

A COMPARISON OF THE MORPHOLOGIC, CULTURAL
AND BIOCHEMICAL CHARACTERISTICS OF
B. ABORTUS AND B. MELITENSIS*

STUDIES ON THE GENUS BRUCELLA NOV. GEN. I

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The problem dealing with the possible pathogenicity of *B. abortus* (Bang) to human subjects investigated in the last ten years from various points of view was placed in a new light when, in 1918, Alice E. Evans¹ demonstrated by morphologic, biochemical and serologic studies a close relationship between the organism responsible for infectious abortion of domesticated animals and the so-called *Micrococcus melitensis*, the cause of the well-known undulant or Malta, or Mediterranean fever in man. Moreover, the peculiar latency in tissues and the apparent ubero- and sexotropic character of the two organisms in cattle and goats, respectively, lend additional support to the above contention. To the bacteriologist, however, who obtains his information mainly from the meager descriptions and accounts given in the usual textbooks instead of from a comparative study of authentic cultures in vitro and in vivo, this correlation of facts appears impossible. We mention in this connection the conservative attitude of a number of English bacteriologists, who place the causative organism of Malta fever with the coccus group and fail to recognize the repeated observation that this organism may appear in smears made from young cultures and even from tissue material as a typical short rod. On the other hand, the small microbes found in some forms of infectious abortion have, since the classic studies of Bang and Stribolt,² been accepted as distinct rods which, however, may occasionally appear in exudates as a "coccobacillus." Furthermore, an analysis of the descriptions dealing with the cultural and biochemical characteristics of the two organisms under consideration reveals only differences of minor importance and adds considerable evidence to the conception of a close relationship of *B.*

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* The letter *B.* is used in this series of papers for the suggested genus *Brucella* Nov. gen. and enhances the suggestions made by A. E. Evans (Footnote 1).

¹ Jour. Infect. Dis., 1918, 22, p. 580.

² Ztschr. f. Thier. med., 1897, 1, p. 241.

abortus and "*Micrococcus*" *melitensis*. Irrespective of the fact, that Alice E. Evans supported her conclusions by the presentation of observations on several strains of the two types of bacteria, a number of bacteriologists have expressed to us their inability to accept this new conception. This in part may be due to the unfortunate mistake which Miss Evans committed in correlating *B. bronchisepticus* with *B. abortus* and with "*Micrococcus*" *melitensis*. As is well known, *B. bronchisepticus* is a motile, strongly alkali-producing rod, which is either related to the pyocyaneus group (Smith),³ or to *B. pertussis* (Ferry and Noble).⁴

In order to verify the various statements and preparatory to a number of experiments to be considered in this series of papers, we studied the morphology and biochemical reactions of 21 cultures, which had been identified by various authoritative laboratories in the United States, England, Algiers and Italy as "*Micrococcus*" *melitensis*. We included in this comparative study 32 cultures of *B. abortus* isolated in this country or in England from aborted fetuses or pathologic discharges, or milk of cattle and hogs.

All the cultures were repeatedly plated on glycerol-peptic digest agar and are kept in triplicate sets on the same medium at room temperature. The tests to be recorded have been repeated at least three times, all the strains being tested for the most part simultaneously. A selected number of strains used in the serologic tests reported in the second paper were studied more extensively after rapid transplantation on the same medium for at least 10 to 15 generations. The inoculated tubes were kept sealed with paraffin wax.

MORPHOLOGY

"*Micrococcus*" *melitensis*.—The stock cultures designated "*Micrococcus*" *melitensis*, when grown on peptic digest agar or broth with a reaction of P_H 7.2-7.4 for 24 to 36 hours at 37 C., revealed in preparations stained with gentian violet short, stumpy, oval or egg-shaped rods frequently tapered at both ends. Identical smears stained by Gram's method and counter-stained with dilute carbol-fuchsin furnished pictures in which the organisms appeared more coccoid in morphology. This observation is quite in accordance with the findings of Fabyean,⁵ made on *B. abortus*; he noted that carbol-fuchsin accentuated the diameter and gentian violet, the length. In hanging drop preparations the organisms are immotile, noncapsulated and appear

³ Jour. Med. Res., 1913, 29, p. 299.

⁴ Jour. Bacteriol., 1918, 3, p. 193.

⁵ Jour. Med. Res., 1912, 26, p. 477.

more like elongated cocci or diplococci. Frequently in the water of condensation or liquid mediums, short chains consisting of from 4 to 10 single, elongated, influenza-like bacillary or stumpy, coccoid elements can be recognized. Strain 2 produces these forms rather frequently; while strains 1, 5, 7, 18, 22 and 26 form single coccoid rods, which are evenly distributed in the stained preparations. Strains 8, 9, 20, 21 and 23 invariably appeared in young cultures as fine small rods in parallel grouping. The individual elements may stain more intensely at both ends and measure from 0.8-1.8 microns in length and from 0.4-0.6 microns in width. The forms most frequently recognized in young cultures on glycerin peptic digest agar, are illustrated in microphotographs 1-6.

It is quite evident that we are unable to recognize the interpretation of Eyre,⁶ who considers these bacillary forms to be staining artefacts. And again the finding of bacilli in 24 hour old cultures on the most suitable mediums with an optimum reaction and oxygen refutes the conception that they are involution forms. We admit, however, the occasional occurrence of a cultural growth after 12 to 18 hours' incubation on suitable solid substratums, which in carbol-fuchsin or thionin preparations consists mainly of coccoid-like elements, indistinguishable from the elements of a young culture of meningococci. A few incomplete tests suggest that definite cyclical changes in the development similar to those described for a variety of organisms by Clark and Ruehl⁷ exist also for the *B. melitensis*. A detailed study of this phase of the problem is in progress.

When stained in thin preparations the organisms of all our strains are gram negative. Repeated tests failed to demonstrate flagella by the method of von Ermengen. Our observations on the morphology of "*Micrococcus*" *melitensis* support, therefore, the finding of Durham,⁸ Galli-Valerio,⁹ Besson,¹⁰ Pollaci,¹¹ and Muir and Ritchie.¹² We therefore concur in the interpretation given by Miss Evans and demand that the generic name "*Bacterium*" be given to the causative organism of undulant or Malta fever.

⁶ Kolle and Wassermann's Handbuch d. pathog. Microorg., 1913, 4, p. 424.

⁷ Jour. Bacteriol. 1919, 4, p. 615.

⁸ Jour. Path. & Bacteriol., 1899, 5, p. 377.

⁹ Centralbl. f. Bakteriologie, I, O, 1904, 35, p. 81.

¹⁰ Practical Bacteriology, London, 1913, p. 475.

¹¹ Centralbl. f. Bakteriologie, I, Ref. 1908, 42, p. 676.

¹² Manual of Bacteriology, 7th Ed., London, 1919, p. 501.

B. abortus.—The morphologic appearance of the various strains of *B. abortus* on the same medium are similar to those of *B. melitensis*. Again, in preparations stained with gentian violet short ovoid or longer rods are demonstrated. The diphtheroid-bacilli-like grouping of the small rods and the indications of granular staining are perhaps more frequently seen in young *B. abortus* cultures, than in those of *B. melitensis*. The length varies between 0.4-2.2 microns and the width between 0.4-0.8 micron. Short chains of coccoid elements are also noted in the water of condensation of young cultures. Recently isolated strains, which are not fully adapted to the new oxygen requirements and the new substratum, appear more coccoid than old, vigorously growing stock cultures. The organisms are always distinctly gram-negative. Microphotographs 7 and 8 illustrate these observations fully (see also, Figs. 1 and 2 on Tafel II, Arb. a. d. k. Gsundhtsamte., 1912, 43, p. 129, and Kolle-Wassermann's Handb. d. pathog. Microog., 1913, 6, p. 299).

In this connection it may appear advisable to recall briefly the various statements relative to the morphologic appearance and the botanical classification of *B. abortus* published in the literature. Preisz¹³ placed the causative organism isolated by him from cases of infectious abortion on account of its irregular staining reaction and its diphtheroid-like grouping with the corynebacteria. It is, however, not unlikely that the organism described by Preisz is not identical with the bacillus of Bang.¹⁴ According to Novak,¹⁵ *B. abortus* resembles the coccobacillus of chicken cholera, and it is therefore grouped with the pasteurella or hemorrhagic septicemia bacilli. Holth¹⁶ considers the organism on "ausgesprochener Kokkobazillus," and Zwick and Zeller¹⁷ noted several strains which possessed a "fast kokken-ähnliches Aussehen," which in turn resembled by dark-field illumination the bipolar bacteria of fowl cholera or swine plague. Fabyean¹⁸ states that "there is some variation in length which in some individuals may be equalled by the diameter, this type suggests a coccus." Our personal observations are therefore fully corroborated by the findings made by other workers. We found it impossible to distinguish *B. melitensis* from *B. abortus* when using cultures with fictitious labels prepared from our stock sets, irrespective of the fact that our constant working with the strains should have impressed on our mind the essential differentiating characteristics. On morphologic grounds the organisms of undulant fever and of infectious abortion of domesticated animals must therefore be considered as identical and must be placed together in the genus bacterium. For reasons to be given in detail in the second paper it is proposed in accordance with the suggestions made by Buchanan¹⁹ of the Committee on Classifications of the Society of American Bacteriologists, that a genus,

¹³ Centralbl. f. Bakteriologie, I, O, 1903, 33, p. 190.

¹⁴ Zwick and Zeller, Footnote 17, p. 5.

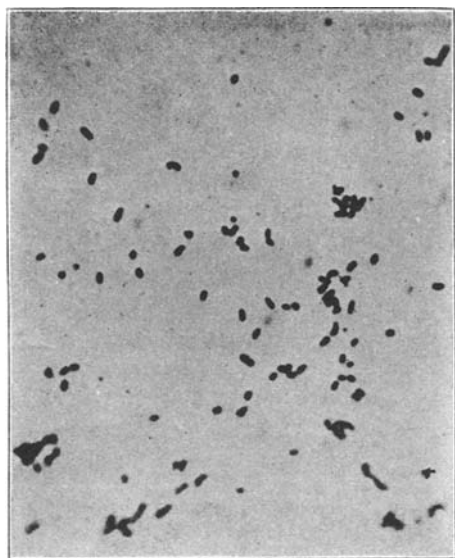
¹⁵ Ann. de l'Inst. Pasteur, 1908, 22, p. 541.

¹⁶ Ztschn. f. Infektionskrankh. parasitärkrankh. u. Hyg. d. Haustiere, 1911, 10, p. 208.

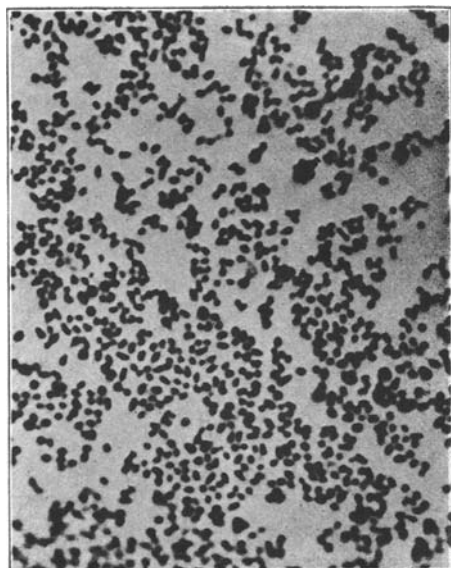
¹⁷ Arb. a. d. Gsundhtsamte, 1912, 43, p. 11.

¹⁸ Jour. Med. Res., 1912, 26, p. 476.

¹⁹ Abstracts Bacteriol., 1918, 2, p. 8.



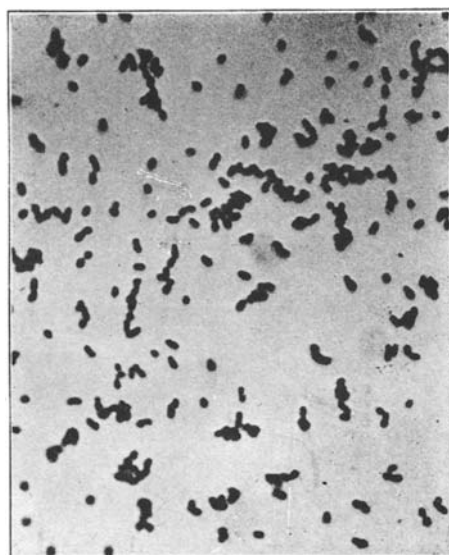
Strain 7.



Strain 1.

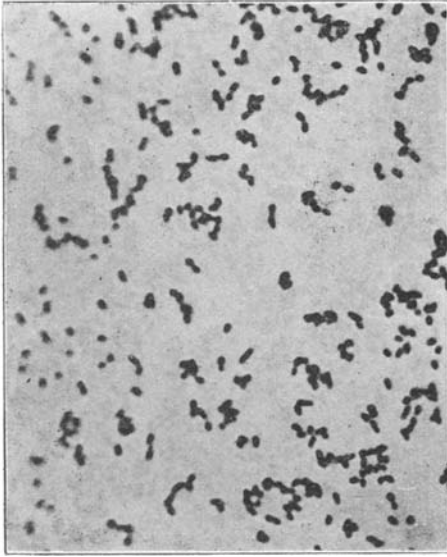


Strain 22.



Strain 18.

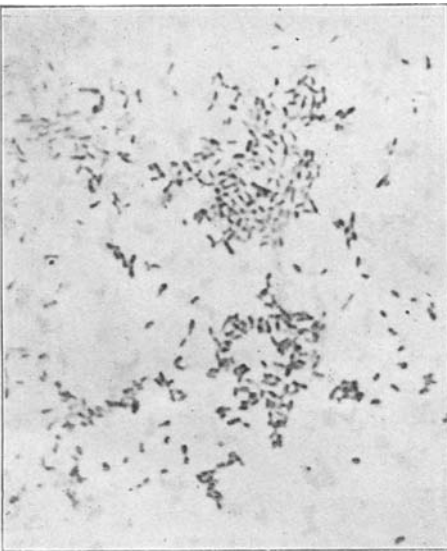
Fig. 1.—*B. melitensis*, $\times 1,500$.



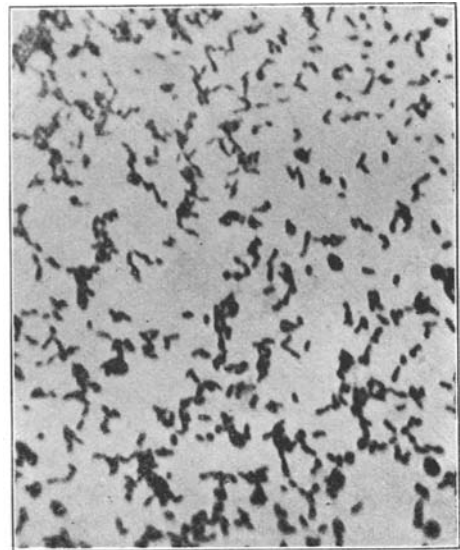
B. melitensis strain 26.



B. melitensis 20.



B. abortus strain 80.



B. abortus 12.

Fig. 2.—*B. melitensis* and *B. abortus*; $\times 1,500$.

for which we propose the name "*Brucella*," be created in the family Bacteriaceae to separate and to distinguish properly these important pathogenic micro-organisms from the other members of the recognized genus bacterium, which is already overburdened with representatives, which have little in common with the *B. melitensis* and *B. abortus*.

CULTURAL CHARACTERISTICS

The descriptions given by Horrocks,²⁰ Eyre,⁶ and others for the growth of *B. melitensis* and by Bang and Stribolt,² Holth,¹⁶ Zwick and Zeller,¹⁷ and Fabyean,⁵ for that of *B. abortus* on various solid and liquid mediums are fully in accord with our own observations, and it is unnecessary to duplicate the recital of facts. We intend to point out chiefly the slight differences which can be noted and perhaps be chosen to separate on cultural grounds the *B. melitensis* from the *B. abortus*.

It is well known that both organisms grow slowly, even on suitable medium visible growth is not recorded before 36-48 hours' incubation at 37 C. It is furthermore established that primary isolation of *B. abortus* from pathologic material on glycerol or serum agar can only be accomplished successfully by either reducing (Bang and Stribolt, Novak and also Fabyean) or increasing (Holth) the oxygen tension of the atmosphere, in which the organism is placed for artificial cultivation. A similar adaptation for *B. melitensis* has not been reported in the literature. It is not unlikely that the usual isolation of this organism from the blood stream of patients in liquid medium or the milk of goats explains the difference. The peculiar adaptation phenomenon of *B. abortus* to varying oxygen tensions, when first isolated from amniotic fluid or uterine material in general is probably merely the result of its intra-uterine existence as clearly demonstrated by McFadyean and Stockman²¹ and also by Holth. Through the observations of Schroeder and Cotton,²² A. S. Evans²³ and Steck,²⁴ who isolated the *B. abortus* directly from milk in ordinary petri-dishes, even in gelatin medium, and the fact that even prolonged sojourn of the organisms in guinea-pig tissues does not confer this adaptation phenomenon again, it is clearly indicated that the adjustment to definite oxygen tensions is primarily a characteristic for the organism living in the uterine cavity. As we had no access to actual cases of undulant

²⁰ Report of the Commission on Mediterranean Fever, London, 1905, Part 1, p. 5.

²¹ Report of Departmental Committee on Epizootic Abortion, Append. to Part I, Lond. 1909, p. 25.

²² Twenty-Eighth Annual Report of the Bureau of Animal Industry, Dept. of Agric. 1911, p. 139.

²³ Jour. Infect. Dis., 1916, 18, p. 437.

²⁴ Schweizer Arch. f. Tierheilk., 1918, 60, p. 547.

fever, we are unable to express an opinion concerning the adaptation of *B. melitensis* to varying oxygen tensions. We noted, however, repeatedly that cultures made from guinea-pig spleens, which had been injected with various *B. melitensis* strains 3 to 4 months previous to the time of necropsy, gave only growth in paraffin sealed blood or glycerin peptic digest agar tubes. Plain agar plates or veal infusion agar slants loosely closed with cotton plugs frequently remained sterile. Some tests indicated that the composition of the medium influences considerably the chances of the primary isolation of *B. abortus* and *B. melitensis*. Our pig or beef-liver peptic digest agar²⁵ is admirably suited for this work. Dr. J. Traum of the University of California isolated on this medium in open unsealed slants *B. abortus* directly from the amniotic fluid in the stomach and from the liver of aborted pig's fetuses. The use of "nutrose," "somatose" and beef serum by English experts on Malta fever is repeatedly recorded, and it is not unlikely that these "growth accessory" substances helped in the primary isolation of *B. melitensis* and acted in a similar manner to our digest agar. The majority of our strains were naturally stock cultures, grew therefore abundantly on all culture mediums and appeared fully accustomed to saprophytic life. The three paramelitensis strains 9, 22 and 23 and strain 18 were shy growers when received and even repeated subculturing only slightly enhanced the cultural vigor in comparison with the other strains. We found the optimum reaction of the medium to be a H-ion concentration of P_H 7.2-7.4. Both types of organisms are slightly more alkali than acid tolerant.

The growth on glycerol peptic digest agar or hormone blood-agar plates appears at 37° C. in form of small convex, glistening, pearly-white, droplet-like colonies, which may develop into colonies of from 2-8 millimeters in diameter. The paramelitensis strains 22 and 23 produced sometimes rather granular, dull, comparatively flat colonies; this phenomenon was particularly marked on dry plates. *B. abortus* colonies cannot be distinguished from those of *B. melitensis*.

On agar slants a fine granular film appears in from 24-36 hours; after 3 days a slight brownish tinge changes the moist, well defined growth. Development continues on peptic digest agar for weeks, even at room temperature, until a stringy, greasy, rather thick layer covers the inoculated agar surface. This growth remains amber or honey-like yellow or perhaps caramel-like brownish (see Fig. 1, Plate 28, twenty-

²⁵ Stickel and Meyer, Jour. Infect. Dis., 1918, 23, p. 68.

eighth annual report of the Bureau A, U. S. Dept. Agri.), for *B. abortus* strains even after six weeks' incubation. *B. melitensis* strains 1, 2, 3, 5, 7, 8, 19, 20, 21, 23 and 24, however, changed their growth to a deep chocolate or dirty chestnut brown, some even to a dull ebony black. This pigmentation of the bacterial layer is usually more marked at the upper portion of the slant and may be accompanied by a slight or pronounced darkening of the agar substratum. Intensive dark pigmentation is regularly observed with the cultures of *B. melitensis* mentioned and differentiates these strains distinctly from all our *B. abortus* strains. The time of incubation to produce this pigmentation is not constant and may vary from 8 to 30 days. It is, however, emphasized that a number of *B. melitensis* cultures (4, 6, 9, 10, 11, 18, 25, and 27), which must be classified serologically as typical strains, have failed to produce a darker pigment than on *B. abortus* cultures and differ therefore in no respect from the latter. Crystals, which are probably due to the increasing alkalinity of the medium, were observed only after two weeks' incubation in our digest agar mediums. In veal infusion agar they may appear on the sixth to tenth day of incubation. Agar shake cultures of all strains fail to show a zone of growth as mentioned by a number of writers; there is a thick growth on the surface, which may also extend slightly beneath the surface.

In gelatin, all our strongly pigment-producing *B. melitensis* strains developed dark brownish granular colonies after incubation of from 10 to 30 days. The *B. abortus* strains acted similarly. The medium was never liquefied.

In veal infusion or digest broth a slight initial turbidity, which is followed by a gradual clearing and by a stringy, tenacious sediment, occasionally with a slight pellicle or ring formation, was noted for all the strains studied after 5 to 10 days' incubation. Our *B. melitensis* strains 9, 18, 22, 33, and *B. abortus* cultures 5, 12, 13, and 19 produced a scaly, powdery sediment with little or no turbidity of the supernatant broth medium.

Cultures on potatoes may give varying results, depending on the age of the tuber and its reaction. On properly chosen, slightly alkaline, moist potato-cylinders the behavior of the majority of our *B. melitensis* strains is in some respect characteristic. Inoculated from a broth culture or the water of condensation, the visible growth was always distinctly amber yellowish or even brownish after 5 to 6 days' incubation. The following strains behaved in this manner: 1, 2, 3, 4, 5, 6, 7,

9, 10, 11, 19, 20, 21, 22, 23, 24, 25, 26 and 27. *B. abortus* strains, however, cultivated simultaneously on the same medium and in the same manner, showed only a faintly yellowish hue. After 3 to 4 weeks of incubation, they may show the well-known glanders bacillus-like appearance (McFadyean and Stockman²⁶). At this period the *B. melitensis* strains mentioned are already deep brownish. Very old *melitensis* cultures demonstrate a more intense pigmentation of the bacterial growth and marked brownish discoloration of the potato itself in contrast to the generally light coloring of that of *B. abortus*. Variations in the shading of the color among the latter strains are not uncommon and again the *B. melitensis* cultures 8 and 18 behaved, when repeatedly tested on potatoes, like the *B. abortus* strains.

Bromcresol purple goat's milk in fermentation tubes is turned slightly alkaline after 5 to 10 days' incubation at 37 C. in the open arm by all the strains tested. The H-ion concentration decreases from P_H 6.6-7.2-7.4. Litmus milk remains unchanged or turns slightly deeper blue in the open arm. Fresh goat's milk with a layer of cream and bromcresol purple as an indicator shows no visible changes even when incubated for three months. In goat's milk litmus whey, the titrable alkalinity of both the *B. melitensis* and *B. abortus* strains varies after 10 days' incubation between 0.2 and 0.5 per cent. of a normal HCl solution. The differences in the final reaction are merely the result of differences in the rate of multiplication of the various strains. Poorly growing *B. melitensis* and *B. abortus* strains produce a small amount of alkali. The absence of changes recorded in the sterile goat's milk stratified with the cream suggests that the alkaline reaction is caused primarily by the oxidation of the salts of citric acid to alkaline carbonates as recently discussed by Ayers and Rupp.²⁷

BIOCHEMICAL REACTIONS

Hiss' serum-peptone-pheno-sulphonephthalein-water, containing 1 per cent. of levulose, galactose, maltose, saccharose, raffinose, mannite, dulcitol or inulin are not fermented by the representatives of the genus "*Brucella*." In glucose and lactose-peptone-phosphate-broth an alkaline reaction develops after 5 to 20 days' incubation at 37 C. The H-ion concentration decreases from P_H 6.8 to 7.6 and to 7.8. This reduction, already observed by Eyre and enhanced by Evans, is con-

²⁶ Report of Departmental Committee on Epizootic Abortion, Append. to Part I, London, 1909, p. 4.

²⁷ Jour. Infec. Dis., 1918, 23, p. 188.

stant for all our strains, when final determinations are made after the twentieth day of incubation. Vigorously growing strains as a rule produce this alkaline reaction in a shorter time interval than the poorly growing types (for example *B. melitensis* 18, 22, 23 and the *B. abortus* 3, 10, 15, etc.). Irrespective of the initial H-ion concentration, 16 strains of the 21 *B. melitensis* studied produced a reduction equal to a P_H of 0.6-0.8, two strains of 0.9, one of 1.0 and two of 0.5. Of 20 *B. abortus* cultures tested, the reduction was: 18 a P_H of 0.7-0.8, and two of 0.9. This important and characteristic reaction emphasized by Miss Evans is therefore confirmed by our tests.

Indol is not produced in Difco-peptone solutions by any of our strains. Only *B. melitensis* strains 10 and 24 and *B. abortus* 80, 8, 32, 33 and 38 gave reactions in nitrate broth, which could be interpreted as indicating the presence of nitrites. Neither Horrocks nor Eyre for *B. melitensis* nor Fabyean for *B. abortus* were able to demonstrate a true reduction of nitrates to nitrites.

Neutral red and lead acetate agar give a slight growth with absence of a reduction of the dye or the chemical.

Following the suggestion of Miss Evans, our cultures were also tested for the production of ammonia in asparagin and urea containing mediums. All our strains of *B. melitensis* and *B. abortus* decomposed urea. *B. melitensis* strains 7, 9, 10 and 24 and *B. abortus* 80, 10, 34 and 40 produced a marked amount of ammonia, about equal to one mgm. in 20 c c of medium. On the other hand, the decomposition of asparagin was irregular and in comparison with the one in urea rather slight for most of the *B. melitensis* strains. In the only complete series in which all the actively growing *B. abortus* strains were tested simultaneously either no reaction or indefinite changes were recorded with Nessler's reagent. The following *B. melitensis* strains decomposed asparagin and gave a distinct ammonia reaction: *B. melitensis* 7, 9, 11, 23, 24, 25 and 26. Even vigorously growing strains of the genus "*Brucella*" may therefore fail to register ammonia production in asparagin solutions.

The viability of the cultures of *B. abortus* and *B. melitensis* in sealed tubes protected from desiccation and kept at a uniform temperature (18-22 C.) Eyre,⁶ Mohler, and Traum²⁸ is well known. We were successful in obtaining viable cultures from agar slants of all the

²⁸ Twenty-Eighth Annual Report of the Bureau of Animal Industry, Department of Agriculture, 1911. p. 154.

strains which had remained unopened at room temperature for 6 and 10 months, respectively, after inoculation.

CONCLUSIONS

A comparative study of 21 cultures of so-called "Micrococcus" *melitensis* obtained from various sections of the world and of 32 cultures of *B. abortus* (Bang) isolated in this country and England justifies the following conclusions:

The causative organism of undulant fever of man and of Malta fever of goats cannot be distinguished morphologically or biochemically from the organism responsible for infectious abortion in domesticated animals.

So-called "Micrococcus" *melitensis* appears in young cultures as a short rod and should therefore be designated as *Bacterium melitensis*.

The pigment production of the majority of actively growing *B. melitensis* strains on glycerol peptic digest agar and on alkaline potato cylinders after 5 days' incubation is more intense than with the strains of *B. abortus*.

Both *B. melitensis* and *B. abortus* cultures produce after 20 days' incubation in glucose and lactose broth an alkaline reaction and a characteristic reduction of the H-ion concentration equal to about 0.6 to 1.0 P_H.