THE CLASSIFICATION OF THE BRUCELLA GROUP: A SYSTEMATIC STUDY.

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THE purpose of the present paper is twofold: firstly to assess the value of the various methods available for the differentiation of members of the Brucella group; and secondly to point out that, though it is possible to distinguish broadly between three main types within the group, there is no hard and fast line of demarcation between them, and the existence of transitional forms is sufficiently frequent to suggest that specific characters are subject to change with environmental conditions. These conclusions are based on a study by a number of different methods of rather over 300 strains collected from different parts of the world.

SOURCE OF THE STRAINS.

For reasons that will be discussed later it is convenient to divide the strains according to their source of origin into two groups. The first, which may be referred to as the main group, includes strains from many different parts of the world; the second, which may be referred to as the Lisbonne-Taylor group, comprises strains that were isolated mainly from human cases of undulant fever in the south-east, east, and north-east of France, and that were very kindly sent me from Montpellier by Prof. Marcel Lisbonne and Dr R. M. Taylor.

MAIN GROUP OF STRAINS.

The strains of the main group are listed according to their final classification, not according to the label with which they were received.

Type	Host	Country of origin	No.
Bovine abortus	Human (32), bovine (34), canine (1), equine (5), caprine (1), and unknown (18)	Great Britain and Ireland, Germany, Denmark, Holland, Switzerland, France (North and Centre), Tunis, U.S.A., and Southern Rhodesia	73
		Unknown	18
Porcine abortus	Human (1), and porcine (13)	Denmark and U.S.A.	14
Melitensis	Human (30), caprine (3), and unknown (10)	England, Malta, Tunis, Palestine, India, South Africa, and Peru	33
		Unknown	10
Para-abortus	Human (1), bovine (2), and equine (2)	Ireland, Holland, and Palestine	5
Para-melitensis	Human (3), caprine (4), and unknown (5)	Malta, Tunis, and Palestine	7
		Unknown	5
		Total	165

TECHNIQUE OF EXAMINATION OF THE MAIN GROUP OF STRAINS WITH THE RESULTS OBTAINED.

Morphology.

The organisms were grown on liver agar for 2–3 days at 37° C. Films were heat-fixed, and stained by dilute carbol-fuchsin. Measurements were at first made under very careful conditions with an eye-piece micrometer, but it soon became apparent that not only was the differentiation of individual strains of the various types impossible by this method, but that within any given strain there was often a wide variation in the shape and size of the organisms. The strains have therefore been grouped (Table I) according to the predominant morphological appearance presented.

Table I. *Predominant morphological appearances presented.*

Type	No. of strains examined	Cocco-bacilli	Mainly coccal forms	Mainly rod forms
Bovine abortus	63	28	6	29
Porcine abortus	13	11	1	1
Melitensis	31	26	3	2
Para-abortus	5	1	4	0
Paramelitensis	12	10	0	2
Total	124	76	14	34

It will be observed that cocco-bacilli, coccal forms, and rod forms may be predominant in both abortus and melitensis strains. While, however, about 46 per cent. of bovine abortus strains consisted mainly of rod forms, only about 6 per cent. of melitensis strains did so. The general conclusion appears to be that, though on the average bovine abortus strains are rather longer than melitensis strains, no reliance can be placed on this criterion in the differentiation of individual strains.

Abundance of growth in culture.

Many statements have been made about the relative abundance of growth of the different types of Brucella on artificial media. In our experience not very much weight can be attributed to this criterion. In each type there are strains that grow fairly rapidly and abundantly, and again there are strains whose development is slower and less pronounced. From any large collection it is not difficult to pick out strains with individual peculiarities. We have observed, however, that the Danish porcine strains grow more poorly than the American porcine strains. Since Kristensen (1931) has made the same observation, it is probable that this characteristic is more or less constant. With this exception it is doubtful whether generalisations on the degree of growth are likely to be of help in type differentiation.

Pigmentation.

The development of a brown colour in agar cultures after prolonged incubation is sometimes considered as characteristic of melitensis strains, and the findings of Kristensen (1931) tend to support this view.

Liver agar, owing to its natural brown colour, is not very suitable for the study of this pigmentation. In our work, therefore, heart extract agar slopes were used, and the cultures were incubated for 5 weeks at 37° C. Bovine abortus strains were given a preliminary incubation in 5 per cent. CO_2, before being transferred to the aerobic incubator. The results are given in Table II.

Table II. *Pigmentation of heart extract agar cultures after incubation for 5 weeks at 37° C.*

Type	No. of strains studied	Pigmentation	
		None, or very slight	Slight, or moderate
Bovine abortus	28	24	4
Porcine abortus	13	12	1
Melitensis	24	23	1
Para-abortus	2	2	0
Paramelitensis	10	3	7
Total	77	64	13

It will be observed that only thirteen out of seventy-seven Brucella strains gave rise to any definite coloration, and of these no fewer than seven belonged to the paramelitensis type. Whether this finding is fortuitous, or whether paramelitensis strains do in fact give rise to a brown coloration more frequently than strains of other types, it is impossible to say, but there is no evidence to suggest that a study of this property is likely to afford any help in the differentiation of melitensis from abortus strains.

Crystal formation.

Huddleson, Hasley and Torrey (1927) and Huddleson and Winter (1927) have observed the development of crystals of ammonium magnesium phosphate in cultures of Brucella on liver agar incubated aerobically, but not in 5–10 per cent. CO_2. They regard their formation as being due to the production of ammonia by the growing organisms, and to its combination with the magnesium phosphate in the medium. In their experience the crystals are formed much more rapidly by melitensis and paramelitensis than by abortus strains.

In an attempt to confirm their observations, cultures on liver agar were incubated aerobically for 18 days at 37° C., and in an increased pressure of CO_2. The results are shown in Table III.

No crystals appeared in the cultures incubated under increased CO_2 pressure, while in cultures incubated aerobically about a quarter of the strains formed crystals. The proportion of melitensis and paramelitensis strains forming crystals was higher than that of abortus and para-abortus

Table III. *Formation of crystals in liver agar cultures incubated aerobically for* 18 *days at* 37° *C.*

Type	No. of strains studied	No. forming crystals	Remarks on strains forming crystals
Bovine abortus	61	10	Mostly old strains of unknown origin
Porcine abortus	13	3	Strains of American origin only
Melitensis	31	10	Strains of varied origin
Para-abortus	4	1	Strain from Palestine
Paramelitensis	11	7	Strains of varied origin
Total	120	31	

strains, but the differences were not sufficient to be of value in the identification of individual strains.

The exact mechanism responsible for the formation of crystals is not very clear. In another experiment in which seventy-seven of the same strains were incubated aerobically in liver agar cultures for 5 weeks at 37° C., not a single strain gave rise to crystals. Possibly the magnesium phosphate content of the medium is important, and small variations may be sufficient to affect the deposition of crystals.

Production of alkali in peptone water.

Assuming the correctness of Huddleson, Hasley and Torrey's (1927) observations on the rate of ammonia production by melitensis and paramelitensis strains, observations were made on the hydrogen-ion concentration of 1 per cent. peptone (Witte) water cultures after aerobic incubation for 1 week at 37° C. Measurements were made by the colorimetric method, using phenol red. The initial reaction of the medium was *p*H 7·0. The results are given in Table IV.

Table IV. *Hydrogen-ion concentration of* 1 *per cent. peptone water cultures,* p*H* 7·0, *after aerobic incubation for* 1 *week at* 37° *C.*

Type	Total no. of strains	No. of strains not growing	No. of strains that grew	Average *p*H of cultures in which growth took place
Bovine abortus	51	9	42	7·42
Porcine abortus				
American	8	0	8	7·75
Danish	5	1	4	7·15
Melitensis	31	11	20	7·29
Para-abortus	2	0	2	7·45
Paramelitensis	11	2	9	7·31
Total	108	23	85	

It is seen that the greatest production of alkali occurred in the American porcine abortus cultures, and the least in the melitensis and Danish porcine abortus cultures. The height of the *p*H seemed to be related to the abundance of growth. The American porcine strains, for example, grew quite well, and gave rise to a *p*H of 7·6–8·0; the Danish porcine strains, on the other hand, grew very poorly or not at all, and hardly altered the *p*H. Apart from this

difference, the estimation of changes in hydrogen-ion concentration does not appear to hold out much hope for differentiation of the types.

Glucose utilisation test.

McAlpine and Slanetz (1928 *a*) found that the Brucella group could be divided into two sub-groups on the basis of sugar utilisation. If grown in 1 per cent. glucose peptone water for 7 days, melitensis and porcine abortus strains are stated to have utilised 4–18 per cent. of the sugar, while bovine abortus strains did not use more than 2 per cent. The majority of workers (Kristensen and Holm, 1929; Meyer and Eddie, 1930; Zeller, 1931; Kristensen, 1931; Olitzki and Bromberg, 1931; Zobell and Meyer, 1932 *a*) have failed to obtain clear-cut results with this test. Olitzki and Bromberg (1931) brought evidence to show that the degree of glucose utilisation was partly dependent on the concentration of peptone in the medium. McAlpine himself (McAlpine, Plastridge and Brigham, 1929) has moreover shown that the ability of melitensis and porcine abortus strains to break down glucose may be lost after long cultivation on certain laboratory media, though later (Plastridge and McAlpine, 1930) it was found that some strains, as the result of passage in liver broth, developed a mucoid variant which was even more active in utilising glucose than the original parent form.

In our experimental work tubes containing 5 c.c. of medium made up with 1 per cent. Witte peptone and 1 per cent. glucose with 0·5 sodium chloride were inoculated with a large loopful of a thick broth suspension of growth off a liver agar slope, and incubated aerobically for 7 days at 37° C. The initial reaction of the medium was *p*H 6·6. The final reaction was tested colorimetrically either by phenol red, brom-thymol blue, or brom-cresol purple. The results are shown in Table V.

Table V. *Hydrogen-ion concentration of* 1 *per cent. glucose peptone water,* p*H* 6·6, *after aerobic incubation for* 1 *week at* 37° *C.*

Type	No. of strains examined	Average *p*H
Bovine abortus	52	6·92
Porcine abortus	13	7·09
Melitensis	31	7·08
Para-abortus	2	7·3
Paramelitensis	11	7·11
Total	109	

Using this method there appears to be remarkably little difference, on an average, between the various types. With few exceptions the final reaction of every culture was more alkaline than at the commencement of incubation. A striking exception, however, was afforded by some, though not by all, of the Southern Rhodesian bovine abortus strains, which reduced the *p*H to between 5·5 and 6·0. Marked alkali production was rarely seen, and the highest *p*H observed was 7·4; this reaction was given by one Southern

Rhodesian bovine abortus, one American porcine abortus, one Palestine melitensis, and one Tunisian paramelitensis strain. These results hold out little hope that the glucose utilisation test is likely to be of much value in the typing of individual strains of the Brucella group.

Sensitivity to carbon dioxide.

The fact that *Br. abortus* could not be isolated on media incubated under ordinary aerobic conditions, but would grow on the surface of the medium if the tubes were sealed, or some distance below the surface in a shake agar medium exposed to the air, led the earlier workers to conclude that this organism required for growth a lower pressure of oxygen than that in the normal atmosphere. Huddleson (1921), however, brought evidence to suggest that carbon dioxide played an important part in the atmospheric requirements of *Br. abortus*, and Wilson (1931 *a*) described a number of experiments from the results of which he concluded that a lowered pressure of oxygen was not specially favourable for growth, but that the best development occurred in an atmosphere containing 21 per cent. oxygen and 5–10 per cent. CO_2. In other papers Wilson (1930, 1931 *b*) was able to show how the conclusions of earlier workers as to the part played by oxygen were probably erroneous, and to explain that all their diverse observations were reconcilable on the assumption that both oxygen and CO_2 were required for the development of bovine abortus strains.

The peculiar CO_2 sensitivity of the bovine type is of some value in identification, since both the porcine abortus and the melitensis types grow aerobically. It is limited, however, by the fact that after sub-culture in the laboratory many bovine strains adapt themselves to aerobic conditions, even though other strains may remain CO_2 sensitive for years.

It has been stated by McAlpine and Slanetz (1928 *b*) that, even when bovine strains have become accustomed to growth in air, they can be differentiated from abortus strains of human and porcine origin and from melitensis strains by their reaction to 10 per cent. CO_2, which stimulates the growth of the bovine, while inhibiting the growth of the other strains.

To test the truth of this statement a number of strains of different types were inoculated very lightly on to duplicate liver agar slopes, one set of which was incubated aerobically, the other in air, 10 per cent. of which had been displaced by CO_2. The resultant growth was estimated after 2 and 3 days. The results are shown in Table VI.

It will be noticed that the effect of 10 per cent. CO_2 on growth varied not only with different types, but with different strains of the same type.

While it is true that the majority of bovine abortus strains grew better in 10 per cent. CO_2, thirteen out of the fifty-two strains examined grew equally well in air, while ten actually grew better in air than in the presence of added CO_2. Most of this last group were old strains of unknown origin, and it is noteworthy that five out of the ten did not produce H_2S. Seventeen of the

bovine type strains were of human origin; of these, twelve grew better in the presence of 10 per cent. CO_2 than in air.

Table VI. *Comparison between amount of growth in air and in 10 per cent. CO_2, after 2–3 days' incubation at 37° C.*

Type	No. of strains examined	Growth: Only in air	Better in air	Equally in air and in 10 % CO_2	Better in 10 % CO_2	Only in 10 % CO_2
Bovine abortus	52	0	10	13	19	10
Porcine abortus	13	1	9	3	0	0
Melitensis	31	0	6	12	13	0
Para-abortus	2	0	0	2	0	0
Paramelitensis	11	0	3	3	5	0
Total	109	1	28	33	37	10

The porcine abortus type seemed to develop better in air than in CO_2, and one strain—of Danish origin—failed to grow at all in 10 per cent. CO_2.

The melitensis type was more variable. Six of the thirty-one strains grew better in air, twelve grew equally well in air and in CO_2, while thirteen were definitely favoured by the presence of 10 per cent. CO_2. A similar behaviour was noticeable with the paramelitensis strains. The melitensis strains whose growth was improved by 10 per cent. CO_2 were of varied origin, though most of them were isolated from human cases of undulant fever in Palestine.

The statement of McAlpine and Slanetz (1928 *b*), that 10 per cent. CO_2 favours the development of the bovine abortus, but inhibits that of the human and porcine abortus and of the melitensis types, while true for a limited number of strains, is certainly not applicable to all strains. Their generalisation is, in fact, altogether too wide, and is definitely misleading.

Since the presence of 10 per cent. CO_2 failed to differentiate between many bovine abortus and melitensis strains, another experiment was performed in which the proportion of added CO_2 was increased to 40 per cent. In this experiment nine bovine abortus strains were added, most of which had been isolated within the preceding 12 months. The results are given in Table VII.

Table VII. *Comparison between amount of growth in air and in 40 per cent. CO_2 after 2–3 days' incubation at 37° C.*

Type	No. of strains examined	Growth: Only in air	Better in air	Equally in air and in 40 % CO_2	Better in 40 % CO_2	Only in 40 % CO_2
Bovine abortus	61	8	28	8	5	12
Porcine abortus	13	4	5	4	0	0
Melitensis	31	3	28	0	0	0
Para-abortus	4	0	0	2	2	0
Paramelitensis	11	3	6	2	0	0
Total	120	18	67	16	7	12

A few remarks may be made. The eight bovine abortus strains growing only in air had all been isolated many years ago; three of them came from

Southern Rhodesia, where, according to Bevan (1930), bovine strains do not require added CO_2—even for primary isolation.

The four porcine abortus strains growing only in air all came from Denmark.

The three melitensis and three paramelitensis strains growing only in air were of miscellaneous origin.

It will be noted that, in contradistinction to the results obtained with 10 per cent. CO_2, no melitensis or paramelitensis strains were favoured by the presence of 40 per cent. CO_2; with only two exceptions they grew definitely better in air.

From these experiments certain limited conclusions are justifiable.

(1) Some bovine abortus strains, particularly those isolated for many years, grow better in air than in 10 per cent. CO_2, and a few may be completely inhibited by 40 per cent. CO_2. The majority of fairly recently isolated strains, however, are definitely favoured in their growth by 10 per cent. CO_2, and some of them will grow only in the presence of added CO_2. Strains growing in 10–40 per cent. CO_2, but not in air, are certainly of the bovine abortus type.

(2) The growth of porcine strains is not improved by the addition of 10–40 per cent. CO_2. Growth is always equally good, and often better, in air. The presence of 10–40 per cent. CO_2 not infrequently inhibits, sometimes completely, the development of porcine strains, particularly those of Danish origin.

(3) The growth of many strains of melitensis and paramelitensis is favoured by the addition of 10 per cent. CO_2, though all of them will grow in air. The presence of 40 per cent. CO_2 almost invariably inhibits their growth to some extent, though rarely completely.

(4) In general it may be stated that, while the inhibition of growth of a given strain by 40 per cent. CO_2 cannot be considered of differential value, the fact that growth in 40 per cent. CO_2 is as good as, or better than, that in air is definitely in favour of its being of bovine abortus type. The occurrence of growth in 10–40 per cent. CO_2, but not in air, almost certainly indicates that the strain belongs to the bovine abortus type.

Hydrogen sulphide formation.

Tests for the production of H_2S were made according to the method recommended by Huddleson (1929). A liver agar slope, *p*H 6·6, was inoculated fairly heavily with the strain to be tested. A piece of filter-paper that had been soaked in 10 per cent. lead acetate solution and subsequently allowed to dry, was inserted between the cotton-wool plug and the test-tube. All but CO_2 sensitive strains were incubated aerobically. Readings of the amount of blackening that occurred were taken after 1, 2, 3 and 4 days, a fresh strip of paper being used to replace the old after each reading. The results are given in Table VIII.

Table VIII. *H_2S production.*

(Production of H_2S for more than 24 hours constitutes a positive reaction.)

Type	No. of strains examined	No. producing H_2S	Percentage producing H_2S	Remarks
Bovine abortus	91	78	86	—
Porcine abortus				
American	9	9	(90–100)	1 strain inconstant
Danish	5	0	0	—
Melitensis	43	0	0	—
Para-abortus	5	5	(100)	—
Paramelitensis	12	0	0	—
Total	165	92		

A positive result has been taken as one in which H_2S was produced for longer than 24 hours. A few melitensis strains and one paramelitensis strain gave off a trace of H_2S during the first day, but during the next 2–3 days no trace of blackening was noticeable. On the other hand, the majority of abortus strains produced H_2S every day for 4 days, the usual amount of blackening being of the order of 1–4 mm., though sometimes it reached 10 or 12 mm. on 1 or more days.

Huddleson (1929) stated that if a given strain produced H_2S to a considerable degree for a period of 4 days, it belonged to the porcine species; if it produced it for only 2 days, it belonged to the bovine species; while if no H_2S was produced, it belonged to the melitensis species. These conclusions are not altogether borne out by the results recorded in Table VIII. It is to be noted that a small proportion of bovine abortus strains, comprising mostly those isolated several years ago, produced no H_2S. The same held true for porcine strains of Danish origin, even when recently isolated. A comparison of H_2S-producing bovine with American porcine abortus strains showed that, though on the whole the porcine strains produced rather more blackening, a number of bovine strains produced equally as much. There was moreover little evidence to suggest that the porcine strains continued to form H_2S for a longer time than bovine strains; indeed, many bovine strains formed their maximum amount on the third and fourth days. With strains of both types the H_2S production was variable. The variation being continuous, the scatter being large, and the arithmetic means being close together, it follows that it is impossible to separate individual bovine and porcine strains by this test. A similar conclusion is reached by Favilli (1930) and by Zobell and Meyer (1932 *b*).

It is interesting to inquire into the mechanism of H_2S production. Huddleson, Hasley and Torrey (1927) are apparently of the opinion that all types of Brucella produce H_2S, but that H_2S can be evolved in the free state only if the medium remains acid. If the reaction becomes alkaline, the H_2S combines with sodium, calcium, and other metals in the medium and is fixed as a sulphide. They maintain that melitensis and paramelitensis cultures become alkaline much more rapidly than do cultures of abortus, so that the

liberation of H_2S by the former types is reduced to a minimum. If, however, the reaction is kept acid by incubating the cultures in 5–10 per cent. CO_2, they state that H_2S is liberated by melitensis and paramelitensis strains and can be detected in the usual way with lead acetate paper. In this connection it will be remembered that the same authors believed that the rapidly produced alkaline reaction in melitensis and paramelitensis cultures was due to the formation of ammonia, and that, since the ammonia frequently combined with magnesium phosphate in the medium, the appearance of crystals of magnesium ammonium phosphate occurred more rapidly in melitensis and paramelitensis than in abortus cultures.

It has already been pointed out (Table IV) that, at any rate in peptone water cultures, melitensis and paramelitensis strains do not become alkaline more rapidly than abortus cultures; in fact the reverse was observed. Nor was any great difference noticed between the frequency of crystal formation by melitensis and abortus strains (Table III). It therefore appeared necessary to ascertain whether the failure of melitensis and paramelitensis strains to evolve H_2S was determined by the reaction of the medium. Accordingly all strains were retested for H_2S production in an atmosphere of 5 per cent. CO_2. Substantially the same results were obtained as in the previous tests under aerobic conditions. A few melitensis strains produced slightly more blackening during the first day in CO_2 than they had done in air, or produced a trace of blackening where previously they had produced none. But examination showed that this was apparently due to the rather better growth during the first 24 hours of these strains in added CO_2 than in air—a fact already recorded (see Table VI). No melitensis or paramelitensis strain produced any blackening after the first day, even in 5 per cent. CO_2, and it is therefore difficult to accept the explanation put forward by Huddleson, Hasley and Torrey.

Another objection to their explanation is furnished by a study of H_2S production over a longer period than the conventional 4 days. By making observations after 6 and 12 days it was found that a few melitensis strains, though not having previously produced any free H_2S, or only a trace during the first 24 hours, formed a small quantity between the third or fourth and twelfth days, at a time presumably when the culture was more alkaline than ever.

It would appear that the blackening of lead acetate paper by abortus and not by melitensis cultures during the first 3 or 4 days of growth is more probably due to a difference in quantitative production of H_2S than to a difference in the reaction of the medium which interferes with its liberation.

Summary and conclusions on H_2S production.

(1) The majority of bovine abortus strains produce H_2S freely for 4 days; the exceptions are mostly strains that have been isolated for several years.

(2) American porcine strains produce H_2S abundantly for 4 days, Danish

porcine strains fail to produce any at all, while according to Favilli (1931) Hungarian porcine strains occupy an intermediate position in this respect.

(3) H_2S production does not afford a reliable means of differentiating between bovine and American porcine abortus strains.

(4) With the exception of a trace in the first 24 hours, no melitensis or paramelitensis strains produce H_2S during the first 3 days of growth. After this time occasional strains produce a trace between the third or fourth and twelfth days.

(5) Reasons are brought for doubting the correctness of the explanation put forward by Huddleson, Hasley and Torrey (1927) with regard to the difference in H_2S production by abortus and melitensis strains. The failure of melitensis and paramelitensis strains to produce blackening of lead acetate paper appears to be due, not so much to the fixation of the H_2S produced by sodium and calcium in an alkaline medium, as to a real quantitative difference in the production of H_2S by melitensis and abortus strains.

(6) While the failure of H_2S production by a given strain cannot be regarded as indicating that it is of melitensis, paramelitensis, or Danish porcine type, the definite production of H_2S can be considered as very strong evidence that it is of bovine or American porcine abortus type.

Dye sensitivity.

The method laid down by Huddleson (1929) was closely followed. Four dyes—thionin, basic fuchsin, methyl violet, and pyronin—all obtained for me from the National Aniline Chemical Company of New York by the kindness of Dr R. M. Taylor—were used in the examination of every strain. Many strains were tested on two or more occasions. The dyes were made up in a 0·5 or 0·25 per cent. solution in distilled water, and added to liver agar *p*H 6·6 at between 90° and 100° C. Every precaution was taken to obtain uniform distribution of the dye throughout the medium, and all glassware, as well as the dye solutions themselves, was heated to prevent precipitation occurring. Inoculations were made with one loopful of a thick suspension of the organism, made by washing off a 2-day liver agar slope culture with about 0·5 c.c. of broth. Each plate was divided into nine more or less equal areas, and on each area a different strain was inoculated. Two concentrations of each dye were employed, namely thionin, 1/30,000 and 1/60,000; basic fuchsin, 1/25,000 and 1/50,000; methyl violet, 1/50,000 and 1/100,000; and pyronin, 1/100,000 and 1/200,000. Besides these eight plates of dye medium, a control plate was inoculated to enable the degree of growth in the absence of dye to be ascertained. The plates were incubated for 3 days at 37° C., either aerobically, or, if the strains were CO_2 sensitive, in 10 per cent. CO_2. The amount of growth was estimated relatively to that on the control plate. Since considerations of space forbid the reproduction of the reactions of individual strains, a synoptic table (Table IX) has been compiled, giving the modal characteristics of the various groups.

Table IX. *Growth in the presence of dyes.*

Type	No. of strains	Thionin 1/60,000	Thionin 1/30,000	Basic fuchsin 1/50,000	Basic fuchsin 1/25,000	Methyl violet 1/100,000	Methyl violet 1/50,000	Pyronin 1/200,000	Pyronin 1/100,000	Remarks
Bovine abortus										
Typical	72	−	−	+++	++	++	+	+++	++	—
Atypical	13	+	−	+++	++	++	+	+++	++	Slightly resistant to thionin
Atypical	6	−	−	± or −	−	± or −	−	± or −	−	Unduly susceptible to all dyes
Porcine abortus										
American	9	++	+	−	−	−	−	−	−	—
Danish	5	±	−	−	−	−	−	−	−	Unduly susceptible to all dyes, but least to thionin
Melitensis										
Typical	25	+	±	++	+	++	+	++	± or −	—
Atypical	18	+ or ±	−	++	+	±	−	±	−	Unduly susceptible to thionin, methyl violet, and pyronin
Para-abortus										
Typical	3	−	−	+++	++	++	+	+++	++	—
Atypical	2	+	−	+++	++	++	+	+++	++	Slightly resistant to thionin
Paramelitensis	12	+	± or −	+++	++	++	+	++	+	—
Total	165									

+++, ++, +, ±, and − represent decreasing amounts of growth.

Before commenting on this table it is necessary to point out that a certain amount of variation of individual strains within a given group was noticeable, particularly with the melitensis type, and that occasionally a strain exhibited a peculiar reaction to one dye which was not, however, considered to be of sufficient importance to justify its being placed in a separate sub-group.

It will be noticed that the majority of bovine abortus strains completely failed to grow in the presence of thionin, but grew well on plates containing basic fuchsin, methyl violet, or pyronin.

Thirteen strains, including four from Southern Rhodesia, four from Germany, four from Great Britain, and one from Holland, were slightly resistant to thionin, generally growing on the 1/60,000, though failing to grow on the 1/30,000 dilution plate. Such a small difference might be considered unimportant, but these strains were further differentiated by the fact that all of them, with one possible exception, grew aerobically on isolation. With regard to the strains in this country exhibiting slight resistance to thionin and absence of CO_2 sensitivity, there is some evidence, which need not be discussed in this paper, that they were really vaccinal strains, being derived from the living vaccine used for protecting cattle against contagious abortion; all of them were isolated from human beings with undulant fever.

Six bovine abortus strains exhibited an unusually high susceptibility to all dyes, either completely failing to develop in the concentrations used, or growing only slightly on the weaker concentrations of basic fuchsin, methyl violet, and thionin. Three of them, the exact origin of which was unknown, could be differentiated by employing higher dilutions of dyes, when the greater susceptibility to thionin became obvious. With the three others, however, the

results with this method were unsatisfactory, practically no growth occurring even when the dyes were used in a concentration only a quarter or an eighth of that usually employed. These particular strains were of bovine origin from the north of France, and were unclassifiable by the dye method.

The American porcine strains grew quite well in the presence of thionin, but failed to grow on the basic fuchsin, methyl violet, or pyronin plates.

The Danish porcine strains had a higher level of susceptibility to all dyes, though their differential susceptibility was similar to that of the American porcine strains. That is to say, though there was no growth in the presence of 1/30,000 thionin, a slight amount of growth occurred in 1/60,000, while in the presence of the corresponding higher dilutions of the other three dyes no growth took place. The Danish porcine strains were further differentiated by their failure to produce H_2S.

The melitensis strains were by no means always easy to classify, there being a considerable amount of individual variation in the presence of different dyes. On the whole they fell into two broad groups. In the first group, comprising strains of varied origin, growth occurred fairly well in the presence of all four dyes, though growth was often inhibited completely or almost completely by the 1/30,000 dilution of thionin and the 1/100,000 dilution of pyronin. In the second group, comprising strains mainly of human origin from Palestine, growth was less abundant, and besides a slightly increased susceptibility to thionin and pyronin, there was a marked increase in susceptibility to methyl violet. Indeed, basic fuchsin was the only dye to which these strains exhibited any marked tolerance.

Only five para-abortus strains were examined. Three of them behaved like typical bovine abortus strains, while two of them, both from cattle in Palestine, showed a slight resistance to thionin.

The paramelitensis strains behaved like the main group of the melitensis strains, though they appeared to have a slightly increased resistance to pyronin.

It will be realised from the foregoing observations that the dye method of differentiating members of the Brucella group, while of very real value, is not devoid of pitfalls. Interpretation of the results obtained requires a considerable amount of experience, and on the basis of dye susceptibility alone it is often quite impossible to be certain as to which type a given strain belongs. For example, bovine abortus strains that exhibit some resistance to thionin are not always easy to differentiate from melitensis strains that are unusually susceptible to thionin, though generally the more abundant growth of the abortus strains in the presence of methyl violet and pyronin will afford assistance. Again, some bovine abortus strains and some melitensis strains exhibiting a fairly high susceptibility to all dyes may be difficult to differentiate. Individual strains of any type reacting anomalously to one or more dyes may also cause confusion. Provided, however, that care is taken in the preparation of the dye plates and in their inoculation, and that suitable

control strains of each type are put up with every batch of fresh organisms examined, it is possible by the dye method to relegate the majority of the strains to their correct type.

Huddleson (1931) has drawn attention to the ability of certain bovine abortus strains to reduce basic fuchsin in liver agar plates. While strains that were not active reducers of fuchsin were of both human and bovine origin, actively reducing strains came only from cattle. We have noticed a similar variation in the ability of different bovine abortus strains to decolorise basic fuchsin, but there has been no apparent relationship between the possession of this property and the source of origin of the strains. Many strains isolated from human beings have reduced fuchsin as actively as strains isolated from cattle. At the moment, therefore, this property appears to have no special differential value.

Mallmann and Gallo (1933), it may be noted, found that rough strains no longer exhibited the differential susceptibility to dyes of the parent forms, and insisted that classification of Brucella strains by the dye method held good only for strains of smooth type. So far, this work has not been confirmed, and the results recorded in the present paper do not seem to support it.

Summary and conclusions on the dye method of differentiating the Brucella group.

(1) By the use of the dye method it is possible to distinguish between three broad groups: (*a*) bovine abortus; (*b*) porcine abortus; (*c*) melitensis and paramelitensis. The few para-abortus strains examined have all behaved like the bovine abortus type, but it is probable that para-abortus strains of porcine origin would behave like porcine abortus strains.

(2) Besides these three main groups, a number of sub-groups have been established, containing strains differentiated from the main group by their undue susceptibility to one or more dyes.

(3) The dye method is of very real value in differentiation, and is the only certain method available for distinguishing between the bovine and porcine abortus types. It not infrequently yields results, however, that demand considerable experience in their interpretation, and it cannot always be relied upon for the correct typing of individual strains.

Thermo-agglutination.

A 48-hour growth on a liver agar slope was suspended in normal saline, and standardised to an opacity corresponding to about 8000 million *coli* per c.c. 1 c.c. was transferred to a glass tube, $13 \times 0{\cdot}8$ cm., which was then immersed in a bath of boiling water. The tube was examined after 5, 10, 15, 30, 60 and 120 minutes. The following notation was used, which, it may be noted, is slightly modified from that of Pandit and Wilson (1932).

Complete failure to show any agglutination within 2 hours	–
Agglutination in 10-120 min., never affecting more than a moderate proportion of the organisms	+
Agglutination in 5–10 min., or very marked agglutination in 2 hours	+ +
Agglutination within 5 min., or complete agglutination in 2 hours leaving a water-clear supernatant fluid	+ + +

The results are given in Table X.

Table X. *Results of thermo-agglutination test.*

Type	No. of strains examined	No agglutination	Agglutination +	+ +	+ + +
Bovine abortus	91	82	2	5	2
Porcine abortus	14	13	0	0	1
Melitensis	43	27	7	6	3
Para-abortus	5	0	0	1	4
Paramelitensis	12	0	0	0	12
Total	165	122	9	12	22

It will be observed that, while nearly all of the bovine and porcine abortus strains reacted negatively to the thermo-agglutination test, sixteen out of forty-three of the melitensis strains showed some degree of thermo-agglutinability. There seems to be little doubt that melitensis strains tend to become rough in the laboratory more rapidly than abortus strains. Two of the bovine, one of the porcine, and three of the melitensis strains showed complete agglutination on being heated in saline. In spite of this, however, it still proved possible to type these strains by the use of monospecific sera, and they have therefore been classified with the other smooth strains. All the para-abortus and paramelitensis strains showed complete or almost complete thermo-agglutination, and, as will be seen later on, failed to react with a smooth abortus or melitensis serum. They may therefore be regarded as completely rough strains.

The thermo-agglutination test appears to be the simplest and most rapid method of detecting antigenic roughness. Any strain that fails to react negatively to this test is unsuitable for the preparation of smooth antiserum or for use in routine diagnostic agglutination work.

Serological differentiation.

Wilson and Miles (1932) brought evidence, based on the study of 118 Brucella strains, that, provided absolutely smooth strains were used for the preparation both of agglutinating sera and of test suspensions, it was possible by the agglutinin-absorption method to differentiate the members of the group into abortus and melitensis types. No distinction could be drawn by this means between bovine and porcine abortus types. Reasons were advanced for concluding that both abortus and melitensis strains contained two qualitatively similar antigens having a different quantitative distribution. On this account it was essential, in order to bring out a difference between the types, to perform a strictly quantitative absorption, adjusting the absorbing dose to the titre of the serum, in such a way as to remove the minor agglutinin

completely, while still leaving a portion of the major agglutinin unaffected. By this means it was possible to produce monospecific sera which could be used for the rapid typing of unknown strains.

Since this work was recorded, Miles (1933) has carried out an antigenic analysis of a small number of Brucella strains by the optimal proportion method of agglutination, and has reached the same conclusions as to the antigenic structure of abortus and melitensis types, and their differentiation serologically, as were drawn by Wilson and Miles (1932). I, too, have re-examined the question, and have performed a fresh series of careful quantitative absorption experiments using some fresh sera and fresh strains, and have been able to confirm completely our previous work.

Recently, however, Olitzki and Gurevitch (1933), working with two melitensis and three abortus strains, have formed the opinion that, though there is a common antigen in both types, each type has in addition a specific qualitatively different antigen. While confirming the results of Wilson and Miles when formolised suspensions were used, they obtained quite different results when washed non-formolised suspensions were employed for absorption. They drew attention to Weil's work (1911) on the inhibitory effect on agglutination of dissolved substances in unwashed bacterial suspensions, and to Olitzki's work (1928) on the destructive action of formol on agglutinins. They are of the opinion that the results obtained by Wilson and Miles were vitiated by the failure of these workers to guard against errors inherent in their technique.

This criticism was so important that it demanded immediate attention, and I therefore undertook a fresh series of quantitative absorption experiments using heat-killed (60° C. for 1 hour), twice-washed, non-formolised suspensions. The results obtained were almost identical with those yielded by heat-killed, 0·25 per cent. formolised, unwashed suspensions, and the same differentiation of abortus and melitensis types was apparent (Table XI).

Table XI. *Quantitative absorption experiments showing that substantially the same results are obtained with* 0·25 *per cent. formolised unwashed and with non-formolised washed suspensions.*

		Bovine abortus serum, K 25, absorbed at 1/40 with	
Antigen	Unabsorbed	Melitensis Phillimore 0·25 % formolised, unwashed 3000 million per c.c.	Melitensis Phillimore non-formolised, twice washed 3000 million per c.c.
Abortus bovine K 25	1800	900	800
Melitensis Phillimore	900	0	0

		Melitensis serum, Phillimore, absorbed at 1/4 with	
Antigen	Unabsorbed	Abortus K 25 0·25 % formolised, unwashed 3000 million per c.c.	Abortus K 25 non-formolised, twice washed 3000 million per c.c.
Melitensis Phillimore	320	90	96
Abortus bovine K 25	180	0	0

Since, in our opinion, the antigens in the two types are qualitatively similar, it should follow that, provided a sufficiently large absorbing dose of the heterologous type in relation to the titre of the serum is used, all agglutinins should be removed. This, in contradistinction to Olitzki and Gurevitch's statements, I found to be true, independently of whether formolised unwashed or non-formolised washed suspensions were used (Table XII).

Table XII. *Quantitative absorption experiments showing that all homologous agglutinins can be removed from a serum by absorption with large doses of a non-formolised, twice washed, heterologous suspension.*

		Bovine abortus serum, K 25, absorbed at 1/40 with					
		K 25 non-formolised, twice washed			Phillimore non-formolised, twice washed		
Antigen	Unabsorbed	3000 million per c.c.	10,000 million per c.c.	100,000 million per c.c.	3000 million per c.c.	10,000 million per c.c.	100,000 million per c.c.
Abortus bovine K 25	1800	100	0	0	640	400	0
Melitensis Phillimore	900	0	0	0	0	0	0

		Melitensis serum, Phillimore, absorbed at 1/4 with						
		Phillimore non-formolised, twice washed			K 25 non-formolised, twice washed			
Antigen	Unabsorbed	3000 million per c.c.	10,000 million per c.c.	100,000 million per c.c.	3000 million per c.c.	10,000 million per c.c.	100,000 million per c.c.	200,000 million per c.c.
Melitensis Phillimore	320	0	0	0	80	40	20	0
Abortus bovine K 25	160	0	0	0	0	0	0	0

Similar findings were obtained when formolised unwashed and non-formolised washed suspensions were used for absorbing one porcine, two other bovine abortus, and two other melitensis sera. In each instance it proved possible to obtain monospecific sera containing only the major agglutinin.

Experiments carried out to test the truth of Weil's findings gave results which were explicable by assuming that Brucella extracts contained a certain amount of dissolved antigen. No evidence of a non-specific inhibiting factor was obtained. As regards Olitzki and Gurevitch's objection to the use of formolised suspensions, it may be remarked that Olitzki (1928) himself found that formol had no action in 0·2–0·4 per cent. concentration unless it was maintained in contact with the serum for several days. Even this action could be definitely substantiated only with flagellated strains. It now appears from the work of Craigie (1931) that the power of formol to inhibit somatic agglutination is restricted to flagellated strains, and that the observations of Olitzki can be better explained on the assumption that the formol acts by hardening the flagella and hence preventing the organisms from coming into close contact, than by directly destroying the toxophore group of the O agglutinin. Since, in our work, formol was never used to preserve antiserum, and

since Brucella strains are devoid of flagella, Olitzki and Gurevitch's criticism hardly seems to be relevant.

There is therefore no reason to doubt that the original conclusions of Wilson and Miles are essentially correct, or that the method of quantitative absorption of agglutinins can be used effectively for differentiating abortus and melitensis strains.

In the present investigation the serological method was employed, every smooth strain being tested against a monospecific abortus and a monospecific melitensis serum. In addition, full quantitative absorption experiments were carried out with a number of specially selected strains, and in every instance the results obtained agreed with those yielded by the use of monospecific sera. Rough strains were excluded by the thermo-agglutination test, used in the way already described. Many strains that were partly rough could still be typed by the monospecific serum method, provided they contained sufficient smooth agglutinogen, though direct absorption experiments could not be relied upon. The results are given in Table XIII.

Table XIII. *Typing of strains by the use of monospecific sera, sometimes supplemented by quantitative absorption experiments.*

Type	No. of strains examined	Agglutinating with: Monospecific abortus serum	Agglutinating with: Monospecific melitensis serum
Bovine abortus	91	90	1
Porcine abortus	14	14	0
Melitensis	43	0	43
Para-abortus	5	Too rough	Too rough
Paramelitensis	12	Too rough	Too rough
Total	165		

It will be noticed that only one strain yielded anomalous results. The exact origin of this strain was unknown, but it had been isolated for many years. It grew better aerobically than in 10 per cent. CO_2, it produced H_2S, and it reacted to dyes like the bovine abortus type. A culture was plated out, six separate colonies were picked off, and all behaved in the same way as the whole culture. A quantitative absorption experiment showed that it did not absorb, in the usual dose, the agglutinins completely from either an abortus or a melitensis serum, though the titre of a melitensis serum was lowered twice as much as that of an abortus serum. It seems most reasonable to conclude that this strain is transitional in nature, not agreeing completely with either of the main types. Gilbert (1930) has recorded the isolation of a similar transitional strain from the foetus of an aborting cow in Palestine.

Summary and conclusions.

By the use of monospecific sera every smooth or relatively smooth strain, with one exception, which appears to be transitional in nature, has been assigned to its correct type.

It is concluded that this method affords a rapid and accurate means of typing individual strains.

Classification of the main group of Brucella strains.

An attempt has been made to classify the main group of strains on the basis of H_2S production, reaction to dyes, and antigenic constitution. The result is given in Table XIV.

Table XIV. *Classification of main group of strains.*

Group	Sub-group	No. of strains	H_2S formation	Reaction to dyes	Serologically	Remarks
Bovine abortus	A 1	64	+	Typical	Abortus	—
	A 2	7	−	,,	,,	—
	A 3	13	+	Atypical	,,	Slightly resistant to thionin, or unduly susceptible to all dyes*
	A 4	6	−	,,	,,	Ditto*
	A 1m	1	+	Typical	Transitional	—
	Total	91				
Porcine abortus	P 1	9	+	Typical	Abortus	American origin
	P 2	5	−	Atypical	,,	Danish origin. Unduly susceptible to all dyes, but with differential susceptibility of main type
	Total	14				
Melitensis	M 1	25	−	Typical	Melitensis	—
	M 2	18	−	Atypical	,,	Unduly susceptible to thionin, methyl violet, and pyronin
	Total	43				
Para-abortus	Pa 1	3	+	Typical	Too rough	—
	Pa 3	2	+	Atypical	,,	Slightly resistant to thionin
	Total	5				
Paramelitensis	Pm 1	12	−	Typical	,,	—
	Total	12				
	Grand total	165				

* Thionin-resistant strains, non-CO_2 sensitive on isolation.

Taking the bovine abortus group, it will be seen that the H_2S test classifies seventy-eight correctly, the dye test seventy-two, and the serological test ninety, out of the total of ninety-one strains. It must be pointed out, however, that most of the strains giving an atypical reaction to dyes could be satisfactorily grouped, and that only nine strains were really unclassifiable by this method.

In the porcine abortus group the atypical reaction to dyes of the Danish strains presented some difficulty, as did many of the unusually sensitive strains in the melitensis group. All of these strains, however, were readily classifiable by the serological method.

Summary and conclusions with regard to the examination of the main group of strains.

(1) An examination has been made of 165 Brucella strains of varied origin, excluding those from the north-east, east, and south-east districts of France.

(2) On the basis of the source of origin, CO_2 sensitivity, H_2S production,

growth in the presence of dyes, and antigenic structure, they have been classified into the following groups:

(*a*)	Bovine abortus with 5 sub-groups	91 strains
(*b*)	Porcine abortus with 2 sub-groups	14 ,,
(*c*)	Melitensis with 2 sub-groups	43 ,,
(*d*)	Para-abortus with 2 sub-groups	5 ,,
(*e*)	Paramelitensis	12 ,,

EXAMINATION OF STRAINS OF THE LISBONNE-TAYLOR GROUP.

The Lisbonne-Taylor strains may be grouped as follows:

Country of origin	Host	No. serologically classifiable	No. too rough to classify serologically
France, S.E.	Human, ovine, caprine and bovine	101	34
France, E.	Human	1	0
France, N.E.	Human, ovine, and bovine	12	6
Unknown, but probably France, S.E.	Human and ovine	1	1
	Total	115	41

Most of these strains were very kindly sent to me by Dr R. M. Taylor of the Rockefeller Foundation, who was engaged in a bacteriological and epidemiological investigation into undulant fever in the south-east of France. His results have already been published (Taylor and Hazemann, 1932; Taylor, Lisbonne and Roman, 1932). Both of us examined the strains for H_2S production and for dye sensitivity, and obtained, so far as the final grouping was concerned, identical results in ninety-four instances; records of Dr Taylor's examination of the remaining strains in this group are not available at the moment.

In addition, I undertook a serological investigation of these strains in order to ascertain whether the grouping by the H_2S and dye methods agreed with that by the serological method. It has already been shown in describing the results with the main group of strains that there was a very good measure of agreement between these three methods, and that the serological method appeared to be the most reliable means of assigning a given strain to its true type. Examination of the Lisbonne-Taylor group, however, has revealed very marked discrepancies between the H_2S and dye methods on the one hand, and the serological method on the other, the chief divergence being that a number of strains, classified as melitensis by the former methods, have reacted serologically like abortus. Many of these strains have been intensively studied, attention being paid not only to their agglutinin-absorption capacity, but also to their power of giving rise to agglutinins on inoculation into rabbits. There seems to be no question that these strains which are of human, caprine, or ovine origin, which form no H_2S, and which grow in the presence of all four dyes, are antigenically similar to the abortus group. Why this divergence should occur only in the strains coming from the south-east, east, and north-

east of France, it is impossible to say, but it is clear that these strains are different from those isolated in other parts of the world. Careful inquiry, carried out by Dr Taylor, failed to reveal any epidemiological differentiation between the strains that reacted serologically like abortus and those that reacted serologically like melitensis.

The complete classification of the Lisbonne-Taylor group is given in Table XV.

Table XV. *Classification of the Lisbonne-Taylor group of strains.*

A. Serologically classifiable group.

Origin	Sub-group	No. of strains	H_2S formation	Reaction to dyes	Serologically	Remarks
Human	A 1	2	+	Typical abortus	Abortus	—
	A 1m	1	+	,,	Melitensis	—
	M 1	15	–	Typical melitensis	,,	—
	M 1a	41	–	,,	Abortus	—
	M 3	22	–	Atypical melitensis	Melitensis	Unduly susceptible to methyl violet
	M 3a	4	–	,,	Abortus	Ditto
	M 1am	3	–	Typical melitensis	Transitional	React with both abortus and melitensis monospecific sera
	M 2	1	–	Atypical melitensis	Melitensis	Unduly susceptible to thionin, methyl violet, and pyronin
	Total	89				
Bovine	A 1	4	+	Typical abortus	Abortus	—
	A 3	1	+	Atypical abortus	,,	Unduly susceptible to all dyes
	A 1m	1	+	Typical abortus	Melitensis	—
	M 1a	3	–	Typical melitensis	Abortus	—
	Total	9				
Caprine and ovine	M 1	4	–	Typical melitensis	Melitensis	—
	M 1a	5	–	,,	Abortus	—
	M 3	3	–	Atypical melitensis	Melitensis	Unduly susceptible to methyl violet
	M 4	2	–	,,	,,	Unduly susceptible to methyl violet, and pyronin
	M 1am	3	–	Typical melitensis	Transitional	React with both abortus and melitensis monospecific sera
	Total	17				
	Grand total	115				

B. Serologically unclassifiable group.

Human	M 1	27	–	Typical melitensis	Too rough	—
	M 3	4	–	Atypical melitensis	,,	Unduly susceptible to methyl violet
	Total	31				
Bovine	M 1	4	–	Typical melitensis	Too rough	—
	Total	4				
Caprine and ovine	M 1	5	–	Typical melitensis	Too rough	—
	M 3	1	–	Atypical melitensis	,,	Unduly susceptible to methyl violet
	Total	6				
	Grand total	41				

Of the 156 strains, 41 were too rough to be serologically classifiable. Of the remaining 115 strains, 27 were partly rough, as indicated by the thermo-agglutination test, but still had sufficient smooth antigen to react to monospecific sera.

Most of the human, caprine, and ovine strains reacted to dyes like the melitensis type, but a considerable number were unduly susceptible to methyl violet. It is interesting to notice that twenty-seven out of thirty-one strains showing this peculiar sensitivity behaved serologically like melitensis, while forty-nine out of seventy-four strains not unduly sensitive to methyl violet behaved serologically like abortus.

Two strains, one of human and one of bovine origin, behaved by the H_2S and dye tests like bovine abortus, yet reacted serologically like melitensis. Both of these strains came from the north-east of France, and were CO_2 sensitive on isolation. Whether they were originally of the melitensis type and were undergoing a transition into the abortus type, it is impossible to say, but it may be noted that in the main group two para-abortus strains, isolated from cattle in Palestine, which had almost certainly been infected with the melitensis type from goats, behaved in a similar way as regards the H_2S and dye tests.

The isolation of three strains from cattle that reacted like melitensis to the H_2S and dye tests and like abortus to the serological test suggests, either that the animals had been infected from sheep or goats carrying the same type of strain, or that they had been infected with typical melitensis strains, which had then undergone an antigenic change in the tissues. It is to be observed that no strain behaving in all respects like melitensis was isolated from cattle.

The single strain of bovine origin, A 3 sub-group, which was unduly susceptible to all dyes, was derived from an animal that had been transferred from the north to the south-east of France, where it was killed. It agreed closely with the three strains already described under the main group, which also came from the north of France.

Of the six strains reacting to both monospecific abortus and melitensis sera, five were plated out, and six single colony sub-cultures were studied from each strain. All the sub-cultures behaved to H_2S like melitensis, while serologically some behaved like abortus, and some continued to agglutinate with both monospecific sera.

No porcine abortus strains were encountered.

GENERAL DISCUSSION.

A number of workers have now recorded their attempts to classify organisms of the Brucella group by means of different tests. It is not proposed to deal with these in detail, because many of the earlier attempts were summarised by Wilson (1931 *c*), and because Habs (1933), more recently, has given a very full review that includes much of the later work.

There is a considerable unanimity of opinion on the interpretation of the H_2S and dye tests. It is true that certain observers, such as Saitta (1929), Marshall and Jared (1930), Meyer and Eddie (1930) and Maggiora-Vergano (1932), have obtained unsatisfactory results by the use of dyes, but the majority of workers, such as Huddleson (1931), Kristensen (1931), Taylor, Lisbonne and Roman (1932), Meyer and Zobell (1932), Grumbach and Grilichess (1932), and Zeller and Stockmayer (1933), who have examined sufficiently large numbers of strains by the technique laid down by Huddleson, have found this one of the most reliable of all methods.

The glucose utilisation test of McAlpine and Slanetz (1928 *a*) has been

reported on unfavourably by most workers who have tried it, and its differential value appears to be very much less than that with which it was originally accredited by its authors.

The results obtained by the agglutinin-absorption method have been variable. Much of the earlier work has to be discounted, since the importance of using smooth strains was not then realised. Probably the same fallacy underlies the work of Plastridge and McAlpine (1932), who failed to obtain a clear differentiation between abortus and melitensis types, and possibly that of Francis (1931), who obtained results that were frequently in disagreement with those yielded by the dye method. Kristensen (1931) failed to obtain satisfactory results, probably through using too heavy an absorbing dose in relation to the titre of his serum. Some workers, however, such as Ross (1927), Bieling (1930), and Olitzki and Gurevitch (1933), have been successful with the agglutinin-absorption method, while Habs (1933) has been able to confirm the findings of Wilson and Miles (1932) on the value of monospecific sera in the differentiation of abortus and melitensis strains.

The result of these investigations has been, as Habs (1933) justly remarks, rather to reveal the occurrence of varieties corresponding to certain geographical situations than to lead to a separation of the two main epidemiological abortus and melitensis types. The truth of this remark is particularly emphasised by the work of Meyer and Zobell (1932), and by the results recorded in the present paper, which render it clear that numerous transitional strains occur, differing in their formation of H_2S, their susceptibility to one or more particular dyes, their sensitivity to carbon dioxide, and their antigenic constitution, or some other less important characteristic. It is specially noticeable in the present work how often these variations are related to some special location, suggesting that organisms of the Brucella group are peculiarly susceptible to environmental change. Future work will necessarily be more and more devoted to a study of the virulence of these different variants, and to ascertaining the importance of their antigenic structure and metabolic activities in determining their invasive powers and general pathogenicity.

General summary.

1. A systematic study has been made of 165 Brucella strains from different parts of the world, and a less intensive study, comprising only the H_2S formation, dye sensitivity, and agglutinin-absorption tests, of a special group of 156 strains from the north-east, east, and south-east of France.

2. For purposes of differentiation little weight can be attached to the use of morphological appearances, abundance of growth in culture, pigment formation, the appearance of crystals in the medium, the production of alkali in peptone water, or the utilisation of glucose.

3. The presence of 5–10 per cent. of carbon dioxide in the atmosphere is essential for the growth of most freshly isolated bovine abortus strains. This concentration frequently favours the growth of melitensis, though not of

porcine abortus, strains. A concentration of 40 per cent. CO_2 almost invariably inhibits to some extent the growth of melitensis and porcine abortus strains, and frequently inhibits the growth of bovine abortus strains. It may be concluded that, while the inhibition of growth of a given strain by 40 per cent. CO_2 cannot be considered of differential value, the fact that growth in 40 per cent. CO_2 is as good as, or better than, that in air is definitely in favour of its being of bovine abortus type, while the occurrence of growth in 10–40 per cent. CO_2 but not in air almost certainly indicates that the strain belongs to this type.

4. Most bovine abortus, para-abortus, and American porcine abortus strains produce H_2S freely for 3 or 4 days, while melitensis, paramelitensis, and Danish porcine abortus strains fail to produce any, or more than a small quantity on the first day only. Though the failure of a given strain to produce H_2S cannot be regarded as indicating that it is of melitensis, paramelitensis, or Danish porcine abortus type, the definite production of H_2S can be considered as very strong evidence that it is of bovine abortus or American porcine abortus type.

5. The dye sensitivity method, introduced by Huddleson, is of very real value in differentiation, and is the only certain method available for distinguishing between the bovine and porcine abortus types. By its means it is possible to divide Brucella strains into three main groups—bovine abortus and para-abortus, porcine abortus, and melitensis and paramelitensis. Not all strains, however, within a given group behave alike, and a number of sub-groups can be established on the basis of special sensitivity to one or more dyes. The method not infrequently yields results that demand considerable experience in their interpretation, and it cannot always be relied upon for the correct typing of individual strains.

6. The thermo-agglutination test is one of the simplest methods of detecting antigenic roughness. Any strain that fails to react negatively to this test is unsuitable for the production of smooth antiserum or for use in routine diagnostic agglutination work.

7. The agglutinin-absorption method, performed by a strictly quantitative technique, enables a differentiation to be made between bovine and porcine abortus strains on the one hand, and melitensis strains on the other, provided that smooth strains are employed both for the preparation of antisera and for absorption. The use of direct agglutination by monospecific abortus and melitensis sera affords a rapid and accurate means of typing individual strains, and may prove of value in the examination of strains which, while partly rough and unsuitable for absorption experiments, still retain sufficient smooth antigen to be agglutinated by one or other serum. It is also of great service in the detection of mixed strains. Both methods yield identical results.

8. In the examination of the main group of strains the serological method proved more valuable than any other method in the correct allocation of individual strains, and the results agreed closely with those afforded by the dye method. In the examination, however, of the special group of strains

from the north-east, east, and south-east of France, there was frequently a marked disagreement between the results of the H_2S and dye tests on the one hand, and the serological method on the other, the chief divergence being that a number of strains reacting by the former methods as melitensis behaved serologically like abortus. It appears as if, in this particular area of France, strains occur having the metabolic properties of melitensis and the antigenic constitution of abortus.

9. A study of the main group of strains by the various methods enumerated enabled them to be classified into the following groups: (*a*) bovine abortus with five sub-groups, (*b*) porcine abortus with two sub-groups, (*c*) melitensis with two sub-groups, (*d*) para-abortus with two sub-groups, and (*e*) paramelitensis.

10. A study of the special group of strains from the eastern districts of France revealed the presence of ten sub-groups. Since it is rather doubtful to what main group many of the strains belong, they have been classified according to the host from which they were isolated.

General conclusions.

The two main conclusions that emerge from this work are:

1. That, besides the existence of three main groups—bovine abortus, porcine abortus, and melitensis—with their subsidiary rough para-abortus and paramelitensis derivatives, there exist within each group a number of sub-groups containing transitional strains, which frequently are associated with some particular geographical location. The suggestion is that members of the Brucella group are relatively labile and respond readily to environmental changes. How far this peculiar lability is responsible for their power to adapt themselves to a number of different hosts and for their varying pathogenicity is for the future to decide.

2. In view of the existence of numerous sub-groups, it is unjustifiable, in the classification of individual Brucella strains, to rely on any single method of examination. Every strain should, if possible, be examined for CO_2 sensitivity, for H_2S formation, for growth in the presence of thionin, basic fuchsin, methyl violet, and pyronin, and for antigenic structure. If reliance is placed on one or two methods only, some strains are bound to be classified wrongly, and erroneous conclusions drawn as to the pathogenicity of the group to which they are allocated.

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