slides from thirty-two blocks of two cows' udders. The slides used for histological examinations and photomicrographic work were obtained from the blocks of the udders of cows 997 B and 805.

As has already been stated the three stains used in staining the whole milk were Wright's, Jenner's and Giemsa's. Wright's and Jenner's were of no value in bringing out the different cells. Giemsa's was found to be the best stain, and from these stained slides the differential counts were made. At least five hundred cells were counted in each case for a differential count of one quarter. The results of these counts are shown in Table XVI.

Table XVI.

Shows the Percent Cells in Whole Milk with
a Differential Stain.

Number	Percent of Various Cells 'in Whole Milk.'				Epithelial Cells	Remarks
	Quarter	Polymorpho-	Lymphocytes	Large	Mast Cells	
Control		nuclear		'Mono-nuclear'		
515	R R	48.74	43.65	2.52	.58	.19
513	R F	57.50	32.94	4.67	.39	.19
528	L R	78.79	18.37	1.51	.57	0
512	L F	63.48	30.86	1.17	.39	.58
Average		62.03	31.45	2.46	.48	.23
						2.96%

R R = Right Rear

L R = Left Rear

R F = Right Front

L F = Left Front

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Before discussing the results obtained it will be necessary to cite the percentages obtained by Dimock and Thompson (129) of the several varieties of leucocytes in the blood of normal cattle.

	Averages	Minimum	Maximum
Leucocytes	54.2%	31%	76%
Large Mononuclear	1.4%	.2	3.3
Polymorpho-nuclears	30.5	13	45.8
Eosinphiles	13.5	3.8	26.5
Mast Cells	.59	0.1	1.2

The results of Table XVI show that in every quarter a higher percentage of polymorpho-nuclears were obtained than the average shown above. This average is over twice as much as for that of cow's blood. The percentage of lymphocytes in the milk which was obtained in this count was about half as much as the average shown above. The writer will not discuss the comparison of the other leucocytes in the milk and in the blood as Table XVI and Dimock and Thompson's percentages of blood show this plainly.

In the opinion of the writer the finding of a large number of polymorpho-nuclear leucocytes does not indicate an abnormal condition because these cells have the power to pass between the cells of the blood vessels

into the epithelial cells of the alveolus where they are secreted with the milk. Should excessively large amounts of lymphocytes, large mononuclears, eosinophiles and epithelial cells have been found it would, in all probability, have indicated a severe inflammation of the udder. It is also the opinion of the writer that no real comparison should be made of leucocytes and other cells of similar nature with that of the blood. The results show that epithelial cells in the milk are not very prevalent, and that the average for the four quarters was 2.96 percent. The origin of the cells in the differential counts made of 997 B shows that they do not come entirely from the epithelium of the alveoli but originate for the most part in the blood stream.

There are, however, certain difficulties met in staining the cells of the milk smears. The casein or some other substance in the milk seems to obscure the cytoplasm of the cells to such an extent that it is very difficult to distinguish by microscopical examination what variety or kind of leucocytes there are present in the field. In some cases it is impossible to classify certain ones. To prove that the casein obscured the cells, blood was obtained and mixed in proportions of

one to five and one to ten, and here the same difficulties of distinguishing them were noticed as were noticed in the whole milk. This accounts for the high counts obtained in the differential counts for certain varieties of leucocytes, as shown in Table XVI.

The writer believes that a differential count cannot be made of milk for diagnostic purposes. It only shows that the origin of the cells in the milk is both the blood stream and the epithelium of the udder.

A further proof that these cells (leucocytes mostly) found in milk are not entirely of epithelial origin, as is claimed by some investigators, will be shown presently. A very careful examination was made of stained slides obtained from two udders, of which mention has already been made. In the lumina of the alveoli in the numerous slides examined from the sixteen blocks of each udder, on many occasions polymorpho-nuclears and eosinophiles were found which were typical or exactly like the leucocytes found in the blood of the cow. Hewlett, Villar and Revis (130) in histological examinations of cows' udders come to an opposite conclusion from that obtained by the writer.

The evidence which they produce to support their views is far from convincing when a careful study is made.

On the next few pages photographs will be shown illustrating polymorpho-nuclear cells. Before photographing these cells in the lumina of the alveoli a very thorough examination was made with the microscope in each case.

Figure 1 represents a photographed section of block 3 of the left front quarter of 805. It will be noticed that the cells are of an entirely different nature as regards size, nucleus and staining properties than the cells of the epithelium. The epithelium of the alveolus is also intact in every place except in the lower left hand corner where the tissue has been slightly torn by the sectioning process

1 = typical polymorpho-nuclear leucocyte.

2 = torn portion of epithelium.

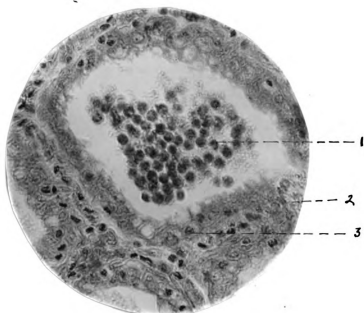
3 = epithelial cell.

Figure 2 represents a photographed section of block 3 of the left front quarter of 805. The photograph shows certain characteristics by a smaller magnification not obtainable in figure 1.

1 = Same as above.

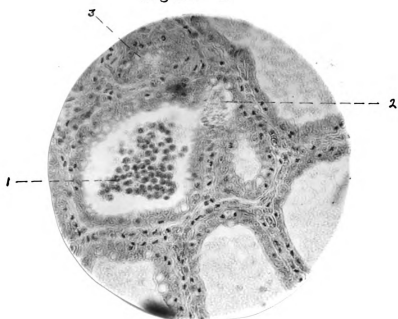
2 = Same as above.

3 = tangential section of alveolus.



x 500 6 u

Figure 1.



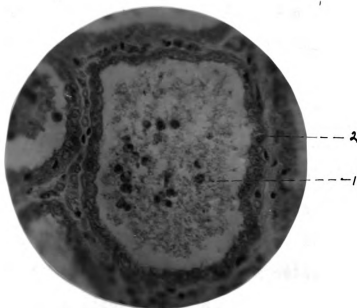
x 300 6 u

Figure 2.

Figure 3 represents a photographed section of block 3 of the left front quarter of 997 B. It will be noticed that the cells are of an entirely different nature as regards size, nucleus and staining properties than the cells of the epithelium. The epithelium of the alveolus is also intact in every place.

1 = typical polymorpho-nuclear leucocyte

2 = epithelial cell.



x 500 6u

Figure 3.

5. The Comparison of the Bacteria in the Fore Milk, Middle Milk and Strippings.

The fifth phase of this problem deals with the bacterial colonies in the three milkings. The literature reviewed has shown that the fore milk always contains more bacteria than does the middle milk or strippings. The work of Harding and Wilson corroborates the results of Stocking in which it is shown that in the three hundred and sixty samples examined there were smaller numbers of bacteria in the middle milk than in the fore milk or strippings.

In this phase it will be shown that the highest count was obtained in the fore milk, the next highest in the middle milk and the smallest count in the strippings both in an apparently normal udder and in an udder naturally infected with Bact. abortus. For the normal animal cow 997 B was taken and for a naturally Bact. abortus infected udder, cow 805.

Table XVII shows the comparison of the bacterial colonies in the three milkings of an apparently normal udder - that of cow 997 B, serum reactions of both the blood and milk and leucocytic counts of the strippings.

Table XVII

Shows the Comparison of the Bacterial Colonies in the Three Milkings of an Apparently Normal Udder - Cow 997 B.

Date	Quarter	Complement Fixation Test	Agglutination Test	'Leucocytic' Counts	'Bacterial Counts of Various Portions of Milk	Remarks
		.1 .04 .02 .005	1-05-085-01-005	'Strippings'	Fore Middle Strippings	
May 5 1921	RR	- - - -	- - - -	800,000	74	2
	RF	- - - -	- - - -	350,000	14	18
	LR	- - - -	- - - -	350,000	1764	18
	LF	- - - -	- - - -	350,000	63	40
May 11	RR	- - - -	- - - -	700,000	54	32
	RF	- - - -	- - - -	600,000	46	9
	LR	- - - -	- - - -	950,000	176	26
	LF	- - - -	- - - -	1,000,000	4	0
May 17	RR	- - - -	- - - -	1016	152	
	RF	- - - -	- - - -	635	54	
	LR	- - - -	- - - -	366	154	
	LF	- - - -	- - - -	168	34	

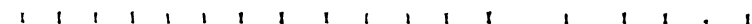


Table XVIII

Shows the Comparison of the Bacterial Colonies in the Three Milkings
of a Naturally Infected Udder - COW 805

Date	Quar-	Complement	Fixa-	Agglutination	Direct	Bacterial	Counts of Vari-	Remarks
	ter	tion Test	tion Test	Test	'Isolation'	'ous Portions of Milk		
	'Milk	'Blood	'Milk	Blood	'Method			
	'Serum	'Serum	'Serum	Serum		'Fore	Middle Strippings'	
July	R R	+	+	+	+	699	511	402
29	'ar F	+	+	+	+	4	5	1
1921	L R	+	+	+	+	450	50	26
	L R	+	+	+	+	219	288	61
	R R	+	+	+	+	1306	754	505
Aug.	R R	+	+	+	+	8	13	20
4	L R	+	+	+	+	1143	128	63
	L R	+	+	+	+	770	145	227
Aug.	R R	+	+	+	+	540	549	435
9	'br F	+	+	+	+	862	432	429
	L R	+	+	+	+	862	146	57
	L R	+	+	+	+	858	161	101
	R R	+	+	+	+	200	16	50
Oct.	R R	+	+	+	+	500	27	19
29	L R	+	+	+	+	8	1	2
	L R	+	+	+	+	6	2	1
	R R	+	+	+	+	13	2	5
Oct.	R R	+	+	+	+	31	30	36
30	L R	+	+	+	+	14	1	1
	L R	+	+	+	+	3	1	2
	R R	+	+	+	+	250	1450	25
Oct.	R R	+	+	+	+	27	56	16
31	L R	+	+	+	+	26	47	8
	L R	+	+	+	+	100	79	35

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1. *Journal of the American Medical Association*, 1997; 277: 1033-1036.

[illegible][illegible][illegible][illegible][illegible][illegible]

100 100 100 100

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1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74 75 76 77 78 79 80 81 82 83 84 85 86 87 88 89 90 91 92 93 94 95 96 97 98 99 100 101 102 103 104 105 106 107 108 109 110 111 112 113 114 115 116 117 118 119 120 121 122 123 124 125 126 127 128 129 130 131 132 133 134 135 136 137 138 139 140 141 142 143 144 145 146 147 148 149 150 151 152 153 154 155 156 157 158 159 160 161 162 163 164 165 166 167 168 169 170 171 172 173 174 175 176 177 178 179 180 181 182 183 184 185 186 187 188 189 190 191 192 193 194 195 196 197 198 199 200 201 202 203 204 205 206 207 208 209 210 211 212 213 214 215 216 217 218 219 220 221 222 223 224 225 226 227 228 229 230 231 232 233 234 235 236 237 238 239 240 241 242 243 244 245 246 247 248 249 250 251 252 253 254 255 256 257 258 259 260 261 262 263 264 265 266 267 268 269 270 271 272 273 274 275 276 277 278 279 280 281 282 283 284 285 286 287 288 289 290 291 292 293 294 295 296 297 298 299 300 301 302 303 304 305 306 307 308 309 310 311 312 313 314 315 316 317 318 319 320 321 322 323 324 325 326 327 328 329 330 331 332 333 334 335 336 337 338 339 340 341 342 343 344 345 346 347 348 349 350 351 352 353 354 355 356 357 358 359 360 361 362 363 364 365 366 367 368 369 370 371 372 373 374 375 376 377 378 379 380 381 382 383 384 385 386 387 388 389 390 391 392 393 394 395 396 397 398 399 400 401 402 403 404 405 406 407 408 409 410 411 412 413 414 415 416 417 418 419 420 421 422 423 424 425 426 427 428 429 430 431 432 433 434 435 436 437 438 439 440 441 442 443 444 445 446 447 448 449 450 451 452 453 454 455 456 457 458 459 460 461 462 463 464 465 466 467 468 469 470 471 472 473 474 475 476 477 478 479 480 481 482 483 484 485 486 487 488 489 490 491 492 493 494 495 496 497 498 499 500 501 502 503 504 505 506 507 508 509 510 511 512 513 514 515 516 517 518 519 520 521 522 523 524 525 526 527 528 529 530 531 532 533 534 535 536 537 538 539 540 541 542 543 544 545 546 547 548 549 550 551 552 553 554 555 556 557 558 559 560 561 562 563 564 565 566 567 568 569 570 571 572 573 574 575 576 577 578 579 580 581 582 583 584 585 586 587 588 589 590 591 592 593 594 595 596 597 598 599 600 601 602 603 604 605 606 607 608 609 610 611 612 613 614 615 616 617 618 619 620 621 622 623 624 625 626 627 628 629 630 631 632 633 634 635 636 637 638 639 640 641 642 643 644 645 646 647 648 649 650 651 652 653 654 655 656 657 658 659 660 661 662 663 664 665 666 667 668 669 670 671 672 673 674 675 676 677 678 679 680 681 682 683 684 685 686 687 688 689 690 691 692 693 694 695 696 697 698 699 700 701 702 703 704 705 706 707 708 709 710 711 712 713 714 715 716 717 718 719 720 721 722 723 724 725 726 727 728 729 730 731 732 733 734 735 736 737 738 739 740 741 742 743 744 745 746 747 748 749 750 751 752 753 754 755 756 757 758 759 760 761 762 763 764 765 766 767 768 769 770 771 772 773 774 775 776 777 778 779 780 781 782 783 784 785 786 787 788 789 790 791 792 793 794 795 796 797 798 799 800 801 802 803 804 805 806 807 808 809 810 811 812 813 814 815 816 817 818 819 820 821 822 823 824 825 826 827 828 829 830 831 832 833 834 835 836 837 838 839 840 841 842 843 844 845 846 847 848 849 850 851 852 853 854 855 856 857 858 859 860 861 862 863 864 865 866 867 868 869 870 871 872 873 874 875 876 877 878 879 880 881 882 883 884 885 886 887 888 889 890 891 892 893 894 895 896 897 898 899 900 901 902 903 904 905 906 907 908 909 910 911 912 913 914 915 916 917 918 919 920 921 922 923 924 925 926 927 928 929 930 931 932 933 934 935 936 937 938 939 940 941 942 943 944 945 946 947 948 949 950 951 952 953 954 955 956 957 958 959 960 961 962 963 964 965 966 967 968 969 970 971 972 973 974 975 976 977 978 979 980 981 982 983 984 985 986 987 988 989 990 991 992 993 994 995 996 997 998 999 1000 1001 1002 1003 1004 1005 1006 1007 1008 1009 1010 1011 1012 1013 1014 1015 1016 1017 1018 1019 1020 1021 1022 1023 1024 1025 1026 1027 1028 1029 1030 1031 1032 1033 1034 1035 1036 1037 1038 1039 104

1. *Journal of the American Medical Association*, 2000; 283: 2686-2692.

Age Group	Percentage
18-24	10%
25-34	15%
35-44	20%
45-54	25%
55-64	20%
65-74	15%
75-84	10%
85+	5%

Table XVIII shows the comparison of the bacterial colonies in the three milkings of a naturally infected udder - that of cow 805. Serum reactions on the blood and milk were made, as well as guinea-pig inoculations, as shown in Table XIX.

Table XIX - See page 81.

(Same as Table VIII).

The results of Table XVII show that the bacterial colonies in the fore milk are always higher than in the middle milk or strippings. The bacterial counts of the middle milk of the various samples is generally greater, as the table shows. The averages of fourteen samples each of fore milk, middle milk and strippings are as follows: fore milk 1718, middle milk 228 and strippings 87 bacterial colonies. These averages show that in the fore milk there are over seven times as many microorganisms as in the middle milk, and slightly over nineteen times as many bacterial colonies as in the strippings. The middle milk contained over twice as many as the strippings.

It is to be noted that with but few exceptions the bacterial counts shown in Table XVII for the fore milk are always higher than in the middle milk and strippings, and those in the middle milk in almost every case were higher than in the strippings.

The averages of seven samples each of fore milk, middle milk and strippings as obtained from Table XVIII are shown in Table XX.

Table XX.

Quarters	Fore Milk	Middle Milk	Strippings.
Right Rear	434	470	204
Right Front	224	84	75
Left Rear	356	54	24
Left Front	281	97	62

The bacterial count of the middle milk of the right rear quarter is greater than that of the fore milk, as will be noted in Table XX, but this is the only exception. With the exception just noted it will be found that in every case the bacterial counts of the fore milk are greater than those of the middle milk or strippings. The middle milk counts are greater in every case than those of the strippings.

The averages of twenty-eight samples, from each of the quarters, of fore milk, middle milk and strippings are as follows: fore milk 2274, middle milk 1233 and strippings 641. From these results it can be seen that the fore milk contained nearly twice as many bacterial colonies as the middle milk and over three times as many as the strippings, and the middle milk contained nearly twice as many

colonies as the strippings.

A greater number of bacterial colonies may be expected in the fore milk, a smaller number in the middle milk and the smallest number in the strippings. The only plausible explanation for this seems to be that in the first milk, or fore milk, coming from the udder after five streams of milk have already been discarded, the largest number of bacteria are found on account of the milk having been stored for some time in the reservoirs and ducts of the various quarters of the udder where the bacteria have had time to multiply. As the milking process continues the greater percentage of the bacteria are washed out, becoming fewer in number until only a small number appear in the last secreted milk of the strippings.

The averages of the fore milk, middle milk and strippings of cow 997 B and cow 805 are: fore milk 1191, middle milk 732 and strippings 369 bacterial colonies. This comparison shows that the fore milk contained over twice as many bacterial colonies as the middle milk and over five times as many as the strippings, and that the middle milk contained twice as many bacterial colonies as the strippings.

The average of the bacterial colonies of the three milkings of cow 997 B is 863, and of cow 805 is 1197. These counts should be considered normal. The results obtained in this phase of the problem are a comparison of an apparently normal udder and of a Bact. abortus infected udder.

6. Types of Microorganisms most Prevalent in Milk.

The types of microorganisms found in samples of milk taken from cows' udders under sterile conditions reveal a large number of species. Among the few isolated by various investigators have been B. coli, Staphylococcus mastitis aureus and Staphylococcus mastitis albus, Streptococcus lacticus, Staphylococcus pyogenes albus, streptococci, Bact. abortus and, finally, a yeast.

Histological investigations have not shown the nature of invasion of microorganisms in the udder and consequently investigators have been forced to go back to cultural findings. Various investigators have shown that in plantings of cultures from freshly slaughtered cows' udders the bacteria are distributed rather evenly in the ducts and other portions of the udder. Bacteria have been found even in the outermost portions of the gland.

It is quite evident that microorganisms are not only free in the lumen of the alveoli but also present on the walls of the ducts of these glands. That bacteria are found on the inside of the epithelium of the udder is very questionable. Only those bacteria free in the secretions would finally be ejected in the milking process.

This phase was undertaken not for the purpose of isolating certain species of bacteria and studying their cultural characteristics, but for the purpose of getting representative colonies from agar plates and transferring these to agar slants and then studying these organisms as to their staining and morphological characteristics. The Breed method was used in studying whole milk smears. To show what microorganisms might be expected in milk from udders, histological examinations were made of stained slides from two udders. For both the histological and bacteriological examinations cows 997 B and 805 were used.

Stains were made from twenty-five cultures of 997 B and examined microscopically. Twenty-three of these slides showed micrococci of which eighty-four were gram-positive staphylococci. The other two stained preparations were gram-positive rods.

Slides were prepared from twenty-five cultures of 805, stained and examined microscopically. Twenty-four of the slides examined showed micrococci, of which ninety-two percent were gram-positive staphylococci. The other stained preparation was a gram-positive rod.

Breed's method was followed in making the smears, stains and bacterial counts of milk samples from both animals. The bacteria were so few in the smears that occasionally micrococci were noticed. The chief reason for using this method was to find out whether any streptococci were present. In the smears examined no streptococci were found. The writer believes that the Breed method is valuable for finding out whether a cow's udder is infected with streptococci.

Histological examinations of stained sections of slides from thirty-two blocks of two udders revealed no bacteria. This was to be expected since the plate counts from the various quarters of these udders, as shown in experiment five of this problem, were low in the case of each of the two animals. Again, the magnification of the microscope being so high it can be readily seen that few if any would be encountered under the above-mentioned condition.

From all these examinations of both the apparently normal udder of cow 997 B and the naturally Bact. abortus infected udder of 805 it is safe to assume that they were normal from the types of organisms found.

7. The Histo-Pathological changes caused by Bact. abortus in an Infected Udder.

The literature has revealed very few data on this particular phase of the problem, the only available data being those of Zwick and Krage.

The work of these investigators will be reviewed very briefly in order that the reader may be enabled to follow this phase of the problem more easily in a later discussion. Zwick and Krage isolated Bact. abortus from the milk of a cow that had aborted thirteen months previously. The infected milk showed no macroscopic changes from the normal. Clinical examinations of the udder and its associated lymph glands showed no changes from normal. These workers conclude that the organism is able to pass through the udder without producing any lesions at all or that the lesions are so small as to be unrecognizable clinically or macroscopically. Two goats were given ten cubic centimeters each of this organism, the latter emulsion injected into one of the goats having half the density of the former. Bact. abortus was isolated for a period of eight weeks. At

the end of this period the two goats were killed. Previous to killing, no macroscopic changes could be noticed in the milk by using the Trommsdorf method of examining leucocytes. Clinical examination showed no noticeable alterations from the normal in the udder and its associated lymph glands.

This phase of the problem was undertaken with a view to showing whether Bact. abortus causes any gross pathological changes in the udder such as macroscopic and microscopic lesions in the udder, oatarrrhal conditions, areas of induration, abscess formation, cell infiltration or any other abnormalities in a naturally infected udder. It was further hoped to show that Bact. abortus could not be found in microscopical examinations of stained slides from various portions of the udder, and its habitat determined in the udder of an infected animal.

The average of the leucocytic counts of the three milkings as shown in the third phase, was normal, and the average of the numbers of bacterial colonies in the three milkings was normal for both animals. Serum tests, as shown in Table VII, and guinea-pig inoculation, as shown in Table VIII for 805 which had been infected for years, were positive throughout

the experiment. Serum tests of 997 B revealed a negative animal. Samples of milk from 805 showed no abnormalities in the hydrogen ion concentration and the percent by volume of carbon dioxide (Table X). The differential counts of 997 B were normal. Types of organisms studied in phase five revealed that no organisms were present which would indicate an inflammatory condition in either animal. Having all these data on hand the two animals were then killed on different dates, 997 B on June 30, 1921 and 805 on November 2, 1921. On these different dates each udder and its associated lymph glands were examined macroscopically to see whether they were normal. These examinations showed no abnormalities and were normal throughout. Sixteen blocks were taken from each udder (four to a quarter), as nearly as possible the same for each animal, and placed in Zenker's fluid and prepared for histological examination as described between pages 32 and 47 in Methods of Investigations.

The writer first studied the slides from 997 B in order to know exactly the histological structure of a normal udder. It may be of interest, however, to note that in examining the slides from the various quarters of the udder all stages of activity in milk secretion were noticed in the epithelium and alveoli. This is of the utmost interest because if these changes are not understood one may very easily mistake a normal

for an abnormal condition. It may be added that the nature of the glandular tissue with these varied changes makes it perhaps the hardest tissue in which to determine any abnormal changes.

Having become familiar with normal udder tissue a very intensive histological investigation of the udder from 805 was started. Several slides from each block of the various quarters of the udder revealed no area of induration, cell infiltration, or any other abnormalities in this naturally infected udder. No organisms were detected in the various tissues of the udder by these histological examinations due to the high magnification of the microscope. This last noted phenomenon could be expected as the microorganism could not be cultured by direct isolation methods.

The failure to obtain any noticeable macroscopic or microscopic changes from normal in this Bact. abortus infected udder has established several points, namely (1) that Bact. abortus though present is able to pass through the udder without causing any macroscopic or microscopic lesions or other changes, and (2) that this study has not established its characteristic location in the udder.

On the following page will be shown photographs of block three of the right rear, right front, left rear and left front quarters of cow 805 and cow 997 B.

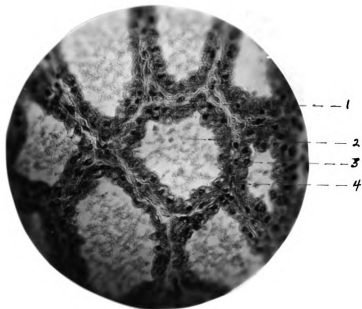
Figure 4 represents a photographed section of block 3 of the right rear quarter of 997 B. From the photograph it will be noticed that the various parts of the section represent normal udder structure.

1 = basement membrane

2 = lumen of alveolus

3 = epithelium

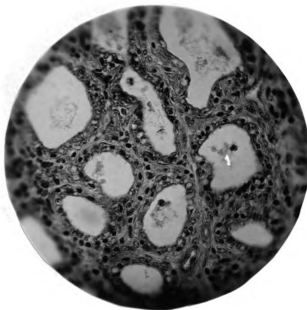
4 = epithelial cells.



x 300 7 u

Figure 4.

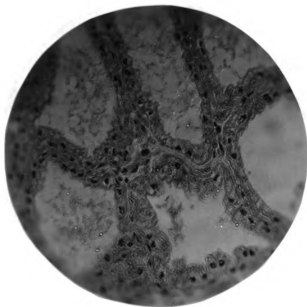
Figure 5 represents a photographed section of block 3 of the right rear quarter of 805. This photograph shows that the epithelium and other structure are normal as compared with Figure 4.



x 300 6u

Figure 5.

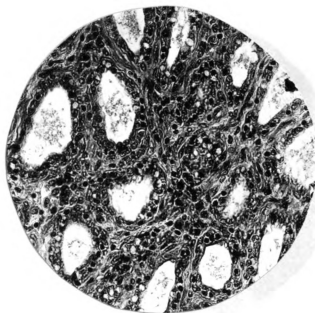
Figure 6 represents a photographed section of block 3 of the right front quarter of 997 B. From the photograph it will be noticed that the various parts of the section represent normal udder structure.



x 500 7u

Figure 6.

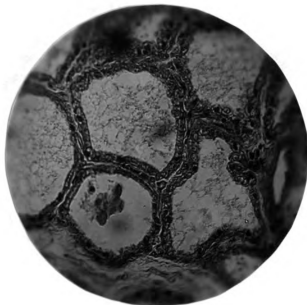
Figure 7 represents a photographed section of block 3 of the right front quarter of 805. This photograph shows that the epithelium and other structures are normal as compared with figure 6.



x 300 7u

Figure 7.

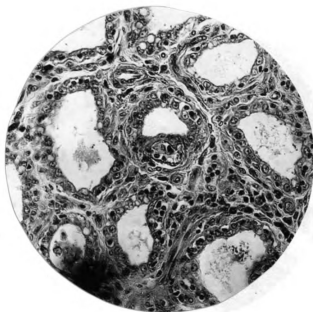
Figure 8 represents a photographed section of block 3 of the left rear quarter of 997 B. From the photograph it will be noticed that the various parts of the section represent normal udder structure.



x 300 7u

Figure 8.

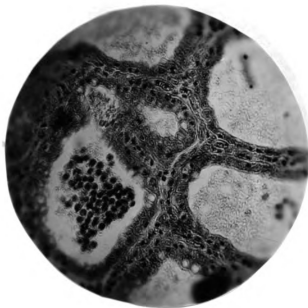
Figure 9 represents a photographed section of block 3 of the left rear quarter of 805. This photograph shows that the epithelium and other structures are normal as compared with Figure 8.



x 300 6u

Figure 9 .

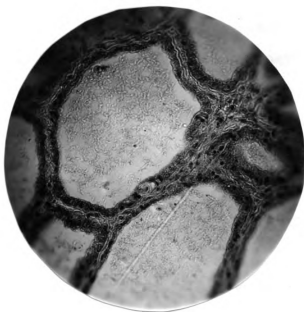
Figure 10 represents a photographed section of block 3 of the left fron quarter of 997 B. From this photograph it will be noticed that the various parts of the section represent normal udder structure.



x 30C 7 u

Figure 10 .

Figure 11 represents a photographed section of block 3 of the left front quarter of 805. This photograph shows that the epithelium and other structures are normal as compared with Figure 10.



x 300 6u

Figure 11.

GENERAL DISCUSSION.

Experiments have been outlined in this problem dealing with different phases of Bact. abortus infected udders.

Phase one of the problem was outlined in such a manner as to show the effect of leucocytes in milk on the hydrogen ion concentration and the percent by volume of carbon dioxide. The artificially infected udder of cow 104 showed that in four samples examined on August 8, October 5, October 9 and December 1 whenever an increased leucocytic count was obtained there was invariably a decreased hydrogen ion concentration and an increased percent by volume of carbon dioxide. The same results were also obtained in the naturally infected udder.

A comparison of the nineteen samples of milk from each separate quarter of the artificially infected udder of cow 104 (phase 2) revealed the fact that with a decreased hydrogen ion concentration an increased percent by volume of carbon dioxide was obtained. In the naturally infected udder of cow 805 the readings were so close to normal (P_H 6.5 - 6.6 and CO_2 volume 10 percent) that no conclusions could be drawn. However, the following is of special interest. The lowest P_H concentration was 7.2 with a

corresponding carbon dioxide volume of 41 percent. This was obtained two days after injection of the various strains of Bact. abortus. This might be explained by the fact that blood serum has a P_H of 7.6 and 65 percent by volume of carbon dioxide due to an inflammation of the udder at this time caused by the introduction of foreign material, and that the greatly increased P_H concentration and percent by volume of carbon dioxide were due in all probability to a direct passage of blood serum through the epithelium of the alveoli into the lumina of the alveoli without being changed to any great extent.

Some very interesting data were obtained in phase three by comparing the number of leucocytes in the fore milk, middle milk and strippings in the apparently normal udder of cow 997 B and the naturally infected udder of 805. The average of four samples collected on various occasions of the fore milk, middle milk and strippings of each quarter shows that a greater number of leucocytes was obtained in the strippings than in the other two milkings, and more in the middle milk than in the fore milk. An average of sixteen samples, from all quarters, of the fore milk, middle milk and strippings (997 B) showed that in the strippings a greater number of leucocytes was found than

in the other two milkings, and a greater number in the middle milk than in the fore milk. The average of six samples, collected on various occasions from each quarter, of the fore milk, middle milk and strippings of 805 showed that the smallest number was found in the fore milk, a larger number in the middle milk and the largest number in the strippings. An average of twenty-four samples, from all quarters, of the fore milk, middle milk and strippings (805) showed a higher count for the strippings than for the other two milkings, and a higher count for the middle milk than for the fore milk. Similar averages were obtained with two other animals.

The higher leucocytic counts obtained in the middle milk and strippings may be due to an irritation of the pliable udder tissue during the milking process. As the milking process continues the irritation and pressure of the outer connective tissue which envelops the pliable udder tissue become greater resulting in an increase in the number of leucocytes, the number being greatest in the strippings.

Another plausible explanation is the close relationship between the blood vessels and the epithelium of the alveoli. The irritant with the pressure of this outer connective tissue covering of the pliable udder

tissue causes a diapedesis of the leucocytes between the endothelial cells of the blood capillaries into the epithelium of the alveoli. This diapedesis increases as the milking process continues, and is greatest in the last secreted milk, the strippings. Another factor which must not be overlooked is the fact that a large number of desquamated epithelial cells should be expected in the strippings thereby causing an increase in the cell count.

The origin of the cells in the different counts made of 997 B in phase four shows that they do not come entirely from the epithelium of the alveoli but originate for the most part in the blood stream, and that the epithelial cells are not very numerous. Certain difficulties were met in staining the cells of the milk smears. The casein, or some other substance in the milk, seemed to obscure the cytoplasm of the cells to such an extent that it was very difficult to distinguish by microscopical examination what variety or what kind of leucocytes were present in the field. In some cases it was impossible to classify certain ones. To prove that casein or some other substance obscured the cells blood was obtained and mixed in proportions of one to five and one to ten with whole milk, and the same difficulties of distinguishing them was noticed as was

noticed in the case of whole milk. This accounts for the high counts obtained in the differential counts for certain varieties of leucocytes as shown in Table XVI.

The writer believes that a differential count cannot be made of milk for diagnostic purposes. It only shows that the origin of the cells in the milk (which certain investigators claim are of epithelial origin) is both the blood stream and the epithelium of the udder. Histological examinations of stained slides from the udders of 997 B and 805 on many occasions showed polymorpho-nuclears and eosinophiles in the lumina of the alveoli. Three photographs of phase four illustrate polymorpho-nuclear cells which is opposite to the results obtained by Hewlett, Villar and Revis. The evidence which they produce is far from convincing when a careful study is made. Especially is their interpretation unusual as regards the epithelial origin of the polynuclear cells. Much more conclusive proof and evidence should be shown before their work can be accepted.

In phase five in the comparison of the bacterial counts in the fore milk, middle milk and strippings it was shown that bacterial counts were highest in the fore milk and next highest in the middle milk, the

strippings containing the smallest number. Table XVII for cow 997 B, and Table XVIII for cow 805 illustrate this.

The average number of bacterial colonies for the three milkings of cow 997 B was 863, and of cow 805, 1197. These counts are normal.

Of the thirty-five cultures stained and examined microscopically in phase six for cow 997 B, thirty-three showed micrococci, belonging mostly to the pyogenic staphylococci group, eighty-four percent of which were gram-positive. The other two preparations were gram-positive rods. Slides were prepared from twenty-five cultures from 805, stained and examined microscopically. Twenty-four of the slides examined showed micrococci most of which belonged to the pyogenic staphylococci. Ninety-two percent of these micrococci were gram positive. The other stained preparation was a gram-positive rod. No streptococci were found in a number of stained milk-smear counts for both animals.

No bacteria were noticed in the histological examinations of slides from thirty-two blocks of these two animals in phase six. This was to be expected as plate counts in phase five were low in both cases. Again, the magnification of the microscope being so high, it can be readily seen that few if any would be

encountered under the above conditions.

In phase seven a macroscopic or clinical examination of the udder and its associated lymph glands revealed no abnormalities. Histological comparisons of stained sections from sixteen blocks of a naturally infected udder (cow 805) and an apparently normal udder were made. No areas of induration, cell infiltration, nor any other abnormalities were detected in the tissues of the udder in these examinations. No organisms were detected in the tissues of this udder by histological examinations.

Several important points have been established by the failure to obtain any noticeable macroscopic or microscopic changes from the normal in this Bact. abortus infected udder, namely (1) that Bact. abortus is able to pass through or reside in the udder without causing any macroscopic or microscopic lesions or other changes, and (2) that the exact habitat of this organism has not been established by this study.

By means of photographs it was further shown that no abnormalities were present in the udder of cow 805 as compared with photographs of 997 B of the same block, number and quarter.

SUMMARY.

An increased leucocytic count in milk of udders, both artificially infected and naturally infected with Bact. abortus was followed by a decreased hydrogen ion concentration and an increased percent by volume of carbon dioxide.

Examination of samples of milk taken from udders artificially and naturally infected with Bact. abortus showed that with a decrease in hydrogen ion concentration an increased percent by volume of carbon dioxide was obtained.

The largest number of leucocytes was obtained in the strippings, the next largest in the middle milk and the smallest number in the fore milk.

The origin of cells in milk is both the blood stream and the epithelium, the majority, however, originating in the blood.

The largest number of bacteria are found in the fore milk, the next largest in the middle milk and the smallest in the strippings.

Morphological examinations of sixty cultures showed that fifty-seven, or eighty-five percent, were micrococci belonging mostly to the pyogenic staphylococci, of which eighty-eight percent were gram-positive. The other

three, or fifteen percent, were gram-positive bacteria.

Bact. abortus does not cause any histo-pathological changes in a naturally infected udder. Two important points, however, are established: (1) that Bact. abortus is able to pass through the udder without causing any macroscopic or microscopic lesions or other changes, and (2) that the exact habitat of this organism has not been established by this study.

ACKNOWLEDGMENT

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2. The second part of the document focuses on the implementation of the proposed changes. It details the steps involved in the transition process, from the initial planning phase to the final execution. This section also addresses the potential challenges and risks associated with the changes, providing strategies to mitigate them.

3. The third part of the document discusses the impact of the changes on the organization's overall performance. It presents data and analysis showing the positive effects of the changes, such as increased efficiency and cost savings. This section also highlights the importance of ongoing monitoring and evaluation to ensure the continued success of the changes.

4. The fourth part of the document provides a summary of the key findings and conclusions. It reiterates the importance of the changes and the need for continued commitment and support from all stakeholders. This section also includes recommendations for future actions and areas for further research.

5. The fifth part of the document is a conclusion that summarizes the main points of the document. It emphasizes the significance of the changes and the need for continued effort and collaboration to achieve the organization's goals. This section also includes a final statement of commitment and a call to action for all stakeholders.

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Note. Reference 112 should have been omitted.

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