BOVINE BRUCELLOSIS: A STUDY OF THE SEROLOGICAL ASPECTS OF THE DISEASE

by L. K. NAGY

SUMMARY

A study of bovine brucellosis was carried out on a herd of mixed vaccination history with a view to evaluating the various methods of diagnosis used under field conditions in Great Britain.

It was shown that the milk ring and whey agglutination tests were largely negative in calfhood-vaccinated brucella-free animals (85.1% and 96.1% respectively). In animals vaccinated as adults or repeat vaccinated, the milk ring test appears to be valueless as an indicator of field infection because of the very high percentage of falsely positive results (47.7%). The whey agglutination test was shown to be as valuable in these cattle as in calfhood-vaccinated cows, provided that the level of significance of whey agglutinins was taken as 1:10. The whey agglutinin titres appear to be influenced by the stage of lactation and there is a rise in the percentage of positives with advancing gestation. It is suggested that the milk ring and whey agglutination tests on them are of little

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value in the detection of infected animals since these are detected more readily by repeat serum agglutination test alone, but they are valuable as moderators in cases where the serum agglutination test gave doubtful results.

The results of the vaginal mucus agglutination test indicate that tis value is very similar to that of the whey agglutination test in that it does not help significantly in the detection of infected animals but does serve to indicate absence of brucella infection in animals giving doubtful serum agglutinin titres.

Over-age vaccination of cattle with Br.abortus S19 diminishes the value of the serum agglutination test to a considerable degree. The vaccinal serum agglutinin titre persists much longer in adult-vaccinated animals than in calfhood-vaccinated stock. There is a marked tendency for the serum titres of adult and also of calfhood-vaccinated animals, born and vaccinated in an infected environment, to show fluctuation of serum titres. One result of this fluctuation is that, on occasions, non-infected animals may show higher titres than animals known to be infected with Br.abortus.

It is suggested that the too rigid application of the accepted criteria to the interpretation of serum agglutinin titres

of adult-vaccinated cows may cause the disposal of many cows which may never constitute any danger for the rest of the herd. The examination of milk and vaginal mucus samples may be of real value in this situation.

The results of cultural and biological examination of milk, vaginal mucus, post-partum and autopsy specimens indicated that the time and expense involved in these tests, purely for the diagnosis of brucella infection, is not proportionate to their value. However, where maximum safety is required their use may be justified.

In the course of the field study on brucella infection of cattle it was observed that a few of the calves, born and suckled by brucella infected cows, gave an inferior response to S19 vaccination at 6 months of age. As the testing for sero-agglutinins is the principal method of diagnosing brucella infection, especially in the bovine, the suppression of agglutinin production, even if partial, could interfere seriously with the diagnostic value of the test. Thus the possibility of aberration of immune response, as a result of brucella infection of the ovine and bovine neonata was examined.

For the first part of these studies new-born lambs were used, more as a result of necessity than choice. They were

exposed daily to viable Br.abortus S19 for the first 65 days of life and their serological response studied to the same and closely related organisms when encountered later in life. When challenged as young adults these lambs showed a very marked suppression of agglutinin formation. The antibody response was of short duration and had been preceded by a lag phase. An increased proportion of non-agglutinating antibodies to agglutinins was noted, which could be demonstrated by the agglutination inhibition test. Complement fixing antibodies were detected in the sera of test and control animals in roughly similar amounts after challenges and were present long after the disappearance of agglutinins.

For the second part of these studies new-born calves were used. They were exposed daily to large oral doses of virulent Br.abortus for the first 15 days of life. At 7 months of age, together with control animals, they were exposed to the same organisms. The results showed that re-exposure of neonatally infected calves to brucella stimulated a serological response without a prolonged lag-phase, but the average sero-agglutinin titres were lower and persisted for a much shorter time than those titres of the control calves experiencing brucella infection for the first time. Complement fixing antibodies were not detectable in the sera of neonatally infected calves during the first 7 months

of life, but appeared after re-infection, at the same time and in comparable quantities to the complement fixing antibody content of the sera of control calves. The complement fixing antibodies persisted in the sera of neonatally infected calves for a much shorter time than in the sera of the controls. Coombs' anti-globulin test detected antibodies in the sera of neonatally infected calves prior to, and after re-exposure to brucella. In the sera of the control calves the Coombs' test detected antibodies for the first time 3 - 4 weeks after exposure, reaching numerically higher values. Suppression of Coombs' titre as a result of neonatal exposure was comparable to that observed in agglutinin titres. From the evidence so far accumulated it is clear that the degree of exposure to brucella applied to very young calves was not sufficient to inhibit their immune response completely. On re-exposure to the same organisms as serologically mature animals, the fact of exposure is detectable for a moderately short time. Whether or not they were free of infection when the titre of the humoral antibody indicated them as such, remains to be established.

In part 3 of this thesis an attempt is described to extract water soluble antigens of various biotypes of Br.abortus in order to subject them to comparative analysis. The aim of the analysis was to detect any antigenic differences which may

exist between the biotypes in order to provide a diagnostic test capable of differentiating vaccinal titres from those caused by superimposed natural infection of cattle. For the comparative analysis, the precipitation reaction in agar gel and the electrophoretic technique were employed.

The application of cold acetone to brucella cells resulted in the release of up to 13 soluble antigens. The quantitative examination of these soluble antigens of the various biotypes of Br.abortus showed that these antigens differed in their relative concentration in the bacterial extract of different origin and that two of the biotypes examined (both melitensis type organisms) lacked one of the antigenic components characteristic of typical Br.abortus. Comparative studies of the precipitate systems of the various biotypes of Br.abortus revealed two antigenic components in some of the virulent strains partially different from those present in the vaccinal strain. Furthermore, the four virulent biotypes of Br.abortus (accounting for over 98% of field infection of cattle in Great Britain) possess at least one antigen which is common to all of them but is absent in the vaccinal strain. The titration of precipitating antibodies in bovine sera directed against these soluble antigens was not always possible due to difficulties inherent in the technique used. Nevertheless, it was shown that the two partially different antigens of the

virulent strains were fairly immunogenic, whereas the extra antigen of the virulent biotypes was not in every individual of the bovine species.

Zone electrophoresis in starch gel and acrylamid gels was employed in an attempt to isolate the extra antigenic component of the virulent biotypes in relatively pure state. One of the numerous buffer systems examined was capable of resolving the soluble fractions of brucella. The comparative electrophoretic patterns of the biotypes revealed an extra component shared by all the virulent strains, but absent in S19 Br.abortus. The elution of the extra component from the gel, however, was not successful, thus the identity of the extra electrophoretic fraction to the extra antigen of the virulent strains of Brucella could not be established.

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GENERAL INTRODUCTION

Historical

Bovine brucellosis as pointed out by Stableforth and Galloway (1959) was known as a disease of cattle in ancient times. As a result of simple observation, the layman had very early recognised the infectious nature of the disease but the learned men of the era were hesitant to adopt this view. However, by the beginning of the 19th century in England, "The Complete Farmer" (5th edition, 1807 - see Stableforth, 1959) expressed no doubts about the contagious nature of the disease. Experimental support of this view was not available in this country until Woodhead et al. (1889) transmitted the disease either by injecting a healthy pregnant cow with the vaginal discharge from a recently aborted one, or by inserting a cotton wool plug, into the vagina of the pregnant cow, which had been introduced into the genitalia of a cow recently aborted. By the same method he demonstrated that the causative agent of "Epizootic Abortion" can bring about abortion in sheep. Although he did not consider his results conclusive as to the infectious nature of the disease, nevertheless he recommended it to be considered as such and put forward some useful practical suggestions to minimise its effects.

Prior to the investigations of Woodhead et al. (1889), Bruce and Nocard (see Bang, 1897) abroad had brought evidence to support the view that Epizootic Abortion was contagious.

The final and conclusive evidence to show that Epizootic Abortion was a bacterial disease was furnished by Bang (1897) who isolated Bacillus abortus in pure culture, from the intestinal contents of a bovine foetus. After some initial failures he succeeded in bringing about abortion in two healthy cows by the injection of a pure culture of abortion bacilli into the vagina of the cows and recovered the same organisms from the aborted foetus.

Some 10 years before Bang isolated and described the causative agent of Epizootic Abortion, Bruce (1887) discovered minute micrococci on stained smears from the spleen of a human subject who died of Malta Fever. Having failed to cultivate the micrococci from the blood of ten infected patients, he finally succeeded in isolating them, often in pure cultures from the spleen of a patient who had succumbed to the disease. Subsequently, (1893) he named these organisms Micrococcus melitensis.

By the beginning of the 20th century, Horrocks (1903) reported the presence of M. melitensis in the milk, blood

and urine of goats around Malta. Zammit (1905) and Kennedy (1905) furnished evidence on the basis of sero-agglutination test as to the status of infection of goat herds in the same region. Shaw (1906) examining the milk of cows in Malta recovered M.melitensis therein and noted the intermittent nature of excretion of these organisms in the milk. Schroeder and Cotton (1911) in U.S.A. inoculating cows milk into guinea-pigs found that almost 14% of the samples tested were infected with Bacterium abortus, and that the same organisms could be demonstrated in the supramammary lymph nodes of the infected cow by guinea-pig inoculation.

The third member of this group of organisms was isolated by Traum (1914) from the foetus of a sow which is the natural host of Br. suis.

It was not, however, until 1918 that Evans recognised that Micrococcus melitensis of Bruce (1887) and the abortion bacilli of Bang (1897) were very similar organisms in their habitats, morphology, biochemical and serological reactions. By cross-absorption tests she noted "that both the abortus and melitensis antisera contain more than one agglutinin, that the agglutinins

in the two antisorums are alike in kind, but differ in proportions and that the corresponding agglutinable substances are present in the bodies of the two species of bacteria in different proportions". She suggested, moreover, the possibility of Bacterium abortus infecting humans but failed to appreciate the real reason why a disease resembling Malta fever is not prevalent in this country despite the reported frequency of virulent strains of Bacterium abortus in cows milk.

Since those times, morphology, cultural characteristics, nutritional requirements, pathogenicity and antigenic structure of the genus, now known as Brucella, has received a great deal of attention resulting in a huge volume of literature on the subject.

The extent of bovine brucellosis and its economical significance in Great Britain.

There are no accurate figures available to show the exact extent of brucellosis in cattle for Great Britain as a whole at the present time, therefore, the assessment of economic losses due to the disease is rather difficult. It is generally agreed, however, that before the introduction of S19 Brucella abortus vaccination in 1944, some 7 - 8% of the cows aborted largely due to Brucella (Stableforth et al. 1959). The situation at present is much improved, the total abortion rate has dropped to 2 - 3%, many of these abortions being for reasons other than brucellosis. It has been stated (Stableforth, 1960) that no more than 0.5% of the total abortions of cattle can be attributed to brucellosis. Although "abortion storms" as were known before the introduction of S19 vaccination are seldom heard of in herds other than non-vaccinated ones, the economic significance of the disease is still of some consequence. Economic losses due to brucellosis in cattle arise from several causes. First the loss of the calf due to actual abortion or birth of a weakling calf (this may be of the utmost importance in beef cattle, the calf being the total production from the cow that year). Loss from the total absence or decreased

milk yield, depending on the time of abortion. Late abortion stimulates milk production but considerably less than a full time calving. Stableforth, et al. (1959) estimates the loss of milk to be in the region of 20% varying between 16 - 33% for late abortion and for early abortion it may be as high as 50%. Severe losses are sometimes experienced in herds which export animals, especially bulls failing the blood serum agglutination test. Loss may also result from reduced fertility subsequent to retained placenta and secondary bacterial infection of the genitalia. The capital value of stock known to be infected is also considerably reduced.

There have been several reports in recent years as to the extent of brucella infection of cattle in different regions of the country. These surveys were based on the examination of individual or bulk samples of milk, largely by the milk ring test (M.R.T.) and/or biological or cultural tests. Stringer (1951) in Hertfordshire found Br.abortus in 6.8% of the milk samples examined. Stableforth (1954) showed that out of 655 samples of individual cows 26.9% were positive to the M.R.T., one quarter of these (approximately 7%) were positive by biological test.

Ferguson and Robertson (1954) in the East of Scotland examined milk from 256 individual cows 10.5% of which were found to contain viable brucella.

From Northern Scotland, Marr and Williams (1958) reported 25% of the examined herds positive to the M.R.T., one quarter of which yielded Br.abortus (approximately 6% of the total number of herds). Kerr, Pearson and Rankin (1958) in Northern Ireland examined 7200 cows in 147 herds. In 6% of the herds they found actual cases of abortion due to brucella and 34 herds (24%) contained animals with positive milk, mucus and blood agglutinations for Br.abortus, without a definite clinical history of abortion at the time of testing.

According to the report of the Public Health Laboratory Service for 1959 in England and Wales out of 11051 herd milk samples (bulked milk of individual herds) 4.5% contained Br.abortus. An analysis of the figures by regions showed that infection was highest in the North West (between 2.8 to 18.8% of samples) and the lowest for the South West (0.7 to 2.5%). The Report advances reasons why the figures show such a low percentage of herd infection and suggest that the true incidence for the whole of the country would be about 16%.

Robertson (see McDiarmid 1961) in 1960 recorded the incidence of brucella in milk samples between from 9% to 30.9% depending on the type of farming. On self contained farms the incidence of infection was much lower than on those where there was a constant influx of replacements bought on the market.

A survey of brucellosis in Oxfordshire and in the Isle of Wight was carried out by McDiarmid (1960, 1961). In the course of this survey 662 farms were examined in Oxfordshire on the basis of milk sampling on a single occasion. The samples were tested by the M.R.T. and whey agglutination test. All the milk samples positive to these tests were biologically examined for brucella and the results showed that 4.4% of the herds were infected. Considering that the herds were sampled on a single occasion when many of the cows may have been dry and when due allowance is made for the intermittent excretion of the organisms in the milk it appears that the true extent of the infection might have been higher, perhaps as high as 10% (McDiarmid, 1961). The total number of infected herds in the Isle of Wight was estimated to be 6.3%.

In the recent national survey of Brucellosis in Dairy Cattle provisional figures seem to indicate that there may be

as many as 74000 infected cows in the national dairy herds (2.32%) and the incidence of herd infection may be as high as 25.- 30%. If we accept the figure of £25 suggested by McDiarmid (1960b) representing the total loss per infected cow per annum then the financial loss due to brucella for all dairy herds in Great Britain would be 74000 x £25 = £1,850,000, a high figure but considerably less than previously estimated (McDiarmid 1960b).

Public health considerations

The frequency of brucella infection in cattle has considerably diminished in Great Britain since the introduction of S19 Br.abortus vaccination and yet brucellosis as a Public Health problem appears to remain the same as it was 20 years ago. Unfortunately the exact evaluation of the occurrence of brucella infection of humans is impossible since England, Wales and Scotland are among those few European countries where brucellosis is not a notifiable disease. Undulant fever in Great Britain is chiefly confined to infection by Br.abortus, only one case of Br.suis infection (Williams et.al. 1957) has been reported in recent years. The frequency of the disease is reported to be between 10 and 32 cases per million with a mortality rate of 1 - 2%. (Bothwell, 1960a; Smith, 1951; Dalrymple-Champneys, 1950).

Smith (1951) working in the North East of Scotland estimated the incidence of the disease to be in the region of 32 cases per million and Bothwell (1960a) on the basis of the Oxfordshire survey put the figure to 9 per million. The latter pointed out (1960a) that although the percentage of sera showing positive agglutinin titres in normal health

has been steadily declining since 1927, from 3.7% to just over 1%, this in effect means that ½ million people may be exposed to infection. If only 1 in 500 contracted the disease there may be as many as 1000 cases per annum in Great Britain.

Dalrymple-Champneys (1950) estimate of human cases in England and Wales in 1950 was 1300 cases per annum.

It is impossible to say what proportion of cases remains undiagnosed, but Bothwell (1960b) suggested that the known cases may be multiplied by a factor of 10 to get the real incidence of the disease. Kerr et al. (1961) found this assessment not unreasonable. The likelihood of latent infection was shown by Wilson (1932) who on examining the sera of 63 practising veterinarians found that 15 out of 63 reacted positively but only one of the reactors gave a definite history of undulant fever. Some of the findings of Elkington et al. (1940) are in good agreement with Wilson's observation.

On the basis of his (Wilson's) own observations and on reports from abroad (Huddleson and Johnson, 1930; Lentze, 1930; Thomsen, 1931) he concluded that persons whose occupation brings them into frequent contact with infective material,

e.g., veterinarians, slaughtermen, laboratory and farm-workers more frequently become infected with brucella than any other people. Thomson (1931) suggested that there may be 400 - 500 cases per annum in Great Britain.

White (see Bothwell, 1960b) in surveying occupational hazards of practising veterinarians in 25 practises found 16 people infected with brucella. Bothwell in a recent paper (1963) considers 2% to be due to occupational contact and in rural areas this figure may be as high as 50%.

Transmission of the disease to humans in this country is predominantly due to the consumption of contaminated raw milk (Paterson and Hardwick, 1938; Elkington, Wilson, Taylor and Fulton, 1940; Cruikshank and Stevenson, 1942; Loys, 1942; Smith, 1951; Wallis, 1957) and to a much lesser degree to direct contact with or ingestion of raw milk products or infected meat. Barrett and Richard's (1953) findings are exceptional, they describe 25 cases of chronic brucellosis, 14 of these were directly connected with either farming or marketing of dairy produce, they all consumed raw milk but were also exposed to the risk of direct infection.

Dalrymple-Champneys (1960) considered that 92% of his Undulant Fever cases were due to milk. In 124 of the

milk infection cases the milk consumed was from T.T. herds. Bothwell (1960) in his survey of the Oxford region described 61 cases diagnosed over a period of 20 years (1939 - 1958). About 80% of the cases appeared to be due to milk infection. In the last 3 years of the survey the follow up of 17 cases brought evidence to the effect that 9 out of 17 cases were due to T.T. milk. Biological examination (guinea-pig vaccination) of bottled milk was carried out in all of the T.T. herds of the county revealing approximately 5% of the samples to be infected. The rural predominance of the disease was indicated by a ratio of 3.5:1 in 49 cases admitted to one of the hospitals. The figure for another hospital in the same region was approximately 2:1 in favour of rural cases. Smith (1951) puts the rural-urban ratio in the North East of Scotland to 1.9:1 and to 2.3:1 for Northern Scotland.

The role of raw infected milk in causing human brucellosis is clearly established in Britain. Raw milk sold to the public makes up 5 - 6% of all supplies (Bothwell, 1963) and this percentage is slowly diminishing. The percentage of milk pasteurised in 1959 was well over 90% in England, Wales and Northern Ireland and approaching 80% in Scotland (Bothwell, 1960b).

It is true that the fairly wide spread use of S19 Br.abortus vaccine controls the incidence of abortion in cattle to a high degree but infected cows may persist in vaccinated herds and excrete viable organisms in their milk (Kerr et al. 1950; Cameron, 1959a, 1960; Bothwell, 1960a; Brinley-Morgan, 1960). No country which has eliminated human brucellosis has done so by pasteurising all the milk consumed, for even if that was possible the problem of contact infection in occupationally exposed people would not be reduced.

Human brucellosis therefore cannot be considered separately from brucellosis in animals and the elimination of human infection in the final analysis depends on the eradication of brucellosis in animals.

Control and eradication of bovine brucellosis.

Methods of control and eradication in different countries are influenced by a number of factors, most important of which is the prevalence of the disease. Whatever the prevailing conditions may be the control and/or eradication may depend on either the prevention of exposure of animals to infection, by the detection and disposal of infected animals or increasing the resistance of the individual animal to infection by immunisation with a suitable vaccine. The combination of the two major policies have often been successfully applied.

It has been shown in some of the Scandinavian countries (Norway, Sweden) that eradication can be successfully achieved by the detection of infected animals by diagnostic tests followed by the segregation and disposal of such animals. This kind of policy, however, can only be adopted economically where the prevalence of the disease is moderate. In herds, regions or countries where the rate of infection is high eradication can be done more safely and economically by the combination of a vaccination policy with Strain 19 Brucella abortus and the disposal of known infected animals (see Denmark, Netherlands, Northern Ireland, etc.).

Vaccination on its own is effective in reducing or even preventing abortion altogether but eradication cannot be achieved by its use alone. If a vaccination policy is to be combined with eradication it is of the utmost importance that its use be limited to calfhood vaccination. It has been clearly demonstrated that after a single dose of S19 vaccine between the age of 6 - 8 months the animal is well protected up to the 5th pregnancy and presumably thereafter (McDiarmid, 1957).

Adult vaccination often results in a persistently high blood titre which can seriously interfere with diagnostic tests. There is no simple method in the meantime to differentiate between titres due to adult vaccination and superimposed field infection.

There are now a number of countries where eradication of bovine brucellosis is completed or has been in progress. Norway 1951, Swedon 1957, eradicated the disease successfully, while in Finland, Denmark and Japan, less than 1% of the herds are infected (Stableforth, 1960). It has been stated for example, that the total cost of eradication in Norway was less than the disease had previously cost each year.

The United States, U.S.S.R., Jugoslavia, Poland, Bulgaria, Holland, Canada, Switzerland, Germany, Northern Ireland, Austria are in the process of eradicating brucellosis.

In Great Britain the policy has been prevention and control by vaccination with S19 Br.abortus since 1944. Although not more than half of the heifer calves in this country are vaccinated in any one year (McDiarmid, 1961), abortion as the clinical manifestation of brucellosis has greatly decreased. Despite vaccination and diminution of the number of clinical cases it has been shown that even in properly vaccinated herds or under experimental conditions, the local infection of the mammary glands by virulent field strains may occur (McDiarmid, 1960b; Kerr, 1958; Bothwell, 1960a) resulting in the insidious spread of the disease to men and animals.

In considering the prospects of an eradication scheme in Great Britain the first thing to be known is the extent of the disease for the whole country. The recently completed national survey of dairy herds should give the answer to that question.

On the basis of the preliminary report of the survey it would appear that due to the extent of infection (25 - 30% of the dairy herds in Great Britain) eradication could not be undertaken economically solely on the basis of detecting and disposing of the infected animals. In order to prevent considerable economic losses, a combined policy of regulated vaccination by S19 Br.abortus, together with the detection and disposal of infected animals would be the more feasible approach. There is no legislation in force regulating vaccination of cattle in this country. Until recently late calfhood and adult-vaccination was a fairly common practice which renders diagnosis unnecessarily difficult. The introduction of compulsory vaccination of all calves under the age of 9 months would be a useful preliminary to an eradication scheme, combined with the marking of vaccinated calves permanently and visibly, and supplying the owner with an approved vaccination certificate. The experimental work of Stableforth (1952) has shown that although eradication of the disease can be achieved without concurrent vaccination of the herds, the maintenance of such herds without serious breakdowns in the scheme would be almost impossible on a national scale where the general incidence of the disease is high.

Concurrently with a period of compulsory calfhood vaccination and abolition of adult vaccination in all but specified cases pilot, brucella eradication schemes could be established in specific districts where the extent of infection is already known from previous investigations. Such a scheme could be based on M.R.T. as a preliminary screening to locate infected herds which would be followed up by blood, whey and vaginal mucus tests to determine which animals are infected and should be removed from the herds. All these tests are essential together with the vaccination history of the animals until the last of the adult vaccinated animals are got rid of. Prior to any eradication scheme even on a pilot scale, the regulations and legislation relating to brucellosis should be brought up-to-date as suggested by the Oxford Working Group (1962) laying emphasis on a). Notification of the diseased animals to the County Health Department thus enabling them to take appropriate steps to prevent human infection, i.e. by issuing pasteurisation orders, the conditions of issuing and removal of which should also be regulated for the whole of the country. b). Compensation for the removal and slaughter of infected animals. c). Marking and registration of infected animals. d). Sale regulations in regard to infected animals. Once prohibition of adult vaccination

and compulsory calfhood vaccination had taken their effects, the pilot eradication areas could be extended into a national eradication scheme.

The education of farming communities in particular is very important in any attempt to eliminate an animal disease. A booklet entitled "What is known about Brucellosis" is published in the U.S.A. and is aimed at disseminating knowledge of this disease among the general public. The publication of this kind of booklet would be valuable before an eradication scheme is started in this country.

PART 2.

A STUDY OF BOVINE BRUCELLOSIS IN THE FIELD

Account of the aims of Brucella eradication in a single herd

Since 1944 bovine brucellosis has been controlled in Great Britain by vaccination. As with other diseases, vaccination does not give complete protection and further progress can only be made by eradication. Eradication presents a number of problems, the majority of which have been commonly experienced by countries which have eradicated this disease. Various factors influence the relative significance of these problems, such as the kind of vaccination policy used, if any; type of husbandry; the intensity of milk production; density of cattle population, to mention only a few. Before any large scale eradication programme can be put into effect, it is desirable therefore to accumulate as much information as possible about the husbandry and technical difficulties encountered under field conditions. More information about relative usefulness of different diagnostic methods would be valuable especially in herds where vaccination had not been restricted to calves but was practised on adult cows often repeatedly. Sweden eradicated bovine brucellosis by using the sero-agglutinin test alone. This may be an economic proposition in a country where no vaccination was carried

out for a number of years prior to eradication. In Britain, however, vaccination with S19 Br.abortus has been used fairly widely and even a few years ago calfhood and/or adult vaccination was a common practice. This resulted in the well known persistent titre cow which presents a major problem in a brucella eradication scheme. For a situation like this there is no single diagnostic test that can be used with reliability.

In this beef shorthorn herd where the eradication was to be carried out, vaccination had not been restricted to calves only, but had been practised on adult cows as well. The problems created by this practice required the diagnosis to be based on as wide a ground as was practicable. There have been numerous tests developed for the diagnosis of brucellosis in the bovine. The most satisfactory method is the demonstration of the causal organism itself. Excretion of brucella in the milk is, however, frequently intermittent. Other sources of isolation such as genital discharge, placental cotyledons, amnio-allantoic fluid are available only at particular times. Nevertheless it was decided that (1) placental - cotyledons and/or amnio-allantoic fluid of all the aborting and calving cows should be collected and tested

for the presence of brucella culturally and/or biologically.

The most readily available source of isolation of brucella in the infected cow is the milk. Therefore (2) milk was collected from all lactating animals at least once in every two months (more frequently if required) and tested routinely by the (a) Milk Ring Test (M.R.T.) and (b) Whey Agglutination Test. Milk samples (3) which were found to be positive by the M.R.T. and whey agglutination test or by the latter test alone were cultured and/or injected into guinea-pigs in an attempt to demonstrate brucella therein.

Mucus samples were collected from all the breeding cows at least once in every two months and (4) tested by the vaginal mucus agglutination test (Kerr, 1955). (5) All the positive samples were tested for the presence of brucella in the same way as the milk samples. (6) Bacteriological examination for brucella was carried out on lymphatic tissues of cattle slaughtered on the premises of the Glasgow University Veterinary Hospital, irrespective of their status of brucella infection. (7) Sero-agglutination test was carried out on the blood samples of every animal in the herd at least once in every two months and more frequently if required.

In addition, information was to be collected on (a) the breeding performance of the animals in the light of their status of infection; (b) the serological response of calves to vaccination; (c) the duration of pregnancy of cows with different histories of brucella infection; (d) the resistance of cattle to field infection with various history of vaccination.

In accordance with the normal management of this herd it was necessary that calves born to infected cows and kept in an isolation unit, should suckle their mothers for the first few weeks of their lives. After this period they need to be transferred to foster cows in the non-infected part of the herd. This transfer may give rise to the possibility of mechanical transfer of infection. To follow up this possibility it was decided that special attention should be paid to such foster cows by more frequent diagnostic tests.

The brucella organisms isolated from the various specimens were to be identified and typed.

By the application of these various tests it was hoped that a correct diagnosis would be arrived at and at the same time, it was also hoped that adequate data would be available to facilitate the evaluation of the relative merits as well as short-comings of the different diagnostic tests.

A. GENERAL METHODS AND ORGANISATION

Milk ring test (M.R.T.)

was carried out on 1 ml. samples of milk in narrow test tubes to which one drop of stained antigen[1] was added followed immediately by gentle mixing. The milk-antigen mixture was left at room temperature for 1 hour and the results read as follows:

Definite blue cream ring; white milk column	= ++++
Definite blue cream ring; slightly blue milk column	= +++
Definite blue cream ring; definite blue milk column	= ++
Cream layer only slightly bluer than milk column	= +
Cream layer same colour as milk	= ±
White or slightly blue cream layer; blue milk column	= -ve.

Whey agglutination test

Quarterly milk samples were collected into sterile universal containers and kept at $4^{\circ}C$ until tested. Before the serological and cultural and/or biological examinations of the samples, the quarterly milk samples of individual cows were pooled. For the whey agglutination test approximately 20 ml. of milk was centrifuged at approximately 1500 r.p.m. for 15 minutes and to the milk from beneath the cream, a few

1. Provided by the Ministry of Agriculture, Fisheries and Food, Central Veterinary Laboratory, Weybridge.

drops of rennet were added and incubated at 37°C until congulation of the casein took place. The clear whey was separated and the agglutination test on it was carried out in the same way as the serum agglutination test with the only difference that the final dilution of whey in the first tube was 1:2 followed by 1:5, 1:10, etc., the samples were titrated to end-point just as in the sero-agglutination test. At interpreting the results due consideration was given to such factors as the stage of lactation and of pregnancy as well as mastitis if present.

Vaginal mucus agglutination test

For the collection of vaginal mucus samples, two different methods were employed during the investigation. The "tampon" method of Szabo (1951) was used introducing a sterile tampon into the vagina through a glass tube. The tampon was left in for about 15 minutes and then removed by means of an attached string which hung out externally and the tampon dropped into a sterile universal container containing 2.5 ml. of physiological saline. The content of the tampon was squeezed out into the saline by means of a sterile spatula before the dilution of the samples was made.

The second method of obtaining mucus samples was by means of oral aspiration, employing the orthodox glass pipette described by Pierce (1949). The mucus was expelled from the pipette into 2.5 ml. of sterile physiological saline and homogenized by vigorous shaking after incubation for 30 minutes at 37°C. Serial doubling dilutions of the samples were prepared but no attempt was made for strictly quantitating the dilutions, otherwise, the test was carried out as the serum agglutination test, titrating to end-point.

Blood serum agglutination test

Following the recognition of the wide divergencies in the use of the serum agglutination test in different countries, in Great Britain in 1933 a dried reference serum was prepared for the standardisation of methods in different laboratories (Stableforth, 1936). The reference serum was adopted by the Office International des Epizootics in 1937 as a standard serum and endorsed by the sixth session of the W.H.O. Expert Committee on Biological Standardisation (see World Health Organisation Technical Report Series, 1953, 67) with the view that international uniformity of the serum agglutination test for brucella should be secured by the use of the standard serum or its counter-part prepared in different national laboratories.

In 1953 (W.H.O. Tech. Rep. Ser. 67) the joint FAO/WHO expert committee on brucellosis restated its view that "published papers including data based on brucellosis sero-agglutination test should always indicate the sensitivity of the test used by stating the titre at which 50% agglutination is obtained when the International Standard Anti-Brucella abortus Serum is tested with a given antigen and method." In 1958 (W.H.O. Tech. Rep. Ser. 148) the Committee accepted the adoption of a unit system expressing the antibody content of the International Standard Anti-Brucella abortus serum in units (1000) and requested that papers which deal with data on serological or milk test for brucella, a statement should be included showing the approximate number of International Units to which a given titre corresponds when their method is used.

The technique used for the tube sero-agglutination test in this thesis was in accordance with the principles laid down in the W.H.O. monograph series No.19 (1953) using serial doubling dilution of the serum starting at 1:5, 1:10, etc., to which an equal volume of the Standardised Br.abortus Agglutination Suspension[1] was added, giving a final volume

1. Standardised Br.abortus Agglutination Concentrate was kindly provided by the Ministry of Agriculture, Fisheries and Food, Central Veterinary Laboratory, Weybridge.

of 1 ml. and a final dilution of 1:10, 1:20, etc., of the serum. The tubes were incubated for 24 hours at 37°C and the result read by ordinary light to end-titre, the results being recorded as follows:

++++ = complete agglutination and sedimentation i.e. 100% or water clear.

+++ = about 75% clearing or nearly complete agglutination and sedimentation.

++ = about 50% clearing and marked sedimentation.

+ = 25% clearing and distinct sedimentation.

In this thesis titres of $1/10^{++}$, $1/20^{++}$, $1/40^{++}$, $1/80^{++}$, $1/160^{++}$, etc., with sera tested indicate approximately 20, 40, 80, 160, 320, etc., units of antibody per ml. respectively.

Interpretation of results for non-vaccinated females, bulls and calfhood-vaccinated (under the age of 9 months) animals 30 months or older was as follows:-

No agglutination or agglutination at 1:10 but less than ++ at 1:20 = pass.

++ (i.e. 50%) agglutination at 1:20 but less than ++ at 1/40 = doubtful (FAO/WHO Expert Committee on Brucellosis Report 1958).

++ (i.e. 50%) agglutination at 1:40 or over = fail.

For adult-vaccinated cattle the interpretation of results was as follows:-

No agglutination or agglutination at 1:40 but less than ++ 1:60 = Pass.

++ (i.e. 50%) agglutination at 1:80 = doubtful.

+++ at 1:80 or over = Fail (if confirmed by either of the following tests: biological positive; whey or mucus agglutinin positive).

Selective medium used for the isolation of brucella

The joint FAO/WHO Expert Committee on Brucellosis in 1958 (World Health Organisation Technical Report Series, 1958, 148) recommended the use of Albimi agar with added ethyl violet and some antibiotics for the isolation of brucella from potentially contaminated materials. However, for certain dye sensitive varieties of brucella this medium was not altogether satisfactory. Drinley-Morgan (1960) compared the suitability of several selective media for supporting the growth of a substantial number of strains of the 3 species of Brucella. He concluded that serum-dextrose -agar plus antibiotics was the only selective medium which supported the growth of all the brucella cultures studied including the more fastidious varieties.

Therefore in this study serum-dextrose-agar plus antibiotics (SDA) of the following composition was used for all the isolation attempts for brucella.

1.5% agar

1% peptone

0.5% sodium chloride

0.5% meat extract

pH adjusted to pH 7.5, autoclaved at 10 lb. per square inch for 15 minutes then cooled to 50°C.

5% inactivated horse serum

1% sterile dextrose

100 mg/l actidione

6000 units of polymixin B/l.

25000 units of bacitracin/l.

were added and poured into Petric dishes - 20 ml. per plate.

Cultural and/or biological examination of specimens for Brucella

All of the mucus and milk samples positive to the agglutination test; all the placentae and/or foetal fluids secured after abortion or parturition and lymphnodes of animals slaughtered on the premises of the Veterinary Hospital, were to be tested for the presence of brucella either culturally or biologically or by both methods when possible.

After having separated the milk for the whey agglutination test the cream and sediment left in the bottle were mixed and kept at $4^{o}C$. If the whey agglutination test was positive at 1:2 or higher dilutions the sample was plated out onto serum-dextrose-antibiotic medium (SDA, Brinley-Morgan, 1960) using 5 plates per sample, each plate inoculated with 2 loopeful of the sample. Positive mucus samples left over after the agglutination test and kept at $4^{o}C$ in the refrigerator were cultured the same way as the milk samples. Amnio-allantoic fluids were collected at abortion or parturition on the farm in sterile universal containers and placental cotyledons into large screw capped bottles and sent to the laboratory. At all times but

during the period of transport (approximately 5 hours) all the specimens were kept in a refrigerator.

The cotyledons were homogenised in 10 parts of sterile Ringer solution v/v in an MSE ATO-MIX homogeniser and the fluid fraction of the homogenate as well as the amnio-allantoic fluid, were plated out onto 5 S.D.A. plates. Lymphnodes of animals (sub-maxillary, supra-pharyngeal, iliac and supra-mammary) collected at autopsy with aseptic precautions were pooled and homogenised as cotyledons for guinea-pig inoculation. All the inoculated plates were incubated in the presence of 10% CO_2 for 6 days at 37^0C then examined for the presence of brucella colonies.

For the biological examination of specimens for brucella, guinea-pigs were used, at least two animals per sample but occasionally as many as twenty. 1 ml. samples of milk-cream sediment, mucus, amnio-allantoic fluid, homogenised cotyledons or lymphnodes, respectively, was inoculated intra-muscularly into guinea-pigs. After a period of six weeks they were killed and an agglutination test was carried out on the sera. The spleen of each guinea-pig, having an agglutination titre of 1:2 or higher, was cultured.

Identification and typing of isolates

After a period of 6 days incubation the SDA plates were examined for the presence of brucella colonies. From any colony resembling brucella smears were made and stained by Gram's method and examined microscopically. If the stained organisms resembled brucella, three serum-dextrose-agar (SDA) slants were inoculated from the colony, one being incubated aerobically, the other two with 10% CO_2. Into one of the two slants incubated with 10% CO_2, a strip of lead acetate impregnated filter paper strip was inserted and looked at every day for 7 days, replacing the lead acetate paper each day if it became blackened as a result of hydrogen-sulphide production.

Dye inhibition test

Serum-dextrose-agar (SD) was used as the basic medium for the dye sensitivity test. To a batch of SD sterile basic fuchsin[1] was added to give a final concentration of 1/25000 and to another batch of SD thiomin[1] was added to give a final concentration of 1/50000 of the dye. From each medium Petri plates were prepared and checked for sterility. Cultures

1. Obtained from National Aniline Division, Allied Chemical Dye Co., New York.

for the dye sensitivity test were grown on SD plates for 3 days and checked for smoothness by the oblique light technique (Henry, 1933). Suspensions were prepared from the cultures to be tested, together with strains of brucella with known dye sensitivity and the opacity of suspension adjusted to a Brown's tube reading of 3, as it had been our experience that a 3-day old culture of such opacity contained approximately 3000 million viable organisms.

The dye plates were marked off into 4 quarters and each of two quarters was inoculated with a known dye-sensitive and a dye-resistant strain, the remaining two quarters were seeded with two different strains under test.

Five strokes were made on each quarter starting at the outer edge of the plate with a loopful of inoculum and progressing inwards without recharging the loop so that the inoculum was lightest nearest the centre of the plate. The plates were incubated at 37°C in 10% CO_2 for 5 days then readings were taken.

Serological test for the typing of a new brucella isolate took the form of slide agglutination test using the same suspension for the agglutination test which was used for the inoculation of dye plates. The Brucella abortus and

Brucella melitensis mono-specific sera[1], were diluted to 1:5 for the slide agglutination test. For positive and negative control Brucella abortus 544 and Brucella abortus type 5 were used.

1. Kindly supplied by the Ministry of Agriculture, Fisheries and Food, Central Veterinary Laboratory, Weybridge, Surrey.

Description of the herd and of some of the policies of management prior to and during the eradication programme

At the beginning of the work in 1960, the herd consisted of 265 head of cattle including some 200 pedigree beef-shorthorn animals, the rest of the herd being cross-bred foster cows. Within the herd of 265 beasts the age and sex distribution was as follows:

Cows (including 38 foster cows)	124
Heifers over one year of age	37
Calves under one year of age	92
Bulls over one year of age	12

These animals were accommodated on two adjacent units of the farm. As the breeding and economic policy of the farm did not allow the disposal of all the infected animals, provision had to be made for the accommodation of such cattle. This necessity was met by the farm management by renting a small farm (isolation unit) some 1 - 1½ miles away from the central unit. A certain amount of traffic was unavoidable between the isolation and the other two units, although this was cut down to as little as possible.

Weekend and holiday duties were carried out by the same personnel on the three units. Calves born to infected cows in the isolation unit had to be transferred to foster cows in the non-infected part of the herd. The danger of mechanical transfer of infection by personnel was minimised by disinfection of boots on leaving the isolation unit. In the case of transferring calves from the isolation unit to the herd a small semi-isolation unit was established on the premises of the central unit for a period of time. Calves having suckled their infected dams for some weeks were transferred here to brucella negative foster cows before being admitted to the brucella free herd.

Records on individual animals in the herd were available at the outset of the work including such information as the vaccination history (in the majority of cases) birth, service, calving and/or abortion dates. The vaccination policy for brucellosis had been unsystematic in as much as in addition to calfhood-vaccination, adult-vaccination was introduced in 1958 for a proportion of the herd, including almost all of the pedigree cows. As a result of this practice the distribution of animals according to their vaccination history was as follows in 1960.

TABLE 1

DISTRIBUTION OF ANIMALS ACCORDING TO THEIR VACCINATION HISTORY IN 1960

Non-Vaccinated (bulls & Calves 6 months old	Calfhood-Vaccinated only	Adult-Vaccinated only	Calfhood & Adult Vaccinated only	Vaccination History not known	TOTAL
68	56	36	50	55	265

The unrestricted vaccination, however, was brought to an end in 1959 when some preliminary brucella survey work was carried out. This brought evidence to the effect that the herd was brucella infected. The evidence was as follows:

1. 11 abortions in 1959
2. Isolation of Br.abortus from several foetuses
3. Isolation of Br.abortus from the semen of one bull
4. Development of sero-agglutinin titre of 1:320 in another bull
5. Demonstration of positive whey and vaginal mucus agglutinins in several cows

Since 1959 strict calfhood-vaccination was adhered to at approximately 6 months of age. Calves were tested for brucella sero-agglutinins just prior to S19 Br.abortus vaccination and thereafter at regular intervals.

The acquisition of foster cows entails a certain amount of danger of bringing in brucella infection. In addition, the vaccination history of such foster cows was often missing. To solve the first problem, at least temporarily, it was decided that only recently calved cows which had passed our diagnostic tests should be purchased. The future policy decided for the farm was to breed their own replacement of foster cows. It was the practice of the farm to take "boarder" animals on the premises (cows and heifers sent to the farm for service - these usually remained for several months) which practice was discontinued.

Organisation of work on the farm and in the laboratory

In order to facilitate the routine testing of the herd once in every 2 months, the animals were divided into 6 groups. The samples on the farm were collected on Monday of each week until the whole herd was sampled, when the routine started anew. This included the collection of approximately 25 - 30 blood, 7 - 12 milk, 12 - 17 vaginal mucus samples each week. The milk samples were collected by the stockmen drawing approximately 20 ml. of milk separately from each quarter of the udder into four different universal containers. The blood and vaginal mucus samples were collected by the veterinary surgeon assisted by stockmen.

The samples collected were kept in a refrigerator on the farm until despatched to the laboratory. They were in transit for approximately 5 hours. On arrival at the laboratory the samples were put into refrigerator ($+4^{o}C$) overnight. The agglutination results on all the samples were read within 48 - 60 hours of being taken; followed by plating onto culture medium or inoculation into guinea-pigs, if necessary.

Although each quarter of the udder was sampled separately, on arrival at the laboratory the four milk samples of each cow were pooled. This procedure was deemed necessary in order to ensure the sampling of each quarter of the udder.

Universal containers for holding the samples together with the vaginal pipettes were sterilised and despatched from the laboratory once a week. A small stock of sterile universal containers and some large waxed carton containers were kept on the farm for the collection of amnio-allantoic fluid and cotyledons respectively, which materials were sent to the laboratory without delay. All the results were recorded on individual cards and evaluated each week taking into account all the known relevant facts about each animal. Reports were sent to the veterinary surgeon of the farm weekly, giving the results as well as comments and recommend-

-ations on the animals tested. In the first year of the eradication programme the veterinary surgeon and the manager of the farm were personally consulted at the completion of each herd test. Later on this meeting took place less frequently as the problems were diminishing.

B. EXPERIMENTAL

The resistance of calfhood and adult-vaccinated cattle to field infection of Brucella

During the period of three years investigation there were 209 cattle in the herd over 1½ years of age with different histories of S19 vaccination. Approximately 15% of these animals were known to have been infected with brucella. As it was shown experimentally (Birch, Gilman and Stone, 1941; Berman, Beach and Irwin, 1952; Plastridge, 1954; Gilman and Hughes, 1955; McDiarmid, 1957) that neither the age at vaccination over 6 months, nor the multiplicity of S19 vaccine administered, resulted in significant differences in immunity it was decided to evaluate the records in order to see if our data supported these findings. Table 2 summarises the results.

TABLE 2

FREQUENCY OF BRUCELLA INFECTION OF CATTLE WITH VARIOUS VACCINATION HISTORY

Vaccination History	Number of animals in group	Brucella infected	Percentage infected
Calfhood-(6-9 months) vaccinated only	125	17	13.6
Adult-vaccinated once	34	0	0
Calfhood and adult-vaccinated	50	15	30.0
Total	209	32	15.3

In evaluating information of this kind obtained under field conditions, the greatest difficulty lies with the fact that the degree and frequency of exposure to field infection is an unknown factor; although all the animals belonged to the same herd. It also has to be borne in mind that the great majority of animals belonging to the adult and calfhood-adult-vaccinated groups had stayed in the herd at a time when segregation of infected animals was not in progress. On the other hand most of the animals in the calfhood-vaccinated group were either born or were young heifers during this time and became cows when all the known infected animals had already been segregated, thus their immunity was exposed to a less severe test. In consequence there is no sound basis for comparing the resistance of calfhood and calfhood-adult-vaccinated groups.

The resistance to infection of the 34 animals in the adult-vaccinated group is rather remarkable. A little less than 50% of them were vaccinated just prior to first service, the rest had calved at least once prior to vaccination. Not only had the majority of these cows been in the herd before eradication commenced, but calves born to infected dams in the isolation unit had been transferred to them for nursing during the 3 years, yet none became infected. There was

no other circumstantial evidence to account for their apparent greater resistance than the fact that almost all of them were dairy type foster cows often cross-bred.

The writer is not aware of any published evidence indicating superior natural resistance of dairy type cattle. Subject to the existence of such evidence a comparison may be drawn between the resistance of adult and calfhood-adult vaccinated animals. The result of this comparison substantiates the findings of Berman et al. (1949, 1952) and Gilman et al. (1955), who found no significant advantage in re-vaccination. It is at some variance with the result of McDiarmid (1957) who found somewhat better protection in multiple vaccinated animals.

The effect of infection on fertility

At the beginning of the eradication work accurate records of services, calvings and abortions were already available from the beginning of 1959, which records were continued until the end of the programme. The possession of these data and the knowledge of the status of brucella infection of cows made the evaluation of fertility possible for brucella infected and non-infected animals.

Investigation of this nature under field conditions calls for extra prudency. Animals in the herd belong to different age groups, different bulls are used for service, artificial insemination and natural mating may be used in combination. As all of these factors influence fertility it is desirable to approach the question from different angles.

The calving index (average interval in days between successive calvings) is one of the accepted indicators of fertility. The following Table (No.3) shows this index for infected and non-infected cattle.

TABLE 3

BRUCELLA ABORTUS INFECTION AND FERTILITY: CALVING INDEX

Status of Brucella infection	Number of animals in group	Total length of time spent as breeding animals (months)	Number of pregnancies conceived	Calving index (days)
NON-INFECTED	129	2868	210	414
INFECTED	35	740	58	386

The number of services required to bring about one pregnancy is another useful clue to fertility. The following Table (No. 4) presents information to this effect.

TABLE 4

BRUCELLA ABORTUS INFECTION AND FERTILITY: AVERAGE SERVICES PER PREGNANCY

Status of Brucella infection	Number of animals in group	Number of pregnancies in 4 years	Number of services	Number of services per pregnancy
NON-INFECTED	129	210	567	2.7
INFECTED	35	58	168	2.9

The third useful measure of assessing fertility is the rate of conception to first service. By first service in this instance is meant either the first mating of a heifer or of a cow after calving. The comparison of brucella infected and non-infected cows is presented in Table No.5.

TABLE 5

BRUCELLA ABORTUS INFECTION AND FERTILITY: FIRST SERVICE CONCEPTION RATE

Status of Brucella infection	Number of first services	Number of successful first services	Conception rate to first services
NON-INFECTED	271	126	46.5%
INFECTED	72	29	40.2%

Infertility has been associated with brucella infection for a long time (Manthei, 1950; Manthei and Carter, 1950; Hendrikse, Joding and Willeng, 1953; Romvary, 1955; 33rd Report N.Z. Dairy Board, 1957). Even vaccination with S19 has at times been suspected of causing infertility. Herds, in general, free from brucellosis are reported to have, apart from the reduction of actual abortions, a better breeding record than infected herds.

Stableforth et al. (1959) examining the breeding records of a herd of 80 cows over a period of 10 years where one half of the herd was re-vaccinated each year and the other half only once as calves, found no evidence of adverse effect of re-vaccination at all. Boyd and Reed (1960) studying the effect of brucellosis on fertility surveyed a substantial number of herds in England and found that although the conception rate of non-infected individuals and herds was somewhat superior compared to infected ones, these differences were not significant.

Kerr et al. (1958) investigating a multiple vaccinated herd with 5 abortions in 6 years found some evidence of infertility in the herd. Hendrikse et al. (1953) examining the effects of brucella infection and of

S19 vaccination on the fertility of cows in the Netherlands, found slightly decreasing rate of conception with increasing numbers of M.R.T. positive animals in the cattle population. At the same time they found no evidence to show that S19 vaccination had any adverse effects on fertility.

In considering the results presented, one can discount the age effect on fertility as the average age of the infected group was 5.3 years and that of the non-infected was 5.6 years. It is true that during these years several bulls were used in the herd, furthermore, artificial insemination was used in combination with natural service. However, these affected the herd as a whole. It is therefore considered that a valid comparison may be drawn between the groups.

The calving index, as it is, is the least reliable measure of fertility being biased in favour of infected animals. It is a well recognised fact that brucella infection of cattle tends to shorten the length of pregnancy or may cause abortion (parturition during the first 240 days of pregnancy). Thus the average length of pregnancy of positive reactors becomes shorter which in turn influences the calving index in favour of infected animals. In this

herd the average length of pregnancy of non-reactors was 267½ days and that of the infected ones 255½ days, a difference of 21 days. Therefore, in order to compare the two groups on a fair basis the calving index has to be corrected. This may be achieved either by substracting 21 days from the calving index of the non-reactors or by adding 21 days to the calving index of the positive reactors. Whichever course is taken the result is the same, i.e. the average interval between successive calvings is still 7 days shorter in favour of infected cattle.

The appraisal of the number of services per pregnancy does not need special qualifications. This is perhaps a more accurate measure of fertility than the calving index and as shown in Table 4 it is slightly in favour of the non-infected animals. The conception rate of non-reactors to first services is also better than that of the infected ones. However, none of these differences are great enough to be of significance especially if found under field conditions where critically influential factors - other than the one under consideration - may not have been entirely even.

The effect of infection on the duration and outcome of pregnancy

It is not unknown in a fully susceptible herd that once brucella infection is brought in it may take a rather acute course causing abortion of the greater part of the herd. This is less common in a S19 vaccinated herd where the protection conferred would take the edge off the severity of an outbreak. It has been stated (McDiarmid, 1960b) that approximately 80% of vaccinated animals would resist a light infection, but when exposed to a heavy infection the effectiveness of protection may drop very appreciably. The outcome of an infection depends on a number of factors such as the degree of exposure, the time at which infection occurrs and the resistance of the animals concerned. A non-vaccinated cow heavily exposed is likely to abort rather sooner than a vaccinated one, usually around mid-pregnancy whereas the vaccinated one might not abort until late in pregnancy or not at all.

On the isolation unit of the farm for infected animals abortions were common, often taking place on grass in the midst of the rest of this part of the herd. It may be therefore of some interest to compare the duration

and outcome of pregnancy of infected cattle to that of the non-infected cows. Table No.6 is the analysis of such a comparison.

TABLE 6

COMPARISON OF DURATION AND OUTCOME OF PREGNANCY

OF INFECTED AND NON-INFECTED COWS

	NON-INFECTED	INFECTED	TOTAL
Number of pregnancies (1959-62)	210	58	268
Number of abortions (premature calvings 240 days (1959-62)	3	17	20
Percentage of abortions	1.4%	29.3%	7.46%
Average length of pregnancies including abortions (days)	276⅝	255⅛	
Average length of pregnancies excluding abortions (days)	276⅛	277	
Average length of pregnancies terminating in abortion (days)	192	200	

The abortion rate of 1.4% of the brucella-free part of the herd compares favourably with the national average of 2 - 3%. The total abortion rate of 7.46%, however, is rather high for a vaccinated herd. Kerr et al. (1958) examined 147 herds in Northern Ireland and found 34 which although containing animals positive to the whey, mucus and serum agglutination test yet had no definite clinical history of abortion. Kerr (1960) investigating 4 infected herds with a total population of 381 animals including 67 infected cows found 5 abortions in all during a period of 3 years.

McDiarmid (1960b) pointed out that if S19 vaccination is properly applied it may reduce the abortion rate to a level of approximately 2%. In this herd the comparatively high rate of re-exposure of infected animals on the isolation unit causing almost one third of the stock to abort each year. Abortion, if it took place, occurred towards the end of the 7th month of pregnancy rather characteristic for vaccinated animals. There was no evidence, however, to show that brucella infection shortened the duration of pregnancy in those animals which did not abort.

Milk tests for the diagnosis of bovine brucellosis

Introduction

Agglutinins in the milk as detected either by the milk ring test (M.R.T.) or by the whey tube agglutination test are commonly used diagnostically in a number of countries including Great Britain.

The M.R.T. has found acceptance primarily as a screening test (W.H.O. Technical Report Series, 1951; Roepke et al. 1958; Stableforth et al. 1959; Cameron, 1960; Kerr, 1960; MacKinnon et al. 1961) being applied to bulk milk samples for the detection of brucella infected herds.

The whey tube agglutination test is mainly used as a quantitative measure of agglutinins in individual milk samples. With certain qualifications a positive whey agglutination test is indicative of localised brucella infection of the udder.

Milk from brucella-free cows in normal lactation does not in general contain detectable antibodies. This applies even to adult-vaccinated cows except for a short

M.R.T. was positive in 92.4% of 304 infected milk samples and the whey agglutination test on the same samples detected 76.0% of them as being positive. From 21 mainly calfhood-vaccinated brucella-free herds 1851 milk samples were examined by the same authors with the M.R.T. and whey agglutination test. 1.6% of the milk ring and 0.6% of the whey agglutination tests showed reactions which were false positive. In repeat adult-vaccinated but non-infected herds the M.R.T. gave 19.7% and whey agglutination test 2.4% false positive readings. The authors concluded that "milk serological tests of samples from individual animals are of limited value and more so where adult-vaccination has been carried out".

Similar results were obtained by McDiarmid et al (1958) who found the detection rate of M.R.T. when applied to individual milk samples of infected cows to be between 85 - 95%. Vaccination at 6 months of age did not interfere seriously with subsequent M.R.T. but if the vaccination was delayed until 18 months of age a considerable proportion ($\frac{1}{3}$ - $\frac{1}{2}$) of the cows free from field-infection gave positive results to the M.R.T. Conversely, many of the calfhood or late calfhood-vaccinated cows after having become infected failed to react to the M.R.T.

Observations on the relative values of M.R.T. and whey agglutination tests in the detection of udder infection

In this investigation observations were made on the relative reliability of the M.R.T. and whey agglutination test when applied to the milk of primarily beef-type cows either calfhood or repeat vaccinated.

In the interpretation of the results of milk tests due allowances were made to the aforementioned stages of lactation or to mastitis when applicable. In the following discussion of the milk tests all the results presented were obtained from mastitis-free cows during - what may be called - normal lactation. The results of tests were disregarded if the milk sample was obtained during the period of drying off or within 10 days after parturition.

Even the earliest results included here were taken 1½ years after the last adult-vaccination.

Table No.7. is the analysis of whey agglutination tests carried out during a period of three years on the milk samples of four groups of animals.

Calfhood-vaccinated Brucella-free	:	highest sero-agglutinin titre in the group is 1:40 all mucus agglutinin negative
Adult-vaccinated Brucella-free	:	highest sero-agglutinin titre 1:80++ : all mucus agglutinin negative
Adult-vaccinated Doubtful	:	highest sero-agglutinin titre in the group is 1:320 : all mucus agglutinin negative
Adult-vaccinated Brucella-infected	:	Brucella has been isolated from the majority of the animals and all of them were positive to the mucus agglutination test.

TABLE 7

WHEY AGGLUTININ TITRES IN RELATION TO STATUS OF VACCINATION AND INFECTION

Status of vaccination and of Brucella infection	Number of animals in group	Sero-agglutinin titres	Number of samples examined	Distribution of whey agglutinin titres			
				2 - 2+	2 - 5	10	10
Calfhood-vaccinated Brucella-free	38	$< 40^{++}$	141	88.7%	7.4%	3.2%	0.7%
Adult-vaccinated Brucella-free	47	$< 80^{+++}$	230	55.9%	38.2%	5.4%	0.5%
Adult-vaccinated doubtful	4	$> 80^{++}$	63	4.7%	61.9%	27%	6.4%
Brucella infected	23	$> 80^{++}$	70	-	2.9%	10%	87.1%

TABLE 9

COMPARISON OF MILK RING AND WHEY TESTS IN CATTLE OF DIFFERENT VACCINATION AND INFECTION STATUS

Status of vaccination and of Brucella infection	Number of animals in group	Sero-agglut-inin titres	Number of samples examined	Results of Ring and Whey agglutination tests			
				M.R.T. ± Whey -	M.R.T. + Whey -	M.R.T. ± Whey +	M.R.T. + Whey +
Calfhood-vaccinated Brucella-free	38	$<40^{++}$	141	84.4%	11.7%	0.7%	3.2%
Adult-vaccinated Brucella-free	47	$<80^{+++}$	238	46.4%	47.7%	-	5.9%
Adult-vaccinated doubtful	4	$>80^{++}$	63	16.6%	50%	1.7%	31.7%
Brucella infected	23	$>80^{++}$	70	1.4%	1.5%	4.4%	92.7%

Table No.8. compares the results obtained by the M.R.T. and whey tube agglutination test when applied to the same milk samples analysed in Table 7. For this comparison any whey agglutinin titre falling under $1:10^{+}$ was considered as negative.

The general points of interest arising from the consideration of these two tables are:

1. Both the M.R.T. and whey agglutination test were by and large negative (96.1%) when applied to the milk of calfhood-vaccinated non-infected cattle provided the level of whey agglutinins was taken as significant from $1:10^{+}$ and higher. In this case, the whey agglutinin test appeared to give a clearer negative picture than the ring test which was falsely positive in 11.7% of the cases. These results are comparable to those of McDiarmid et al. (1958) and of Boyd et al. (1960) who found that vaccination at 6 months of age did not seriously affect subsequent milk agglutination tests in the third lactation. The 3.9% positive whey agglutination tests in the calfhood-vaccinated brucella-free group may have arisen from an arrested field infection or from the fact that the stage of lactation in these cases might have been misjudged and those cows were going dry earlier than expected.

One of the serious drawbacks milk tests suffer from is that it becomes unreliable with advancing lactation or gestation. In dairy breeds the stage of lactation of individuals is better known and generally last longer than in beef breeds. Thus, not only is the availability of milk for testing limited in beef cattle but the judgment of their exact stage of lactation more difficult.

2. In the adult-vaccinated Brucella-negative group of cows the whey agglutination test is as reliable as in the calfhood-vaccinated group (Table 8) if whey agglutinin titres were taken as significant at 1:10 or higher.

It is noticeable though that the proportion of low whey agglutinin (1:2 - 1:5) positive samples (Table 7) increased sharply in comparison to that of calfhood-vaccinated cows. This finding is at some variance with that of Kerr and co-workers (1958), who reported that 13 heifers and 35 cows with serum titres of 1:20 or less when vaccinated with S19 vaccine returned to completely negative whey titres 12 weeks after vaccination. The usefulness of the M.R.T. was extremely limited because of the high proportion of positive results (47.7% - Table 8) in this group of cows. McDiarmid et al. (1958) reported that in a group of cattle vaccinated

at 18 months of age 194 out of 327 milk samples (59.3%) were positive to the M.R.T. when examined towards the end of their third lactation. McKinnon et al. (1961) found 7.3% false M.R.T. positive milk samples in a group of calfhood-vaccinated cows. In their adult-vaccinated group 19.7% of the results of M.R.T. were falsely positive, considerably less than that shown by McDiarmid et al. (1958) or the results presented here. These findings are in contrast to Marr's and Williams' (1958) who found that S19 vaccination of adult cattle did not affect the M.R.T. carried out on their milk over a period of 15 weeks subsequent to vaccination. Boyd et al. (1960) also showed that adult-vaccination was associated with a higher incidence of ring test positive tests and this difference was satisfactorily significant.

3. In the very small adult-vaccinated group of cattle of doubtful status of brucella infection one third of the milk samples were positive to both milk ring and whey agglutination tests (Table 8). The milk of these cattle was examined culturally and by guinea-pig inoculation for the presence of brucella. Eight milk samples from each cow were examined in the course of 8 weeks but none of the milk samples in this group yielded brucella.

4. In the brucella-infected group 92.7% of the samples were positive to both milk ring and whey agglutination tests, a result almost identical to those obtained by Blake and co-workers (see: W.H.O. Technical Report Series (1953) 67 p.26) and somewhat higher proportion than shown by McKinnon et al. (1961) who found 76.8% of milk samples positive to the whey agglutination test at $1:10^{++}$ or higher in brucella infected cattle.

The effect of stage of lactation and of gestation on the whey agglutination test

The results of whey agglutination tests accumulated during this investigation are comprised in 3 tables (10, 11 and 12: Table No.9 was omitted as it did not add materially to the information contained in Table 10) in an attempt to analyse the effect of the stage of lactation and of pregnancy on the whey agglutination test.

Table 10 shows the effect of the stage of lactation on the whey agglutination test of all adult-vaccinated brucella-negative cows. The point of interest in Table 10 is the sharp drop of whey agglutination positive samples around mid-lactation.

Table 11 shows the results of whey agglutination tests of calfhood-vaccinated brucella-negative cows with reference to the stage of lactation.

It will be seen in Table 11 that the fall in whey positive milk samples around mid-lactation does not apply to the milk of calfhood-vaccinated animals. The highest percentage of whey positive milk samples was obtained during the first weeks of lactation. Two of these samples were taken within 10 days after parturition.

TABLE 10

MILK TEST AND LACTATION

Adult vaccinated Brucella-negative cows.

Weeks of lactation	1 - 8	9-16	17-24	25-32	33	Total
No. of tests	57	52	62	86	61	318
No. of +ve*	15	10	0	5	8	38
% of +ve*	26	19	0	6	13	12

* Agglutination at 1:10 or higher

TABLE 11

MILK TEST AND LACTATION

Calfhood-vaccinated Brucella-negative cows.

Weeks of lactation	1 - 8	9 - 16	17 - 24	25	Total
No. of tests	46	29	37	37	149
No. of +ve *	4	1	1	1	7
% of +ve*	8.6	3.4	2.7	2.7	4.6

* Agglutination at 1:10 or higher

In this group of animals there was again no evidence of rising whey titres with advancing lactation.

Table 12 shows the effect of the stage of gestation on the whey agglutination test of milk of calfhood-and adult-vaccinated cows.

TABLE 12

WHEY TEST AND GESTATION

All calfhood and adult-vaccinated cows other than those from which *Br. abortus* was isolated.

Weeks of Gestation	1 - 8	9 - 16	17 - 24	25 - 32	Totals
No. of tests	89	122	65	30	306
No. of +ve*	6	11	12	7	36
% of +ve*	6.7	9	18.4	23.3	11.7

* Agglutination at 1:10 or higher

It is apparent from Table 12 that with advancing gestation the proportion of whey agglutinin positive milk samples increases. During the second half of pregnancy the percentage of positive samples exceeds the average (11.7%) and the highest proportion of whey agglutinin positive milk samples were obtained towards the end of gestation. (This finding is in good agreement with Boyd et al. 1960; and supports the view of Stableforth et al. 1959). However, there was no evidence whatever to show that in the later stages of lactation séro-globulins would infiltrate into the milk influencing its antibody titre. It may be that the whey agglutination test as it was performed was not a sufficiently sensitive method of showing a slight rise in the antibody content of the milk. The very high proportion of milk samples positive to the M.R.T. throughout the whole of lactation made the use of this test, as an alternative to the whey agglutination test, impracticable for the examination of this point.

Conclusions

Considering the value of the milk ring test or whey agglutination tests, when used parallel to repeat serum agglutination tests for the detection of infected individuals it may be said that their usefulness was limited. Only in a single case did the whey agglutination test indicate a cow to be infected with a borderline serum and negative mucus agglutinin titre. All the rest of the reactors were shown as such by repeat serum agglutination tests alone. There was some evidence to show that the later stages of gestation influenced the whey agglutinin titres resulting in some rises therein but the stage of lactation did not seem to affect whey agglutinin titres.

If only the milk ring and whey agglutination tests were relied upon for diagnosis, over 97% of infected animals would have been eventually detected, a very good record, but at the same time they would have incriminated 3.9% of calfhood and 5.9% of adult-vaccinated animals free of field infection. It has to be pointed out, however, that the shortage of milk samples from most of the beef cows - resulting from the short period of lactation - would have seriously delayed making a diagnosis possible

and this would have rendered milk tests as the only means of diagnosis impracticable. The whey agglutination test was more useful as a moderator especially at interpreting the serum agglutination titres of adult and calfhood-adult-vaccinated cows. A high proportion of these cows had a serum titre >80++ at one time or another. In such a case a whey agglutinin titre of <1:10 prevented the cow being sent to the isolation unit for infected animals. Repeat blood serum, whey and mucus agglutination tests usually proved such a moderation justified.

The vaginal mucus agglutination test for the diagnosis of bovine brucellosis

Introduction

Jepson and Vindekilde (1951) examined fluid from the uterine mucosa of several brucella-infected cows and found a higher agglutinin titre therein than in their blood serum. A technique for collecting cervico-vaginal mucus for test by the vaginal tampon was first described by Szabo (1951) and the method was referred to in the 3rd report of the W.H.O. Technical Report Series (1958).

In Great Britain the vaginal mucus agglutination test for the diagnosis of bovine brucellosis was described by Kerr in 1955. He reported that hyper-immunisation of cows by intramuscular injection of either live or alcohol precipitated S19 vaccine did not cause antibodies to appear in the mucus of the uterus or of the vagina except maybe for a few days around parturition. However, the introduction of S19 vaccine into the uterus induced the local production of antibodies which could be demonstrated in uterine washings or in the vaginal mucus by the agglutination test. As the vaginal mucus

agglutination test remained negative after vaccination with S19 he suggested that a positive reaction indicated field infection with virulent organisms. His findings were confirmed later by both Romvary (1955) and Schmid (1957).

The vaginal mucus agglutination test was subsequently widely applied in Northern Ireland. It was shown (Kerr et al. 1958) that its main value is as a positive indicator of field infection for a negative test cannot be taken as evidence of freedom of field infection. It does not suffer from the limitations the whey whey agglutination test does (stage of lactation or of gestation, mastitis, etc.) and a positive test almost certainly indicates that the uterus and its associated lymphatic glands are infected by a field strain.

Results and Discussion

In the course of the investigation reported here the vaginal mucus agglutination test was routinely used for diagnosis. For obtaining the samples the vaginal tampon method of Szabo (1951) was used during the first year, whereas during the last two years the vaginal pipette method of Pierce (1949) was adopted for use.

As a result of certain difficulties, the collection of mucus from infected animals was brought to an end after the first year and those few samples collected later were from cows which became infected after the first year.

Table 13 summarizes the results of mucus agglutination tests during the period of 3 years of herd testing.

The following points arise from Table 13:

1. During the first year when mucus samples were collected by the vaginal tampon from the calfhood and adult-vaccinated brucella-free groups over 10% of the tests were falsely positive to the agglutination test.

TABLE 13

RESULTS OF MUCUS AGGLUTINATION TESTS OF BRUCELLA-FREE AND INFECTED CATTLE

Status of vaccination and of brucella infection	No. of animals in group	Sero-agglutinin titres	No. of samples examined		Results of v.mucus agglutination test			
					1st year *		2nd & 3rd year ***	
			1st yr.	2nd - 3rd year	-ve	+ve	-ve	+ve
Calfhood vaccinated Brucella-free	38	< 40++	168	165	86.9**	13.1	99.4	0.6
Adult-vaccinated Brucella-free	47	< 80++	189	171	89.3	10.7	99.42	0.58
Brucella-infected	25	> 80++	175	0	23.5	76.5	0	0

* = percentage: ** = sample obtained by v.tampon: *** = sample obtained by v.pipette

2. During the same period in the infected group 23.5% of the mucus samples were negative to the agglutination test. However, almost one half of this figure represents false negative titres, for it was a common occurrence to obtain a negative titre between two positive ones for the same cow within a period of a few months. Using the vaginal tampon for obtaining mucus samples it was necessary to transfer the tampon into a measured quantity of sterile saline to prevent drying in transit to the laboratory. As a result of this practice it was impossible to ascertain in the laboratory how much mucus if any was contained in the tampon. Thus the false negative mucus agglutinin titres of uterine infected cows was almost certainly brought about by the shortage or complete lack of mucus in the tampon.

3. During the 2nd and 3rd year the proportion of false positive results in the two brucella-free groups of cows fell to a negligible level (0.60 and 0.58% respectively - Table 13) as a result of obtaining the samples by the vaginal pipette.

This method of collecting samples suffers from apparent inefficiency. The number of mucus samples

collected from either the calfhood or from the adult-vaccinated cows during the last 2 years were fewer than those collected by the vaginal tampon from the same groups in the first year.

Conclusions

Assessing the value of the mucus agglutination test as applied in this herd parallel to repeat serum agglutination tests for the detection of infected cattle, it may be stated that its use was rather limited. Samples collected by the vaginal tampon were unreliable giving too high a proportion of false positive titres in non-infected cattle. Attempts to obtain mucus by the vaginal pipette method were often unsuccessful although if a sufficient quantity was secured it was a reliable specimen for serological examination giving only 2 false positive reactions out of 336 tests. The great majority of infected cows (20/30) gave a positive mucus agglutinin titre, although repeat serum agglutinin test alone furnished sufficient evidence of field infection before a positive mucus titre was obtained.

The ratio of whey and mucus agglutinin positive cows in the infected group was 1:1 although 3 heifers

became mucus agglutinin positive 1 - 4 months prior to parturition.

The real value of the mucus agglutination test, just like that of the whey test, becomes apparent in evaluating the sero-agglutinin titre of adult-vaccinated cows. Serum titres of the individual in this class may exceed the 1:80++ mark periodically then fall to a non-reactor level after a while. Such changes in the serum titres together with negative mucus and whey titres ($\angle$1:10) did not appear to be of concequence in this herd.

Serum agglutination test for the diagnosis of bovine brucellosis

Introduction

In 1897 Wright and Smith introduced the serum agglutination test for the differentiation of Malta, Typhoid and Malaria fevers. The technique soon found acceptance in the diagnosis of brucellosis (Zammit, 1905; Kennedy, 1905) and for a long time it has been one of the major means of detecting brucella infection in man and animals.

The international uniformity of the test as applied to bovine brucellosis was secured by the adoption of reference serum (W.H.O. Technical Report Series (1953) 60) thus results of serum agglutination tests obtained in different parts of the world become comparable. Although the means of diagnosis has remained a matter of choice, in Great Britain it was the tube serum agglutination test which found the widest acceptance. As a result of much research work significant levels of serum agglutinin content of cattle of different categories have been determined and agreed upon by the joint FAO/WHO Expert Committee on Brucellosis (Third Report 1958).

The detection of brucella infected cattle in a non-vaccinated population is a comparatively simple matter, the serum agglutination test alone may be sufficient. To make an accurate diagnosis on blood test alone is virtually impossible in herds where uncontrolled adult or multiple-vaccination with S19 has been carried out (Kerr, 1960).

The age at vaccination affects the persistence of agglutinin titres. Hardenbergh (1939) showed that in the absence of a natural exposure less than 0.5% of heifers vaccinated when 6 - 8 months old reacted positively 2 years after vaccination. The observations of Haring and Traum (1941) are relevant here. They investigated the sero-agglutinin reactions of a substantial number of animals vaccinated at different ages. Two years after vaccination the results were as follows:

Vaccinated at 4 - 8 months	99% negative
Vaccinated at 8 - 12 months	91% negative
Vaccinated at 12 - 16 months	83% negative
Vaccinated over 16 months	50% negative

In a later experiment (Haring and Traum, 1943) they vaccinated 752 animals at various ages. The animals were divided into 5 age groups at the time of vaccination as summarised below:

In the adult-vaccinated group approximately one third (30%) of the animals had titres in excess of $1:80^{+}$ one year after vaccination which proportion remained virtually unchanged (30.7%) at the end of the second year.

The highest proportion of persistent titre cows was found in the calfhood and adult-vaccinated group, approximately half of them being positive (48%) to the agglutination test two years after vaccination.

Fluctuation of serum agglutinin titres of cattle

Although a proportion of each vaccination group remained serum positive for a long time after vaccination their titres were not consistently positive as there was some degree of fluctuation of titres.

Table 16 shows the degree of changes in sero-agglutinin titres for the different vaccination groups during the 3 years of testing. Results for this table were disregarded until the serum titre of the individuals fell to their lowest level after vaccination and changes occurring only after such time were taken into account.

TABLE 16

FLUCTUATION OF SERUM AGGLUTININ TITRES OF CATTLE VACCINATED AT DIFFERENT AGES (ALL FREE OF FIELD INFECTION)

Vaccination History	No. in groups	Changes of serum titres in terms of doubling dilutions						
		0	<1	1	>1-<2	2	>2-<3	3
Calfhood (1)	37	*70.4	13.5	5.4	2.7	0	0	0
Calfhood (2)	37	16.2	0.1	8.1	29.8	13.5	18.9	5.4
Adult	33	18.2	12.1	15.1	24.3	9.1	18.2	3.0
Calfhood and Adult	29	0	0	6.9	55.1	17.3	17.3	3.4

Calfhood (1) = born and vaccinated after segregation of infected cows.

Calfhood (2) = born and vaccinated before segregation of infected cows.

* = percentage of total agglutination tests obtained in 5 years, showing a particular degree of fluctuation of titres.

0 = no change in titre.

<1 = changes in serum titres amounting to less than one fold of serum dilution.

1 = changes in serum titres amounting to one fold of serum dilution.

>1-<2 = changes in serum titres amounting to more than one fold but less than two folds of serum dilution, etc.

TABLE 17

THE EFFECT OF THE STAGE OF GESTATION ON THE SERO-AGGLUTININ TITRES OF ADULT-VACCINATED BRUCELLA FREE COWS

Time selected during pregnancy		No. of pregnancies	1st half of pregnancy				2nd half of pregnancy			
			Serum agglutinin titres							
1st Half	2nd Half		$10-20^{++}$	$20^{+++}-40^{++}$	$40^{+++}-80^{++}$	$80^{+++}-160^{++}$	$10-20^{++}$	$20^{+++}-40^{++}$	$40^{+++}-80^{++}$	$80^{+++}-160^{++}$
Highest one	Highest one	40	15*	32.5	35.0	17.5	17.5	12.5	47.5	22.5

* = percentage

The points of interest arising from Table 16 are as follows:

1. Calfhood-vaccinated cattle born in a brucella free herd exhibited the fewest changes in their serum titres, the great majority of them (78.4%) showing no fluctuation.

2. Calfhood-vaccinated animals brought up in an infected herd exhibited much the same degree of fluctuation of serum titres as did the adult-vaccinated group.

3. Most of the changes in the frequency and the extent of fluctuation were recorded in the calfhood and adult-vaccinated group. None of the 29 cows had a constant titre but 93.1% of them had changes in their serum titres amounting to more than one fold of serum dilution.

The effect of the stage of gestation on sero-agglutinin titres

To determine whether there was any change in serum agglutinin titres with stage of gestation Table 17 was compiled. There was not enough information available in each case to present data for short intervals of pregnancy and consequently information is presented for the 1st and 2nd calves of gestation. The highest serum agglutinin

TABLE 18

COMPARISON OF THE HIGHEST TITRE OBTAINED FROM NON-INFECTED ADULT VACCINATED COWS WITH THE LOWEST TITRE FROM INFECTED VARIABLY VACCINATED COWS.

	Total	Number of cows with titres of																	
		1:10		1:10		1:20		1:40		1:80		1:160		1:320		1:640		640	
		No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Non-infected adult-vaccinated highest titre	44	0	0	8	18.1	3	6.9	9	20.5	17	38.7	5	11.3	2	4.5	0	0	0	0
Infected variably vaccinated lowest titre	38	0	0	0	0	0	0	2	5.3	3	7.9	6	15.8	13	34.2	10	26.3	4	10.5

titres occurring during the first and during the second half of pregnancy were taken for 31 animals with 40 pregnancies.

On the basis of a comparison of this kind it would appear that there was no significant increase in sero-agglutinin titres with advancing gestation. During the first half of pregnancy 17.5% of the titres were in excess of 1:80^{++} and the proportion of titres in the same category increased 22.5% during the second half of gestation.

Overlapping of sero-agglutinin titres of brucella-free and of infected cows

It has been demonstrated that a considerable proportion of adult-vaccinated cows become so-called persistent titre cows with sero-agglutinin titres which may fall and rise at different times. To underline the difficulties of interpreting titres of such cows a comparison of the minimum and maximum sero-agglutinin titres of brucella infected and non-infected cows (all adult-vaccinated) are summarised in Table 18.

In the adult-vaccinated brucella negative group of cows (all of them consistently negative to the whey and

vaginal mucus agglutination test, vaccinated at least 1½ years prior to this investigation) the ~~lowest and~~ highest serum agglutinin titres of each individual were taken as they occurred during a period of 3 years. In the infected group only titres obtained after the cows were known to be infected were considered.

Table 18 clearly shows the degree of overlapping of sero-agglutinin titres of adult-vaccinated brucella-free, and of infected cows. Of 44 adult-vaccinated cows 54.5% gave a serum titre of 1:80 or higher at one time or another during the period of 3 years. During the same time 29% of 32 brucella infected cows gave serum titres of 1:160 or less as the lowest value in the range of their sero-agglutinin titres.

Discussion and summary of results

In the foregoing section an account has been given of the serum titres obtained in animals vaccinated with Strain 19 at different ages. It was shown that a brucella infected environment had a measurable effect on the persistence of sero-agglutinin titres of calfhood-vaccinated cattle. This observation supports the findings of Birch et al. (1944) who reported that post-vaccination serum titres persisted for more than 9 months in 22.2% of 45 calf-vaccinated heifers which were kept experimentally in an environment which provided frequent exposure to virulent Br. abortus.

Wight (1942) found that under field conditions in 260 infected herds on an average 4.1% of calf-vaccinated cows were positive when tested after their first calving. Haring et al. (1943) writing about the persistence of agglutinins in the sera of calfhood vaccinated animals remarked that "if the animals had been in herds free from brucella infection the percentage of those whose titres become negative may have been greater". Whether calves infected before reaching sexual maturity can remain permanently infected is not quite certain. Rettger et al. (1918) reported that calves up to 8 months of age, with few exceptions, are resistant to field infection. Resistance

in unvaccinated heifers then gradually decreases as they reach sexual maturity. Carpenter (1924) was able to obtain brucella from the excretions and from the tissues of calves fed on infected milk. Such calves, however, usually came clear of infection within a few months after the feeding of brucella contaminated milk ceased.

Information relevant to this question is unfortunately very limited in this herd. Eight animals in all were present in the herd which were born 8 months and vaccinated 2 months prior to the removal of all the known infected cows. In other words these calves were born and vaccinated in an infected environment but unlike most of such other calves their serum agglutinin titre was tested regularly, shortly after vaccination and they were just 8 months old when the removal of brucella positive cows was carried out. The likelihood of their being exposed to a heavy infection of Brucella abortus after 8 months of age thus became rather remote. Five of the eight calves lost their vaccine titre and fell just under 1:40^{++} one year after vaccination. The other three maintained a borderline or periodically positive titre right through from after vaccination but their whey and

Vaccinated at 4 - 6 months	100% negative 2 years after vaccination
Vaccinated at 6 - 8 months	90% negative 2 years after vaccination
Vaccinated at 8 - 10 months	75% negative 2 years after vaccination
Vaccinated at 10 - 16 months	65% negative 2 years after vaccination
Vaccinated over 16 months	15% negative 2 years after vaccination

It is apparent from this summary that with increasing age at vaccination the proportion of positive titres increases. Over 8 months of age at vaccination a significant proportion of animals may maintain a positive titre long after vaccination. The problem of persistent vaccinal reaction resulting from over-age vaccination has been widely appreciated. (Huddleson, 1942; Cameron and Kendrick, 1957; Plastridge, 1954; McDiarmid, 1957 and 1960; Kerr et al. 1958; Kerr, 1960; Stableforth et al. 1959; Stableforth, 1960).

It has been a continuing source of confusion whenever serum agglutination test has been used for diagnosis in late calfhood or adult-vaccinated herds. It has been pointed out (Kerr, 1960) that a single blood test in the presence of vaccination is of very limited use. Unless a series of agglutination tests on the sera of such animals are carried

out to determine the possible loss of titre an accurate assessment of their state of infection cannot be reached and even then doubt exists as to their titre being vaccinal or field strain in origin.

In the herd, during the three year period of eradication the distribution of animals according to their history of vaccination was as follows:

Calfhood-vaccinated only	125
Adult " "	34
Calfhood and adult-vaccinated	50

The mixed nature of vaccination provided an opportunity to study the effects of vaccination of different age groups on the serum agglutinin titres and the degree of interference in diagnosis adult-vaccination may bring about.

Influence of environment on the persistence of sero-agglutinins of calfhood-vaccinated cattle

As previously stated, vaccination of animals over 9 months of age was brought to an end 1½ years prior to the beginning of eradication. Thus in the herd, the calfhood-vaccinated animals could be sub-divided into two groups. To the first group belong those animals which were born and vaccinated while there was wide spread infection in the herd. The second group consists of animals which were born and vaccinated after the segregation of all the known infected cows.

Table 14. is an attempt to show the influence of environment on the sero-agglutinin reactions of calfhood-vaccinated animals.

It seems quite clear from Table 14 that the infected environment had a profound influence on the persistence of sero-agglutinins of calfhood-vaccinated animals.

The titres of all of the 38 calves, born and brought up in a herd free of clinical brucellosis, fell under 1:40*** six months after vaccination and remained negative thereafter. About one quarter (22.3%) of those calves which were brought

TABLE 14

SERUM AGGLUTINATION REACTIONS RESULTING FROM CALFHOOD-VACCINATION IN BRUCELLA INFECTED AND BRUCELLA FREE ENVIRONMENT

BORN AND VACCINATED		Time after vaccination											
		2 - 4 weeks			4 months		6 months		1 year		2 years or over	
		$< 160^{++}$	160^{++} to 640^{++}	$> 640^{++}$	$< 40^{++}$	$> 40^{++}$	$< 40^{++}$	$> 40^{++}$	$< 40^{++}$	$> 40^{++}$	$< 40^{++}$	$> 40^{++}$
before segregation of infected cattle	No.	-	-	-	9/14	5/14	14/18	4/18	42/48	6/48	44/52	8/52*
	%	-	-	-	64.3	35.7	77.7	22.3	87.5	12.5	84.6	15.4
after segregation of infected cattle	No.	7/65	40/65	18/65	44/48	4/48	38/38	0/38	32/32	0/32	4/4	0/4
	%	10.8	61.5	27.7	91.6	8.4	100	0	100	0	100	0

- = Not known

* = Highest sero-agglutinin titre in these groups is $1:80^{++}$ with -ve whey and mucus agglutinin titres.

up in an environment where exposure to field infection was a very likely possibility retained a positive titre 6 months after vaccination. Even at the time of first calving over 15% of these cows gave a low positive serum titre without any other evidence of their being infected by a field strain of Brucella abortus.

Serological response to S19 vaccination of calves neonatally exposed to virulent brucella under field conditions

There was another aspect of serological response following S19 vaccination of calves. During the time of this investigation 7 heifer calves were born to, and suckled, infected cows in the isolation unit. All of these calves had very high titres (1:1280 or higher) in the neonatal period and three of them had titres ranging from 1:10 to 1:40 just prior to vaccination. On injection with S19 vaccine the serological response of these calves was somewhat inferior ranging from 1:40^{++} to 1:160^{+} when tested repeatedly during the 30 days following vaccination. These titres fell to 1:20 under two months after vaccination. The other four calves born to infected cows had no sero-agglutinin titre at vaccination and gave an agglutinin titre

on vaccination comparable to that of those calves born to brucella negative cows. To follow up the histories of two of the three calves which responded poorly to vaccination became impossible as they were sold. It may be, however, of importance that the third one remaining in the herd aborted towards the end of the 7th month of gestation. Unfortunately no post-partum materials were available for bacteriological examination thus the nature of abortion could not be ascertained. Serologically this cow remained a non-reactor having had a titre of less than 1:10 prior to abortion which rose to 1:10^{++} a few days after abortion. As the question of exposure of young calves to field infection and its consequences could not be satisfactorily examined under field conditions it became the subject of an investigation which will be described later.

Persistence of sero-agglutinins resulting from vaccination of cattle at different ages

To compare the persistence of sero-agglutinins of cattle vaccinated at different ages Table 15 was compiled. All the cows included in the 3 groups (Calfhood, Adult and Calfhood-Adult vaccinated) were believed to be free of field

TABLE 15

PERSISTENCE OF SERO-AGGLUTININS RESULTING FROM VACCINATION OF CATTLE AT DIFFERENT AGES

Vaccination History		Time after vaccination			
		1 year		2 years	
		-ve[x]	+ve[xx]	-ve[x]	+ve[xx]
Calfhood Vaccinated	No	74/80	6/80	48/56	8/56
	%	92.5	7.5	85.8	14.2
Adult Vaccinated	No	7/10	3/10	18/26(+)	8/26(+)
	%	70-	30-	69.3	30.7
Calfhood and Adult vacc.	No	N	N	13/25	12/25(·)
	%	N	N	52	48

x $<1:40^{++}$ and $<1:80^{++}$ for calfhood and adult-vaccinated animals respectively.

xx $>1:40^{++}$ or higher and $>1:80^{++}$ for calfhood and adult-vaccinated animals respectively.

N = Not known

(+) = Maximum sero-agglutinin titre is $1:160^{++}$ with consistently negative whey and vaginal mucus agglutinin titres.

(·) = Maximum sero-agglutinin titre $1:320^{+}$ with consistently negative whey and vaginal mucus titres.

infection with consistently negative whey and vaginal mucus agglutinin titres.

It was shown in Table 14 that calves born and brought up in an environment free of the dangers of gross exposure to field infection lost their serum titres comparatively quickly falling under 1:40++ six months after vaccination and remaining negative thereafter.

In Table 15 the calfhood-vaccinated group contains all the calfhood-vaccinated animals irrespective of whether they were born prior to or after segregation of infected cattle. As a result of this grouping 7.5% of calfhood-vaccinated animals are positive to the serum agglutination test one year after vaccination.

Two years after vaccination the proportion of positives to the agglutination test doubles (14.2%). The explanation for this increase lies in the fact that whereas 38 out of 80 animals examined one year after vaccination were born after the eradication of brucellosis, only four of such animals could be included in the group examined two years after vaccination.

vaginal mucus titres remained negative. It would seem therefore that these 3 calves may have become infected under the age of 8 months, resulting in a persistent serum titre but without any evidence of clinical infection.

The analysis of the persistence of sero-agglutinin titres of cattle vaccinated either as calves or as adults or repeatedly as calves and adults gave comparable results to those summarised by Stableforth et al. (1959).

More than 92% of calfhood vaccinated animals gave titres less than 1:40^{++} one year after vaccination. 70% of cows vaccinated only once as adults became non-reactors one year after vaccination, but those which were positive at this stage remained positive even 2 years after Strain 19. vaccination. The highest proportion of persistent vaccinal titres were found in the repeat-vaccinated group. Almost 50% of these had a borderline or low positive titre two years after vaccination.

Cows showing a vaccine titre also showed titre fluctuation. The least fluctuation was shown by calfhood-vaccinated cows reared in a brucella infection free herd. Of 37 such cattle 8.1% had changes in their titre amounting to one or more than one fold of serum dilution. In contrast 75.7% of calfhood-vaccinated cows brought up in a brucella

infected herd had rises and falls in their serum titre amounting to one or more than one fold of serum dilution. Cows vaccinated once as adults were not appreciably different (69.7%) from the latter calfhood-vaccinated group but 100% of the repeat-vaccinated cows fluctuated in their serum titre one or more than one fold of serum dilution.

These changes in the blood titre were a matter of concern and a source of anxiety during the time of the eradication. Fluctuation of titre often caused an adult-vaccinated cow with a borderline titre to become a positive reactor. If this rise of titre coincided with advanced gestation it was necessary to regard it as a possible indication of impending abortion and this in turn necessitated the isolation of such cattle either until after calving or until a fall of titre occurred bringing it back to a non-reactor level.

Goode et al. (1954); Cameron and Kendrick (1957) and Korr (1960) appreciated the fact that titres of adult-vaccinated cows undergo changes periodically which may lead to false impressions. Mingle (1955) in the United States pointed out that temporary rises of residual vaccine titres was a disturbing factor and seriously interfered with diagnosing the exact brucella status of herds.

A considerable proportion (29%) of brucella infected cows at one time or another gave serum agglutinin titres comparable to those given by more than 54% of adult-vaccinated non-infected cows when the latter gave the highest of their residual blood titres. From this considerable degree of overlap alone the disadvantage of adult-vaccination and the resulting persistent titre cow will be readily appreciated. Kerr's analysis (1960) of the problem of persistent titre cows is relevant here. He pointed out that this problem assumes importance at the terminal stages of eradication programmes where adult-vaccination has been carried out. "In a brucella infected herd the persistent titre cow is assumed to be one where there is a balance between the disease progressing and of its being completely overcome. In the vaccinated animal, the superimposition of a light infection appears to arrest the slow fall in the blood titre which should normally occur, with the result that the titre remains relatively constant for 12 to 18 months, without evidence of agglutinins in the milk or mucus". "The majority of the animals would eventually lose their persistent titres without shedding the organisms, but some after a long period, perhaps two years, suddenly show a sharp rise in titre with evidence of the organisms in the milk or uterus or in both. Very closely connected with the possibility

of the latent infected persistent titre cow is the state produced by vaccinating the adult cow with Strain 19. An initial sharp rise in antibody formation occurrs following the multiplication of the vaccine organism in the body, but the titre may not fall so rapidly as in calfhood-vaccination or reach as low a level. In some cows it persists at a high level, e.g. 1:80 and over and may fluctuate at certain periods, e.g. at one test a rise of one or more dilutions may take place then the titre falls again in the next test".

Such an interpretation of the persistent titre cow whether field or vaccinal strain in origin would seem to explain the serological behaviour of many of the vaccinated cows. Even a complete understanding of the underlying causes resulting in the persistent titre cow does not minimise the dangers inherent in this situation. A rigid application of the criteria as applicable to the interpretation of serum titres may be too costly, causing the slaughter or disposal of many adult-vaccinated cows which might never constitute any danger for the rest of the herd. The examination of milk and vaginal mucus samples may be of considerable help in this situation although their becoming positive might come too late after the damage had been done by a brucella abortion.

Therefore in order to bring an end to the anomalies resulting from over-age vaccination one of the following procedures must be adopted:

1. Adult-vaccination must stop and S19 vaccine be used only for calves under 9 months of age.

2. A non-agglutinogenic vaccine giving at least as good immunity as S19 must be found which may be used for cattle of all ages.

3. A method, serological or otherwise, must be evolved capable of differentiating between vaccine titres and those resulting from superimposed field infection.

Non-specific anamnestic reaction in brucella agglutinating system

Introduction

It was shown in Table 16 that the sero-agglutinin reactions of cattle with residual titres resulting from Strain 19 vaccination exhibited a certain degree of fluctuation. It was also shown (Table 17) that advancing gestation did not appear to be a major factor involved in these changes although a rise of titre did often coincide with advanced pregnancy. Pregnant animals (among others) with an earlier non-reactor or inconclusive titre often became positive reactors in this way. From the practical point of view a rising titre, even if the increase was moderate, had to be regarded as a possible sign of impending abortion, which in turn necessitated the segregation of such cattle from the rest of the herd.

Rising antibody titres specific to a pathogenic agent have been regarded as evidence of active infection (Carpenter, 1956). Most of these rising titres occurring in the herd, however, fell to a non-reactor level when tested at a later date. Although this was the general tendency exceptions did occur when rising titres did

indicate impending abortion and only removal of the cow prevented abortion taking place among the brucella-negative cows. In this case the titre rose to a higher degree than the one to two folds of serum dilution characteristic of fluctuation.

An experiment was set up to examine the role of one of the possible causes for these changes in serum antibody content. The aim of the experiment was to eliminate the possibility of chronic Strain 19 infection of vaccinated cattle being the cause of fluctuations. It was assumed that by injecting into cattle a substance antigenically unrelated to brucella the envoked serological response, once levelled out, could be used as a control system for the interpretation of changes in the brucella titres. It was reasoned that by testing the agglutinin titres of the two unrelated systems parallel to each other the tendencies in rises and falls in titres would throw light on the problem.

If, for example, rises and falls of titres in the two agglutinating systems ran parallel to each other it would be taken as an indication that factors other than chronic Strain 19 infection were responsible for the

fluctuations. Alternatively, if a consistent slow fall of titre in the control system was accompanied by rises and falls in the brucella system the possibility of Strain 19 infection being responsible for fluctuating brucella titres could not be excluded.

Experimental methods

Antigen

For the antigen in the control serological system B.cereus M.8 (Mahood, 1955) was chosen. To obtain young vegetative cells the organism was grown on nutrient agar for 8 hours and then harvested in 0.1% formol saline. The cells were washed in formol saline before use. For the immunisation of rabbits and cattle and for carrying out "H" agglutination tests, the cells suspension of B.cereus M.8. was adjusted to Brown's opacity tube No.8 and No.2 respectively. Standard brucella antigen at 1:10 dilution was used in the brucella agglutinating system as well as for testing cross-reactions with anti-sera produced against B.cereus M.8.

Animals

For the cattle experiment 4 calfhood and 5 adult-vaccinated cows were selected all of which had shown some degree of fluctuation in their brucella serum titre. The immune serum against B.cereus M.S. was prepared in two adult rabbits.

Immunization

Rabbits were immunised with 6 injections of 0.5 ml. each of B.cereus M.S. suspension. The injections were given intramuscularly at 3 day intervals. Seven days after the administration of the last injection the rabbits were bled from the ear vein and the separated sera were pooled.

The nine cows received two intramuscular injections, each of 5 ml. of B.cereus M.S. 25 days apart. Just prior to the first injection they were bled from the jugular vein and blood samples were similarly collected 7 days after the first injection and then weekly over a period of 2½ months.

Serological test

For checking the possibility of cross-reactions between the two systems the agglutination test was used. Serial doubling dilutions of B.cereus M.G. (agglutination titre for the homologous antigen 1:5,000) and of Brucella abortus (Burroughs Wellcome & Co., agglutinin titre 1:320) antisera were made in formol saline in duplicate. To each serum dilution an equal volume (0.5 ml.) of the heterologous antigen was added to give a final dilution of 1:5 in the first tube. Duplicate sets of tubes were prepared, the first set being incubated at 56° centigrade for four hours in a water-bath and the second set at 37°C for 24 hours in the incubator.

For testing the bovine sera the agglutination test was carried out in the conventional manner used in the diagnosis of bovine brucellosis except for the fact that each serum dilution was made up in duplicate. One of them receiving the standard brucella antigen and the other receiving B.cereus M.G. antigen. The tubes were incubated for 24 hours at 37°C and the result recorded as described

on page 30

TABLE 19

SERUM AGGLUTININ TITRES OF CATTLE (INJECTED TWICE WITH B.CEREUS M.8 ANTIGEN) TO BR.ABORTUS AND THE HOMOLOGUS ANTIGEN.

Cow No.	Status of brucella vaccination	Serum titres to:	Weeks 0	1	2	3	4	5	6	7	8	9	10
1	C	Br.abortus	10+	10+	10+	10+++	10±	80+++	80+	40+++	40+	20+++	20+++
		B. cereus	20±	40+	40+	40++	40++	80+	40+++	40+	40++	40+++	40++
2	C	Br.abortus	10+++	20++	10+++	10+++	10++	40+++	20+	20+	20++	20+	20+
		B. cereus	10+	40+	80++	40+++	40++	40+++	80+	40+	40+	20+++	10+++
3	C	Br.abortus	20+++	20++	20+	20+++	20+	ND	20++	20+++	40+	20+	20++
		B. cereus	<10	10++	20+	20+++	40+	ND	80+	40++	40+	40+	10++
4	C	Br.abortus	20++	10+++	20+	20++	20+	40+	40+++	40+++	80+	40+	20+++
		B.cereus	<10	40++	40+	40+	40+	40+++	40+++	40+++	40+	40+	10+++
5	A	Br.abortus	40++	40+	20+++	40++	20+++	40+	80+++	40+++	40++	40+	40+
		B.cereus	<10	40+	40++	20+++	20++	40+++	40+++	40++	20+	20+++	10++
6	A	Br.abortus	80+++	80++	80+	80+	80+	80+++	40+++	80+	80+	80++	40+
		B.cereus	<10	40++	40+	10+++	20+++	80+	80+	20+++	20+++	40++	20+++
7	A	Br.abortus	160+++	160+++	80+++	160+++	160++	320+	160++	160+	160++	160+++	160+
		B.cereus	<10	80++	40+++	10+	20+++	80+	40+++	20+++	20+++	20+++	20++
8	A	Br.abortus	20+++	20++	10+	20++	20+	40+	20+	10+++	20++	20+	10+
		B.cereus	10+	40+++	20+++	20+++	40+	40+++	80+	20+++	20+++	40+	20+++
9	A	Br.abortus	40+	20+	20+++	20++	20+	40+++	40+	40++	40+++	40++	20+
		B.cereus	20+	80+++	40++	40++	40+	40+++	80+	20+++	20+++	20+++	20+++

x = injection of B.cereus M.8. antigen
N.D. = not done
C = Calfhood-vaccinated
A = Adult-vaccinated

Results

Cross-agglutination tests carried out between B.cereus M.0 "H" antigen, and Brucella abortus antiserum on one hand and between Brucella abortus antigen and B.cereus M.0. antiserum on the other, did not reveal any signs of antigenic relationship between the two antigens. The results of the parallel agglutination tests for Br.abortus and B.cereus M.0. antibodies in bovine sera are recorded in Table 19.

The first injection of B.cereus M.0. did not elicit a significant agglutinin response to the homologous antigen. It was decided therefore to administer a second dose of the same antigen which was carried out between the third and fourth bleeding of the 9 cows (week 4 in Table 19). The serological response to the second injection was unexpected. It will be seen in the table that the agglutinin titre to the homologous antigen was only very slightly affected resulting in a one fold increase in serum titre or even less than that. The unexpected outcome was an almost general rise in the agglutinin titres in the brucella agglutinating system. The exception was cow No.7 where no significant changes in the brucella titres were noted. The most

significant change in the brucella titres occurred in cow No.1 where a rise of over 4 folds of serum dilution was recorded. For the rest of the cows the increases ranged between from 1 to 2 folds of serum dilution. As a result of these changes in brucella titres all four of the calfhood-vaccinated cows with non-reactor (less than 1:40^{++}) levels of sero-agglutinin titres prior to the second injection of B.cereus gave inconclusive (cow No.3) or positive titres (greater than 1:40^{++}) for varying lengths of time. Of the 5 adult-vaccinated cows, 3 had titres in the non-reactor (less than 1:80^{++}), one in the inconclusive (1:80^{+++}), and one in the positive reactor class respectively. After the second injection despite rises in the brucella sero-agglutinin titres only one of these cows (No.6) had a change of titre sufficiently high to place her from the non-reactor to the brucella-reactor class.

By the 5th week after the second injection of B.cereus M.6. antigen all the brucella agglutinin titres began to decline and at 6 weeks they had returned virtually to the levels existing before the B.cereus injection. The only exception to this was cow No.1 her titre took

twelve weeks to fall to pre-injection level. The weekly testing of sera of the 9 cows was ended ten weeks after the experiment began and thereafter the agglutination test for brucella only was carried out at two monthly intervals for at least one year. No after effects of the B.cereus injections on the brucella agglutinin titres were noted during this time.

Discussion

The choice of a B.cereus agglutinating system as a serological control for testing one of the likely causes of fluctuation, observed in the brucella agglutinin titres proved in a way to be unfortunate. Despite two injections of B.cereus antigen the serological responses evoked to this organism were poor. To secure long lasting high serum titres to B.cereus a course of injections would have been necessary. This could not be carried out without endangering, for an indefinite period of time, the use of the serum agglutination test for the diagnosis of Brucella abortus infection in these cattle.

The experiment, however, brought to light a non-specific anamnestic reaction manifested by increases in the brucella sero-agglutinin titres of cattle injected with B.cereus M.O. antigen. The injection of an apparently unrelated antigen causing an increase of circulating antibodies against an earlier immunising agent is not unknown phenomenen. Dreyer and Walker (1909) reported that the injection of killed staphylococci caused an increase in anticoli titres in 10 rabbits previously inoculated with E.coli. Carpenter (1966) enumerated examples of non-specific anamnestic reactions including infectious mono-nucleosis producing strongly positive Wassermann reactions in human patients showing no history of syphilis. Other non-specific anamnestic stimuli include injection of milk, casein, gelatine and peptone. The possible beneficial effects of the once employed non-specific protein therapy of certain chronic diseases like gonococcal arthritis and rheumatoid arthritis have been attributed to foreign substances inducing, fever, leucocytosis and general inflammatory reactions together with renewed formation or release of antibodies.

The observations reported here would fulfill the criteria of non-specific anamnestic reaction inasmuch as

the stimulating agent (B.cereus M.8) is apparently unrelated to Br.abortus and the increase of titre to brucella was short-lived. Elder and Rodabough (1951) reported that the experimental feeding of certain trace minerals did not affect the blood titre of cows to Babortus S19. Scheidy and Live (1957) studied the effects of injection of Leptospira pomona bacterin into Strain 19 vaccinated cattle. They concluded that such injections did not illicit an amnestic reaction as manifested in rise of titre to Br.abortus. Berman (1956) reported diagnostically significant increases in titres of agglutinins for Br.abortus following immunisation with P.multocida types C and D in cattle which had been vaccinated with S19. He suggested that decisions can be made on the significance of such rises in brucella titres if the sera are tested about one month after exposure to pasturella antigen. Simon (see Berman, 1956) found agglutinins to Br.abortus in sera of non-vaccinated cows inoculated intravenously with viable Vibrio fetus, but not in the sera of animals infected via the vagina.

Since the diagnosis of bovine brucellosis is largely based upon the serum agglutination test it is of importance to determine whether an injection of a given biological

product other then brucella vaccine, or infection with a pathogenic agent other than brucella may stimulate the production of agglutinins which could react with brucella antigens.

Cultural and biological examination of milk, vaginal mucus, post-partum material and autopsy specimens

The most satisfactory method of diagnosis of brucella infection, as in many other diseases, is the isolation and identification of the causal organism. Efforts were therefore made to carry out as many cultural and biological examinations of samples from the herd as was practicable.

Preliminary trials carried out on the selective medium (serum-dextrose-antibiotics agar, Brinley-Morgan, 1960. ~~See also page~~) containing 25000 u/l. of Bacitracin (Glaxo) showed that this medium did not support the growthe of all the biotypes of Br.abortus (S19, types 1, 2 and 3) examined. Replacement of the Glaxo preparation of Bacitracin (25000 u/l) by Bacitracin manufactured by Burroughs Wellcome and Co., however, overcame this difficulty ensuring as good a growth of the 4 biotypes of Br.abortus as was seen on the basic serum-dextrose medium without added antibiotics.

Cultural and biological examination of whey agglutinin positive milk samples for the presence of Brucella

Introduction

It is well recognised that one of the most important predilection sites for the localisation of brucella in cattle is the udder and the supramammary lymph nodes (Manthei and Carter, 1950; Stableforth, 1954; Stableforth et al. 1959; McDiarmid, 1957; and Kerr et al. 1958). Kerr (1960) has shown a ratio of infection of the udder to infection of the uterus of approximately 3:1 in cows. As a result of udder infection brucellae may be excreted in the milk for various lengths of time. Most infected animals excrete the organisms in the colostrum or in the milk shortly after abortion or normal parturition. However, excretion may soon become intermittent or may eventually cease altogether. Morgan and McDiarmid (1960) made cultural examinations of milk samples from 45 experimentally infected cows during their post-infection lactation. They found that, in general, excretion of the organism was more consistent and abundant during the later part of lactation although exceptions occurred. The frequency of the excretion of the organisms varied greatly from

6 to 84% of samples yielding the organism. Five of the 45 cows never excreted brucella in their milk. The same workers also examined 10 infected cows during their second post-infection lactation and found 5 of the 10 still excreting brucella. There is no close correlation between blood and whey titres and excretion of brucella (Huddleson, 1942), although on the whole the frequency of excretion rises with increasing titres (Stableforth et al. 1959). Occasionally brucella may be isolated from whey agglutinin-negative milk (Huddleson, 1942; Cameron, 1958; Kerr et al. 1958).

Intensive biological examination of the milk of 16 cows for the presence of Brucella

At the outset of this work it was envisaged that all the milk samples positive to the whey agglutinin test at a dilution of 1:2 or higher would be examined culturally or biologically or by both methods. It soon became apparent that a large proportion of agglutinin positive milk samples would, in fact, have low titres in the region of 1:2 to 1:5, occasionally rising to a titre of 1:10. In order to obtain some information as to the significance of these low titres and the liklihood of such milk

TABLE 20

BIOLOGICAL DEMONSTRATION OF BR.ABORTUS IN THE MILK OF KNOWN INFECTED COWS AND OF COWS WITH DOUBTFUL TITRES

Identification Number	Status of Vaccination	Serum agglutinin Titre	1st Week		2nd Week		3rd Week		4th Week		5th Week		6th Week		7th Week		8th Week	
			guinea-pig No.infected/ No.inoculated (1)	Whey aggl. titre (2)	(1)	(2)	(1)	(2)	(1)	(2)	(1)	(2)	(1)	(2)	(1)	(2)	(1)	(2)
1	C*	1:640	2/2	1:160	0/2	1:40	2/2	1:160	1/2	1:10	2/2	1:1280	2/2	1:640	2/2	1:640	1/1	1:640
2	A**	1:5120	2/2	1:640	0/2	1:320	0/2	1:320	1/2	1:160	0/2	1:320	1/2	1:320	1/1	1:320	2/2	1:320
3	A	1:640	0/2	1:160	1/2	1:160	0/2	1:40	1/2	1:80	0/2	1:160	0/2	1:160	1/2	1:160	0/2	1:160
4	C	1:320	0/2	1:40	0/2	1:160	0/1	1:40	0/2	1:80	0/2	1:80	0/2	1:160	0/2	1:80	0/2	1:160
5	A	1:160	0/1	1:20	0/2	1:20	0/2	1:20	0/2	1:10	0/2	1:10	0/2	1:10	0/2	1:10	0/2	1:10
6	A	1:80	0/2	1:5	0/2	1:5	0/2	1:2	0/2	1:2	0/2	1:5	0/2	1:5	0/2	1:10	0/2	1:5
7	A	1:80	0/1	1:10	0/2	1:20	0/2	1:2	0/2	1:5	0/2	1:5	0/1	1:10	0/2	1:10	0/2	1:10
8	A	1:80	0/2	1:5	0/2	1:5	0/2	1:2	1/2	1:5	0/2	1:2	0/2	1:5	0/2	1:10	0/2	1:5
9	A	1:40	0/2	1:5	0/2	1:5	0/2	1:2	0/2	1:2	0/2	1:2	0/2	1:2	0/2	1:5	0/2	1:2
10	A	1:160	0/2	1:10	0/2	1:5	0/2	1:2	0/1	1:5	0/2	1:10	0/2	1:5	0/1	1:10	0/2	1:10
11	A	1:80	0/2	1:5	0/2	1:2	0/2	1:2	0/2	1:5	0/2	1:5	0/2	1:5	0/2	1:5	0/2	1:5
12	A	1:80	0/2	1:5	0/2	1:5	0/2	1:5	0/2	1:2	0/2	1:5	0/2	1:5	0/2	1:10	0/2	1:5
13	A	1:40	0/2	1:5	0/2	1:10	0/2	1:5	0/2	1:2	0/2	1:2	0/2	1:5	0/2	1:40	0/2	1:10
14	A	1:80	0/2	1:5	0/2	1:5	0/2	1:2	0/2	1:2	0/2	1:5	0/2	1:10	0/2	1:10	0/2	1:5
15	A	1:160	0/2	1:5	0/2	1:5	0/1	1:10	0/2	1:10	0/2	1:10	0/2	1:10	0/2	1:10	0/2	1:10
16	C	1:40	0/2	1:10	0/2	1:10	0/2	1:5	0/2	1:10	0/2	1:20	0/1	1:40	0/2	1:80	0/2	1:20

* = Calfhood-Vaccinated ** = Adult-vaccinated

containing brucella an intensive biological examination of milk samples from some of the cows was carried out. During a period of 8 weeks these cows were sampled weekly and composite milk samples of four quarters of the udder collected. Each sample was examined by the whey agglutination test as well as being injected into two guinea-pigs.

For the purpose 16 cows were selected. Four of them (Nos. 1 - 4) were known to have been clinically infected, they were to serve as positive controls. Five cows (Nos. 5, 7, 10, 15 and 16) had borderline or slightly positive blood titres and milk titres ranging between 1:2 and 1:20 prior to this intensive milk testing. The remaining 7 cows (Nos. 6, 8, 9, 11, 12, 13 and 14) had borderline or negative blood titres and low whey agglutinin titres of 1:2 to 1:5. The detailed results obtained are shown in Table 20.

It will be seen in the table that none of the clinically infected cows excreted brucella in their milk consistently, indeed one of them did not excrete the organism at all despite a comparatively high whey agglutinin titre. All of the cows exhibited a certain degree of whey

titre fluctuation in the course of 8 weeks. The 5 cows (Nos. 5, 7, 10, 15 and 16) had whey titres ranging from 1:2 to 1:80 but none of them yielded brucella. The whey titre of the remaining cows fell between 1:2 and 1:5 dilution and from one of these milk samples brucella was isolated (No.8 on the 4th week). In order to see how frequently brucella was excreted in the milk of this cow, each of the 10 weekly milk samples was inoculated into 6 guinea-pigs. None of these 10 samples yielded brucella.

Cultural and biological tests for the isolation of brucella from 272 milk samples

As a result of isolating brucella from the milk of a cow with a borderline blood and a whey titre of 1:5 it was felt that the cultural and biological examination of all the whey agglutinin positive milk samples was justified and this was carried out throughout this investigation. Of 521 milk samples examined by the whey agglutination test, 251 were found to be positive at a dilution of 1:2 or higher. All these positive samples as well as some 21 additional ones giving an indefinite reaction were examined culturally or biologically and

TABLE 21

CULTURAL AND BIOLOGICAL EXAMINATION OF MILK SAMPLES, POSITIVE TO THE WHEY AGGLUTINATION TEST, FOR THE PRESENCE OF BRUCELLA

Whey agglutinin titre	Whey agglutination positive milk samples examined for the presence of brucella				Total	% +ve
	Only Culturally	Culturally & Biologically		Only Biologically		
	No.positive/ No.examined	No.positive/ No.examined	No.positive No.examined	No.positive/ No.examined	No. +ve No.exam.	
2 - 5	0/37	0/29	0/29	1/83	1/149	0.67
1:10	1/10	0/5	0/5	1/33	2/48	4.6
20 - 40	0/8	0/2	0/2	0/13	0/23	0
1:80 or higher	2/9	0/15	2/15	13/28	17/52	32.7
Total	3/64	0/51	2/51	15/157	20/272	7.3

in some cases by both methods. The results of these isolation attempts are summarised in Table 21.

Although the number of samples in each catagory is comparatively small some conclusions may be drawn with reasonable assurance.

1. Brucella may be isolated from a milk sample even though the whey agglutinin titre of that milk is lower than the generally accepted positive level for adult-vaccinated cattle.

2. There was a certain degree of positive correlation between the height of the whey titre and the frequency of brucella isolation. This was particularly noticeable at whey titres of 1:80 or higher. The organism was recovered on an average from 1.3% of milks with a whey agglutinin titre lower than 1:80, but from 32.7% of milks with titres greater than 1:80.

3. Our results are too few to enable a comparison to be made between the relative efficiency of the cultural and biological methods, but they seem to indicate that the biological method is slightly more efficient.

Summary and Conclusions

Milk samples showing any degree of agglutination to the whey agglutination test were examined culturally or biologically and in some cases by both methods for the presence of brucella. The results showed that the shedding of the organisms in the milk was intermittent indicating that a single negative biological test is of very limited value. Multiple biological, or cultural examinations are essential in order to establish freedom from infection. The whey agglutinin titre is not a wholly reliable guide to the presence or absence of brucella in the milk. Brucella abortus may be isolated from milk with a very low whey agglutinin titre although the frequency of excretion does rise with increase in titres. Cameron (1958) and Kerr et al. (1958) reported the isolation of brucella from whey agglutinin negative milk samples. These findings would indicate that if maximum safety is required eliminating the possibility of udder infection either on an individual or on a herd basis, multiple cultural or biological examinations of all the milk samples are essential.

Cultural examination of vaginal mucus samples

Owing to the brucella survey being conducted by the Ministry of Agriculture, guinea-pigs were in very short supply and those which we were able to secure were preferentially used for the biological examination of milk and post-partum specimens. Thus the bacteriological examination of vaginal mucus samples was carried out almost exclusively by cultural tests. In the course of this work 174 vaginal mucus samples were found to be positive to the agglutination test. Approximately half of these samples were collected by the vaginal tampon method resulting in many falsely positive titres. Only those vaginal mucus samples which appeared to be genuinely positive to the agglutination test were examined culturally. (Details of technique are on pages 33)

Out of 116 isolation attempts 3 vaginal mucus samples yielded Br.abortus. The serum agglutinin titres of each of the three cows involved were well in excess of 1:80. The first of the brucella isolates was recovered from the vaginal mucus of a cow 9 days prior to abortion, the second from a cow 14 days after full-time calving, and the third animal was a three-year old cow which had aborted 6 months prior to the isolation and did not subsequently become pregnant despite numerous services.

In the case of uterine infection of the cow brucellae are usually present in large numbers in the discharges at parturition. The numbers of organisms usually decrease quickly in the ensuing weeks and excretion seldom persists for longer than a few weeks or exceptionally for a few months after parturition. If the animal becomes pregnant, excretion ceases as a result of the establishment of the cervical seal but infection may persist in the gravid uterus. (Stableforth, et al. 1959).

Kerr and co-workers (1958) maintained that the liklihood of isolating brucella from the vaginal mucus of cows later than 3 weeks after calving is remote. Results obtained in the course of this investigation support this view. The great majority of samples examined were from cows either bacteriologically proven brucella positives or having persistently high vaginal mucus agglutinin titres and yet no brucella was isolated from these samples except shortly before or shortly after calving. There was one exception to this but the fact that this animal did not become pregnant might explain the long lasting excretion of Brucella abortus.

Cultural and biological examination of post-partum specimens

Payne in 1959 described the progress of brucella infection in pregnant cows experimentally infected with Br.abortus. Bacteriological examination of maternal and foetal tissues collected at autopsy at various intervals after infection indicated that the placentae, uterine exudate, allantoic and amniotic fluids were better sources of isolation of brucella than were foetal tissues. Among the foetal organs most consistently infected was the spleen. The infection of the stomach and lung appeared to take place just before or at abortion, probably via the amniotic fluid.

Examination of amnio-allantoic fluid and cotyledon samples for Brucella

Guided by the results of Payne's work it was decided that samples of all the cotyledons and of vaginal discharges ("amnio-allantoic" fluid) secured at abortion or at normal calving would be examined culturally as well as biologically, irrespective of whether the animals were considered brucella-negative or brucella-positive. Thus 52 amnio-allantoic fluids and 60 cotyledons were examined. Table 22 summarises the results.

TABLE 22

ATTEMPTS TO ISOLATE BR.ABORTUS FROM POST PARTUM SAMPLES

SERUM TITRE	"Amnio-allantoic" Fluids		Cotyledons	
	Cultural x	Biological xx	Cultural x	Biological xx
	No.positive/ No.attempted	No.positive/ No.attempted	No.positive/ No.attempted	No.positive/ No.attempted
< 80++	0/24	0/24	0/36	0/36
80++ - 320++	0/17	0/17	0/14	0/14
> 320++	3/11	5/11	1/10	8/10
TOTAL	3/52	5/52	1/60	8/60

x = 5 SDA plates/sample ; xx = 2 guinea-pigs/sample

The relative efficiency of cultural and biological examination of brucella infected post-partum specimens

From the 60 post-partum specimens 9 strains of Br.abortus were isolated. To show the exact source of isolation of these strains as well as the relative efficiency of the cultural and biological methods of detecting brucella in these specimens Table 23 was compiled.

From these tables (22 and 23) the following observations may be made:

1. No brucella infected cows were detected by cultural and biological examination of post-partum specimens which had not been known to be infected by blood titres alone.

2. The biological method of detecting brucella in post-partum specimens was more efficient than culture tests especially when applied to cotyledons. From 8 biologically positive cotyledons only one yielded the organism on culture.

3. From the very limited number of brucella positive cases where both cotyledons and amnio-allantoic fluids were available for examination the two samples proved to be equally good sources of Br.abortus.

TABLE 23

THE EFFICIENCY OF CULTURAL AND BIOLOGICAL EXAMINATIONS

OF BRUCELLA INFECTED POST-PARTUM SPECIMENS

OUTCOME OF PREGNANCY	Amnio-allantoic Fluid		Cotyledons	
	Cultural x	Biological xx	Cultural x	Biological xx
	No.positive/ No. used	No.positive/ No. injected	No.positive/ No. used	No.positive/ No. injected
Normal calving	ND	ND	0/5	2/2
"	0/5	2/2	0/5	2/2
"	0/5	2/2	0/5	2/2
"	5/5	2/2	ND	ND
"	ND	ND	0/5	1/2
Abortion	5/5	2/2	5/5	2/2
"	ND	ND	0/5	2/2
"	ND	ND	0/5	2/2
"	4/5	2/2	0/5	2/2
TOTAL	3/5	5/5	1/8	8/8

x = 5 SDA plates/sample ; xx = 2 guinea-pigs/sample ; ND = not done

4. From those 4 cases of abortion where a sample of after-birth was secured brucella was invariably isolated.

5. Brucella can be excreted in post-partum material after a normal parturition.

Summary and Conclusions.

Sixty post-partum specimens from cattle of various brucella infection status were examined culturally and/or biologically for the presence of brucella. Although no new cases of brucella infection were discovered in this way, nevertheless, the results, though limited in number, indicated that either samples of cotyledons or vaginal discharges including amnio-allantoic fluid may be used with good effect instead of the examination of the aborted foetus for diagnostic purposes. The use of 5 S.D.A. plates per sample was almost totally inadequate, but the injection of two guinea-pigs per sample would seem to be adequate as well as desirable in the case of every parturition where the blood titres are not negative.

Biological examination of lymphatic tissue collected at autopsy

Stableforth et al. (1959) stated that in non-pregnant cows the predilection site of brucella infection is the udder and the supra mammary lymph-nodes, whereas in the pregnant cow it is the uterus and its associated lymph-nodes. We therefore collected lymph-nodes at slaughter and attempted to demonstrate the presence of Br.abortus by guinea-pig inoculation. The results are presented in Table 24.

It will be readily appreciated from the table that the efficiency of this technique was extremely poor. From the 13 adult-vaccinated cows positive to the whey and vaginal mucus agglutination test 7 had been experimentally infected with approximately 1 x 10^{11} Br.abortus 544 or type II (Wilson) by the subcutaneous route from 4 to 36 days prior to being slaughtered. The sub-maxillary, supra-pharyngeal, iliac and supra-mammary lymph-nodes of each animal were pooled and homogenised. The homogenate from each of 4 animals was inoculated into 4 guinea-pigs, while the fifth sample was inoculated into 10 guinea-pigs,

TABLE 24

RESULTS OF ATTEMPTS TO ISOLATE BR.ABORTUS

FROM LYMPH-NODES COLLECTED AT AUTOPSY

Serum agglutinin titres	Status of vaccination		
	Calfhood (Whey and v.mucus agglutinin)	Adult (Whey and v.mucus agglutinin)	
	+ve	-ve	+ve
10 - 20++	0/4 0/4 0/4 0/6 0/2	0/4 0/4 0/4 - -	- - - - -
20+++ - 40++	0/4 0/4 0/4 0/4 0/4 0/15 0/20	0/4 0/4 0/20 - - - -	- - - - - - -
40+++ - 80++	0/6 0/8	0/4 -	- -
80+++ - 160++	0/6	0/4	0/20
160+++ and higher	- - - - - - - - - - - -	0/20 - - - - - - - - - - -	0/2 0/3 0/3 1/4 0/4 0/4 0/4 0/6 1/6 0/10 0/20 0/20

and the sixth and seventh samples into 20 guinea-pigs. In only one of these cases was brucella recovered and then only in one of the 4 guinea-pigs injected. This was from a cow infected with Br.abortus Type II, 10 days prior to slaughter.

The last cow slaughtered towards the end of the eradication programme was an udder excretor. Her supra-mammary lymphatic-node was not pooled and homogenised with the other lymph-nodes, (sub-maxillary, supra-pharyngeal, iliac) but prepared separately while the other three lymph-nodes were homogenised together. Each of the two homogenates was inoculated into 6 guinea-pigs. Six weeks after inoculation none of the 6 guinea-pigs which received the pooled homogenate of lymph-nodes was infected, while one of those which was injected with the supra-mammary node homogenate was infected.

Kerr (1960) examined the lymphatic tissues of 131 brucella reactor cows and heifers collected at autopsy. Ninetyfour strains of brucella were isolated but in no case was brucella recovered from other than the iliac or supra-mammary lymph-nodes. It is probable that our lack of success was due partly to dilution of infected material

with non-infected nodes and greater success might have been achieved by taking nodes from the site of infection only. Unfortunately, in field infected cases the site of infection is not known with certainty and we therefore took several nodes in order to ensure collection of material from the most probable regions.

Payne (1959) was able to recover Br.abortus readily from head lymph-nodes (supra-pharyngeal and sub-maxillary) of cattle experimentally infected via the conjunctiva but in field cases, Kerr (1960) failed to recover the organism from any nodes other than supra-mammary and iliac. A further factor affecting the recovery rate might be that, due to circumstances associated with the slaughter of the cattle and beyond our control, the homogenised tissues frequently had to be held overnight at 4°C. As previously described the homogenisation was carried out in Ringer solution, and we now have evidence that this solution has a markedly adverse effect on the viability of Br.abortus. This effect is shown with all strains so far tested but its severity differs between strains. Thus Strain 19 appears to be more sensitive than 544. This somewhat surprising effect is being investigated more fully but it is possible now to state that it is unrelated to changes in pH.

Sources and identity of brucella strains isolated

For the identification of brucella isolated, the conventional tests for species identification were used as described in W.H.O. Monograph series No.19 (1953). These included:

1. the need for added CO_2 for growth on primary isolation
2. the production of H_2S
3. differential growth on dyes basic fuchsin and thionin
4. agglutination in monospecific sera

It has been a well recognised fact that the differences based on these tests are rather quantitative than qualitative but when the methods are standardised and the cultures are tested in the S-phase of growth the great majority of isolates fall into one of the three recognised species of Brucella, i.e. abortus, melitensis or suis.

In the identification of any of the strains, care was taken to ensure that isolates were in the S-phase of growth. In performing the dye sensitivity tests three known strains of Br.abortus were used as positive and negative controls.

These were:

Br.abortus 544 (thionin sensitive, basic fuchsin resistant)

Br.abortus Type II (both thionin and basic fuchsin sensitive)

Br.abortus Type 5 (both thionin and basic fuchsin resistant)

(Additional details of techniques of identification are described on pages 35-36)

Table 25 shows the sources of isolation and the characteristics of the 25 strains of brucella isolated.

It will be seen in the table that 22 out of 25 isolates were typical strains of Brucella abortus. The three dye-sensitive varieties of brucella actually represent one genuine and one laboratory strain of Br.abortus Type II. Those two organisms isolated from milk and from vaginal mucus respectively originated from one and the same cow. It may be of some interest to note that this particular animal was imported from abroad and yielded the only field strain of Br.abortus Type II. For the third dye-sensitive variety of brucella recovered was from an animal artificially infected prior to slaughter with this strain of the organism.

TABLE 25

SOURCE AND CHARACTERISTICS OF 25 BRUCELLA ISOLATES

Sources of isolation	No. of isolates	CO_2 requirements	H_2S production	Growth in presence of		Agglutination in monospecific sera	
				Basic fuchsin 1/25000	Thionin 1/500000	abortus	melitensis
MILK	11	+ (10)	+	+	-	+	-
		+ (1)	+	-	-	+	-
V. Mucus	3*	+ (1)	+	+	-	+	-
		+ (1)	+	-	-	+	-
Post-partum specimens	9	+ (9)	+	+	-	+	-
Lymphatic tissue	2	+ (1)	+	+	-	+	-
		+ (1)	+	-	-	+	-

* One of these strains has always been in the 'R' phase of growth and thus could not be tested.

SUMMARY AND CONCLUSIONS OF PART I.

The more important points arising from the work which has been described may be summarised as follows:

1. The Dairy-type foster cows appeared to become infected with brucella much less readily than the beef cows, although there is no reason to suppose that they were exposed to less risk. Indeed, since some of them suckled calves born to infected cows it might be thought that they were in greater hazard.

2. There was no evidence to show that calves born to and suckled, for a few days, by infected cows, on transfer, would cause clinical infection of vaccinated foster cows.

3. Infection with Brucella abortus appeared to have no adverse effect upon the establishment of pregnancy.

4. The effect of brucella infection of pregnant cows was either to cause a markedly premature termination of their pregnancy or to have no influence on its duration. Approximately one third of the pregnancies of infected cows terminated in abortion.

5. a) The milk ring and whey agglutination tests were by and large negative in calfhood-vaccinated brucella-free animals. The whey agglutination test gave even fewer false-positive results than the milk ring test (3.9% and 14.9% respectively).

b. The use of these milk tests is limited in beef cattle due to the shortness of lactation and the consequent non-availability of milk for much of the year. It also follows that the use of the milk tests as the only means of diagnosis in beef cattle is impracticable.

c. The milk ring test appears to be valueless as an indicator of field infection in animals vaccinated as adults or repeat vaccinated. The whey agglutination test, however, is as valuable in these cattle as in calfhood-vaccinated cows provided the significant level is taken as 1:10. Low whey agglutinin titres (1:2 or 1:5) are very frequent in adult and repeat-vaccinated cows.

d. Approximately 93% of infected cows are shown as such by the use of the milk ring test and whey agglutination test unsupplemented by any other investigations.

e. The whey agglutinin titres appeared to be uninfluenced by the stage of lactation but there was a rise in the percentage of positives with advancing gestation.

It would appear that the use of M.R.T. and the whey agglutination test did not facilitate the detection of infected animals, since these were revealed more readily by repeat serum agglutination tests alone. The milk tests were, however, very valuable as moderators in cases where the serum agglutination test gave doubtful results. Negative milk tests in these animals allowed many of them which might otherwise have been discarded, to remain in the herd.

6. a. Approximately 10% of vaginal mucus agglutination tests were falsely positive and the same proportion flasely negative when the mucus was collected by tampon.

b. Very few false positives were obtained when the mucus was collected by the vaginal pipette, but unfortunately when using this method there were large numbers of animals from which samples could not be obtained.

c. The great majority (20/23) of infected cows reacted to the vaginal mucus agglutination test, although repeat serum agglutination tests alone furnished sufficient

evidence of field infection before a positive mucus agglutinin titre was obtained.

The real value of the vaginal mucus agglutination test would appear to be very similar to that of the whey agglutination test in that it did not help significantly in the detection of infected animals but did serve to indicate absence of brucella infection in animals giving doubtful serum agglutinin titres.

7. The vaginal serum agglutinin titre persisted very much longer in repeat-vaccinated, adult-vaccinated animals than in calfhood-vaccinated stock. Furthermore, it was found that calfhood-vaccinated animals born and vaccinated before segregation of infected cattle had more persistent titres than did similar animals born and vaccinated after segregation of infected cattle.

8. Some calves which came into contact with Br.abortus during the neonatal period still had serum titres at the time of vaccination and these tended to give a poor titre response to vaccination.

9. There was a marked tendency for the serum titres of repeat, adult and also of calfhood-vaccinated animals, born and vaccinated in an infected environment, to show

fluctuations of the serum titres. One result of this fluctuation was that on occasions non-infected animals would show higher titres than animals known to be infected with Brucella abortus.

10. There was no evidence to show that the fluctuation of serum titres was connected with the stage of gestation.

11. The injection of a dead agglutinogenic agent, antigenically unrelated to brucella, into cows with brucella serum agglutinin titres brought about a marked but temporary rise in brucella titres.

Over-age vaccination of cattle with S19 Br.abortus diminished the value of the serum agglutination test in a considerable degree. The rigid application of criteria as applicable to the interpretation of serum agglutinin titres of adult-vaccinated cows may cause the disposal of many such cows which might never constitute any danger for the rest of the herd. The examination of milk and vaginal mucous samples may be of real value in this situation, although their becoming positive might come too late after the occurrence of Brucella abortion.

12. At the beginning of the work there were difficulties in culturing Brucella abortus due to the use of Bacitricin of a particular brand, when another manufacturers product was substituted this difficulty was overcome.

13. Brucella abortus could only be demonstrated intermittently in the milk of known infected cows, and following a period of intensive testing, the organisms were not demonstrated in the milk of the great majority of cows of doubtful status of infection.

14. Of 9 animals whose post-partum material yielded brucella only four had aborted, the other five having carried their calves to full term. This serves to underline the fact that the cow which carries her calf to full-term can be a dangerous spreader of the organisms.

15. Three vaginal mucus samples yielded Brucella abortus. Two of these were samples collected within a few days of calving, but the 3rd was obtained 6 months after abortion in an animal which failed to become pregnant subsequently.

16. Very little success was achieved in isolating Brucella abortus from lymph-nodes collected at autopsy, in fact, in the whole course of the work the organism

was demonstrated in such material from only one herd animal and one artificially infected beast although many known infected animals have been examined.

17. The use of 5 S.D.A. plates per sample for isolation of brucella from suspected specimens proved to be inadequate missing approximately two thirds of the organisms demonstrated by the injection of two guinea-pigs per sample.

18. By the use of cultural and/or biological examination of specimens, only a single case of brucella infection was discovered which had not been detected by the application of the various serological tests.

Time and expense involved in the cultural and biological examination of specimens, purely for the diagnosis of brucella infection did not appear to be proportionate to their value. However, where maximum safety is required their uses may be justified.

By the repeated application of the serological and bacteriological tests to the various specimens of cattle it is believed that finally a correct diagnosis was arrived at in most of the cases. With so many adult-vaccinated animals

in the herd giving serum titres within the normally accepted doubtful range it is impossible to state with certainty that no infected animals remained in the herd. The eradication programme, it is understood, did not interfere too much with the normal management of the herd, although certain changes and provisions had to be made on the organisation and management side (ensuring isolation units, some re-organisation of labour force, cutting down traffic between various units of the farm, hygienic precautions, etc.) to ensure the success of the programme.

Finally to name a single factor causing most of the difficulties in diagnosis the choice unquestionably fell on over-age vaccination. While it is in practice, diagnosis remains laborious, time consuming and uncertain. Therefore to bring an end to this situation one of the following procedures must be adopted:

1. Adult vaccination must be brought to an end and S19 vaccine be used only for calves under 9 months of age.

2. A non-agglutinogenic vaccine, giving at least as good immunity as S19 does, must be found which may be used for cattle of all ages.

3. A method, serological or otherwise, must be evolved capable of differentiating between vaccine titres and those resulting from superimposed field infection.

PART 2

THE EFFECT OF BRUCELLA INFECTION OF THE OVINE AND BOVINE NEONATA, THEIR SEROLOGICAL RESPONSIVENESS TO RE-EXPOSURE IN LATER LIFE

Introduction

In Part 1 of this thesis it was shown that a few of the calves, born to and suckled by brucella infected cows, gave an inferior response to S19 vaccination at about 6 months of age. As the dams of these calves were udder infected animals it was likely that they ingested large numbers of brucella at a very early age, which in turn might have affected their serological capacities to respond to these organisms in later life. The question of exposure of young calves to field infection and its consequences could not be satisfactorily examined under field conditions thus the matter became the subject of the following studies.

The possibility of calves becoming infected with brucella by the ingestion of milk from infected cows and the liklihood of such calves becoming chronic carriers received attention in the earlier parts of this century.

As early as 1916 Huddleson collected data from experiments indicating that calves were capable of resisting infection. In the course of these early experiments he (Huddleson) exposed calves to infection by either nursing

them by udder infected cows or by adding live organisms to their milk ration. Despite such treatment the calves did not develop antibodies actively against brucella. The results of another experiment conducted by the same writer (Huddleson & Hasley, 1924) supported his earlier conclusion, for no evidence of persisting infection was found when 11 animals, which had been exposed to brucella infection during the nursing period, were slaughtered either during first pregnancy or after parturition. In regard to antibody production by these calves he concluded "Agglutinating and complement fixing bodies for Bact.abortus are very rarely demonstrated in the blood of calves as a result of ingesting naturally infected milk".

Schroeder and Cotton (1911) in their investigations regarding the transmission of brucellosis from infected dam to offspring, either did not find agglutinins in the sera of such calves, or if antibodies were acquired passively by the ingestion of colostrum these titres declined rapidly.

Quinlan (1923) made an extensive study on 41 calves some of which were born to infected cows and reared by them, others were removed from such cows within a few hours after birth and were fed on non-infected milk. Yet another group consisted of calves born to non-infected cows which were brought up on infected milk. The calves were exposed to infection

for various lengths of time, some of them for as long as 9 months. Only 25 out of 41 calves showed agglutinins in their blood, but these antibodies disappeared from the sera after exposure was brought to an end. In his conclusions Quinlan remarks "That it appears to be a perfectly safe procedure to feed calves upon naturally infected raw milk up to the age of six to seven months without danger of their becoming chronic carriers of infection.

Carpenter (1924) added important information to our knowledge of brucella infection of young calves by demonstrating that these organisms can be recovered from many organs of calves during and shortly after the feeding of live organisms in the milk. Five weeks after the discontinuation of exposure via the infected milk it was no longer possible to recover brucella from the tissues.

Fitch et al. (1941) collected over 50 calves of brucella infected dams to determine whether such calves, if infected, would carry infection through to sexual maturity and suddenly manifest evidence of infection. These calves were exposed to infection only at birth and during the first week of life ingesting infected milk. The animals were observed over a period of from one to six parturitions. No Brucella abortus was isolated from any of the colostrum, milk, placental membranes, vaginal discharges, foetuses or calves

that were examined shortly after birth. They concluded (Fitch et al.) that a clean herd can be built up from an infected one if the calves are not allowed to remain with infected animals after the first week of life and are brought up in a non-infected environment.

Considering the results of all of these investigations together one cannot escape the conclusion that calves up to a certain age possess a high degree of resistance to brucella infection.

However, there are two very important possible differences in the status of calves exposed and not exposed to infection in early life. The first of these differences is their susceptibility to infection in later life and secondly their capacity to react immunologically on re-exposure to infection.

In connection with the aberration of immune response Van Waveren (1960) reported that in the Netherlands it became necessary to apply the complement fixation test even to agglutinin negative serum samples of cattle, for experience has shown that older cows with negative titres can be brucella carriers. Kerr et al. (1958) discussing the diagnostic use of serum agglutination test for brucellosis of cattle stated, that although the main problem in using this test is the falsely

positive titre arising from vaccination, falsely negative titres do occur but not in large numbers. Manthei and Carter (1950) reported the case of a cow experimentally infected with brucella which has not had an agglutinin titre higher than 1:50 although brucella was recovered from the uterine material at the time of normal calving. In the herd subject of the first part of this thesis there was a bacteriologically proven brucella infected cow which having had a very high agglutinin titre (1:10000) became almost negative to the agglutination test but her c.f. titre remained positive at 1:160 serum dilution and the anti-globulin test of Coombs' revealed antibodies up to 1:20000 dilution of the serum.

Falsely negative agglutinin titres and the pre-zone phenomena in the agglutination test of brucella infected subjects are well known in human medicine. (Huddleson and Johnson, 1933; Evans, 1934; Parson and Poston, 1939; Robinson and Evans, 1939; Wallis, 1957; Kelly et al. 1960).

Glenchur et al. (1961) experimenting on the significance of blocking antibodies in brucellosis found direct correlation between the quantity of killed brucella administered to rabbits and the appearance and extent of blocking antibodies.

The possibility of the aberration of immune response to microbial agents or to their derivatives have been indicated by Burnet and Penner (1948). Such an aberration of the immune response appears to be greatest in diseases like brucellosis where the infection of the neonate does not usually terminate in early death, and the new-born may, under natural conditions, be exposed to large numbers of the organism via the colostrum and milk from the very first hours of life. Testing for sero-agglutinins is the principle method of diagnosing brucellosis especially in bovine, where considerable importance is attached to the titre of agglutinins in calfhood and adult-vaccinated cattle. Suppression of agglutinin production, even if partial, could interfere seriously with the diagnostic value of serum agglutination test in case of such animals.

A. EXPERIMENTAL BRUCELLA INFECTION OF YOUNG LAMBS, THEIR SEROLOGICAL RESPONSIVENESS TO THE SAME AND CLOSELY RELATED ANTIGENS IN LATER LIFE.

For the first part of these studies on the brucella infection of the neonate, new-born lambs were used more as a result of necessity than choice.

The accommodation of large experimental animals as well as the financial resources available were both in short supply rendering the use of cattle at that time impossible.

MATERIALS AND METHODS

Animals

For the experiment involving early exposure to brucella infection, seven black-face lambs (later referred to as Test Lambs, TL) 1 - 2 days old were bought from a commercial farm after having received some of their dams' colostrum. They were bottle-fed for approximately 9 weeks and thrived normally throughout the experiment. One of them (TL 1/7) died on the 191st day of the experiment, but the cause of death had no connection with the experiment.

At a later stage of the work a further six lambs (later referred to as Control Lambs, CL) were acquired from the same flock. They were tested three times for brucella sero-agglutinins over a period of two weeks before being

taken into the experiments. The sera of all these lambs were completely free from brucella agglutinins.

Preparation of antigens

For the agglutination, agglutination inhibition and complement fixation (c.f.) tests the cell suspension used was provided by the Ministry of Agriculture, Fisheries and Food, Central Veterinary Laboratory, Weybridge. The sensitivity of each batch of concentrated suspension was tested against a Br.abortus reference serum and the batch diluted accordingly. Dilution ranged from 1:10 to 1:12.5 of the concentrated antigen suspension. For the agglutination and agglutination inhibition tests the agglutinogenic concentrate was diluted in formol saline (0.1 % formaldehyde in 0.85% saline), for the c.f. test, dilutions were made in physiological saline.

For the Escherichia coli and Salmonella gallinarum agglutinating systems the antigens were prepared the following way: Nutrient broth media were inoculated with the appropriate cultures and incubated overnight at 37°C. The cultures were centrifuged and the sedimented cells suspended in 0.1% formol saline. After a second centrifugation the washed cells were re-suspended in 0.1% formol saline to give an opacity reading equivalent to No.3. tube on the Burroughs Wellcome opacity scale.

For the skin sensitivity test, the antigen was prepared as follows: Br.abortus Strain 19 was grown on serum dextrose agar (Brinley Morgan, 1960) and incubated at 37°C for 7 days. Growth was harvested in 0.1% formol saline and left for a few hours at room temperature. The suspension was then centrifuged and washed twice in physiological saline. The packed bacterial cells were re-suspended in 20 - 30 times their own volume of acetone at - 20° and kept at this temperature overnight. The suspension was then lightly centrifuged, the supernatant poured off and the cells re-suspended in the same quantity of cold acetone. After a further 2 - 3 hours standing in the cold, the suspension was finally centrifuged and the cells dried in a vacuum desiccator over calcium chloride for 10-15 hours.

To 100 mg. of acetone-dried cells, 1 ml. saline containing 1:10,000 thiomersalate was added and the pH adjusted to 9. The cells were carefully suspended and incubated for 30 minutes at 56° in a water bath and the suspension was then centrifuged for 1 hour (R.C.F. = 12,000). The supernatant was collected and stored at -20°. Such a brucella extract gave six to nine precipitin lines when

tested against homologous and heterologous brucella antisera in the Ouchterlony gel-diffusion tests.

Preparation of Br.abortus S19 inoculum

The bacteria were grown on SD agar (Brinley Morgan, 1960) for 3 days and then harvested in sterile Ringer's solution. The total number of organisms per cc suspension was established by means of the Burroughs Wellcome opacity tubes. The number of viable cells of such suspension was determined by plate counts on S.D. agar.

Inocula were made up once a week and viability counts were carried out on each batch of freshly prepared inoculum which were then kept at 4°C in refrigerator and used through the week for the daily inoculation of milk-feed for the lambs.

Sera

Blood samples were collected from the lambs by the jugular vein and sera separated, thiomersalate was added in 1:10,000 concentration. All the serum samples were stored at -20°. Br.abortus S19 antiserum (in the text referred to as No.340 reference serum) was prepared in rabbits. A pool of rabbit antiserum was divided into

1 ml. quantities and stored at -20°C until used. A fresh tube of reference serum was used on each occasion. The agglutinin titre of No.340 reference serum, incubated at 37° for 24 hours, was from 1:2 to 1:320++++; 1:640+++ (see under 'Serological Techniques'); incubated at 56° in the water bath for 3 hours, the agglutinin titre was from 1:2 to 1:320++++, 1:640+.

The homologous rabbit antisera to E.coli and Sal.gallinarum were obtained by the courtesy of Dr. R. Morrison of the Department of Bacteriology, University of Glasgow.

Brucella agglutinin free ovine sera were collected from ten adult sheep the sera of which had been tested repeatedly for agglutinins to brucella.

Serological techniques

1. Tube agglutination test

For this test the dilution of serum started at 1:2, 1:5, 1:10, etc., with a final volume of 1 ml. of antigen-serum dilution mixtures. The end titre was read after 24 hours at 37° in the incubator. At each dilution of the sera the degree of agglutination was recorded as follows:

100% of antigen agglutinated,			water clear supernatant after gentle mixing	++++
75%	"	"	very slightly hazy after gentle mixing	+++
50%	"	"	hazy after gentle mixing	++
25%	"	"	very hazy after gentle mixing	+
A minor indication of agglutination				±
Complete lack of agglutination				-

The appropriate controls were set up for each set of agglutinations carried out. Each agglutination was carried out in duplicate and when the degree of agglutination at any one dilution of a serum under test differed more than ++ (such as 1:80+, 1:80++++) the test was repeated. All the agglutination tests with sera from experimental animals were done within 24 hours of the blood sample being taken.

2. Complement fixation tests

The sera were diluted in physiological saline, starting at a dilution of 1:5. Each sample was set up in duplicate.

The complement fixation (c.f.) test was carried out as described by Carpenter (1956) using 5% sheep red cell suspension and two minimal sensitizing doses of amboceptor

(reagents obtained from Burroughs Wellcome & Co. London). Serum from brucella-free guinea-pigs was used as complement. The last dilution of serum in which the complete fixation of two minimal haemolytic doses of complement was effected was taken as the c.f. titre of the serum. For checking the sensitivity of the system No.340 reference serum was used at each of the tests.

3. For the agglutination inhibition tests two methods were tried. The first of these was carried out as follows:

Method 1.

Reference serum No.340 was set up for agglutination in the conventional manner and the tubes were incubated at 56°C in the water bath. After 3 hours of incubation the highest dilution of the serum in which complete agglutination of the brucella antigen was affected was taken as containing one minimal agglutination dose of antibodies (1:320).

From heat-inactivated ovine serum suspected of containing blocking antibodies serial doubling dilutions were made in 0.5 ml. quantities. To each of these dilutions 0.1 ml. of 1:29 dilution of No.340 reference serum was added followed by the addition of 0.9 ml. of the brucella antigen.

This final dilution of No.340 serum was 1:319 in each dilution of the ovine serum. The tubes were incubated at 56°C in the waterbath for 3 hours and readings on the progress of agglutination were recorded periodically.

Method 2

The second method employed was carried out in the following way: serial doubling dilutions of No.340 reference brucella antiserum were set up in duplicate. In one set of dilutions the diluting agent was 0.1% formol saline; in the second set the diluting agent was the heat-inactivated serum of a lamb suspected of containing incomplete or blocking antibodies. (Sera were inactivated at 56° for 15 minutes in the water bath.) To both sets of dilutions 0.5 ml. of the dilution of the standard concentrated brucella antigenic suspension was added giving a final volume of 1 ml. of reagents in every tube. Incubation was at 56° in the water bath. Readings of the progress of agglutination were taken at 30, 60 and 180 minutes. The degree of agglutination in each of the two sets (saline and serum as the diluting agents) were compared and recorded.

Sera from ten sheep known to be free of brucella infection were also checked for their effect as diluents of the reference (No.340) serum.

Skin sensitivity test

The wool over the right scapula of each lamb was clipped, shaved and disinfected with 'Phisihex' (Bayer Products Ltd., Kingston-upon-Thames). The skin thickness was measured and 0.1 ml. of the soluble brucella antigen preparation was injected intradermally. Fifteen centimetres from the site of the first injection, 0.1 ml. of thiomersalate saline (1:10,000) was injected as a control. The first post-injection measurement of skin was made 30 minutes after injection and was repeated at short intervals for 4 hours. Three further measurements were made after 24, 48 and 72 hours respectively. The reaction to the injections was measured in terms of changes in the thickness of the skin as well as the extent of oedema calculated in square centimetres.

For control purposes, lambs of the same age and breed as the experimental animals but free from brucella infection were tested for skin sensitivity to soluble brucella antigen.

Adsorption of brucella agglutinin-positive and negative lambs' sera

This was effected by adding 1 volume of packed Br.abortus S19 cells (brucella cells in the standard antigenic suspension) to 3 volumes of the appropriate sera and incubating for 4 hours at 37°C. After centrifugation, the supernatant serum was mixed again with the same quantity of antigen and incubated at 4°C for 24 hours. After thorough centrifugation the serum was tested for presence of agglutinins. Absence of agglutination at 1:2 dilution was accepted as an indication of efficient adsorption.

For the agglutinin-negative sera the same procedure was used for the adsorption of antibodies, with the same quantities of serum and antigen.

RESULTS

Schedule of exposure of the test and control lambs to Brucella

The seven test lambs were divided into two groups according to the route of exposure to brucella infection.

Test Lamb Group 1 (TL 1). Consisted of four lambs marked: TL 1/1, 1/2, 1/3 and 1/7.

Test Lamb Group 2 (TL 2). Consisted of three lambs marked: TL 2/4, 2/5 and 2/6 respectively.

Both groups of lambs were fed on milk contaminated with Br. abortus S19. Approximately $0.5 - 1 \times 10^6$ organisms were added to the daily milk ration of each lamb from the first or second day of life over a period of 65 days.

TL 1 in addition, received four subcutaneous (s/c) injections of Br.abortus S19 vaccine. (Standard vaccine supplied by the Ministry of Agriculture, Fisheries and Food, Central Veterinary Laboratory, Weybridge.)

After a period free of exposure to brucella, the two groups of lambs (TL 1 and TL 2) along with the Control Group (C) were challenged twice on the 153rd (first challenge)

TABLE 26

SUMMARY OF EXPOSURE OF THE TEST AND CONTROL LAMBS TO BR.ABORTUS OVER THE WHOLE PERIOD OF THE EXPERIMENT

Age (days)	Test Lambs		Control Lambs
	TL 2/4, 2/5, 2/6	TL 1/1, 1/2, 1/3, 1/7	C8,9,10,11,12,13
1-65	0.5-1 x 10^6 viable Br. abortus S19 administered daily by the oral route	Same as for TL 2 but in addition: 0.5 ml. Br.abortus S19 Vac.on 1st 2.0 " " " " " 3rd 2.0 " " " " " 15th day of life injected subcutaneously	No exposure to Brucella
66-94	No exposure to Brucella for 67 days	No exposure to Brucella for 29 days	
95		2.0 ml. of Br.abortus vacc. s.cut.	
96-132		No exposure to Brucella for 37 days	
133	60x10^9 Br.abortus 544 subcutaneously	60 x 10^9 Br.abortus 544 s.cut.	60x10^9 Br.abortus 544 s.cut.
134-197	No exposure to Brucella for 64 days	No exposure to Brucella for 64 days	No exposure to Brucella for 64 days
198	50 x 10^9 Br.abortus S19 s.cut.	50 x 10^9 Br.abortus S19 s.cut.	50 x 10^9 Br.abortus S19 s.cut.

TABLE 27

BRUCELLA SERO-AGGLUTININ TITRES OF TL 2 GROUP FOR THE FIRST 132 DAYS OF LIFE

Days of life	Exposure to Br.abortus S19	Identity numbers of lambs		
		TL 2/4	TL 2/5	TL 2/6
1-2	0.5-1 x 10^6 viable Br.abortus S19 orally for the first 65 days of life	<5*	<5	<5
9		<5	<5	<5
16		5+; 10-20+	<5	<5
23		<5	5±	<5
30		<5	<5	<5
38		<5	<5	<5
45		<5	<5	<5
52		<5	5++	<5
59		<5	<5	5±
65		<5	5-20±	<5
108	No exposure to Brucella	<5	<5	<5
118		<5	<5	5±
127		<5	<5	<5
132		<5	<5	<5

* Reciprocals of serum dilutions.
< No agglutination at the dilution of serum.
± Traces of agglutination but less than 25% of antigen agglutinated.
\+ 25% of antigen agglutinated.
++ 50% " " "
+++ 75% " " "
++++ 100% " " "

TABLE 28

BRUCELLA SERO-AGGLUTININ TITRE OF TL 1 GROUP FOR THE FIRST 132 DAYS OF LIFE

Days of life	Exposure to Br.abortus S19	Identity numbers of lambs			
		TL 1/1	TL 1/2	TL 1/3	TL 1/7
1-2	0.5 ml. Br.abortus S19 vacc.	<2*	<2	<2	<2
3	2 ml. Br.abortus S19 vacc.	<2	<2	<2	<2
10		<2	<2	<2	<2
15	2 ml. Br.abortus S19 vacc.	40+++	5+	40++	40+
23		160+++	80+++	320+	160+++
30	In addition to these three subcutaneous injections	320+	160+	320++	320+++
37		40+++	40+	80+	80+
44	0.5-1 x 10^6 viable Br.abortus	40+	20++	40+	40+++
51	S19 orally during the first	10++++	10++	20+++	20++
60	65 days of life	5±	<2	5+	<2
65		<2	<2	5+	<2
67		<2	<2	5±	<2
80		<2	<2	<2	<2
86		<2	<2	<2	<2
95	2 ml. Br.abortus S19 vacc. s.c.	<2	<2	<2	<2
100		10++	5++	5+++	10+++
109		5++	5-10±	5-20±	20±
119		2±	2-5±	2±	<2
123		<2	<2	<2	<2
128		<2	<2	<2	10-20±
132		<2	<2	<2	<2

* Reciprocals of serum dilutions.
< No agglutination at the dilution of serum.
± Traces of aggl. but less than 25% of antigen agglutinated.
\+ 25% of antigen agglutinated.
++ 50% " " "
+++ 75% " " "
++++ 100% " " "

and on the 198th (second challenge) day of life respectively. Blood samples were collected from the jugular veins at 1 - 7 days interval as required throughout the experiment.

Table 26 summarises the exposure of test and control lambs to brucella over the whole period of the experiment.

Serum agglutination test

The results of the tests of TL group 2. for the first 132 days of life are summarised in Table 27. Table 28 contains the same information for TL group 1.

It is apparent from Table 27 that the three lambs that were exposed to brucella infection only by the oral route gave only a very mild serological response to the ingested organisms. The lambs of Group TL 1 (Table 28) on the other hand responded with definite agglutinin production. At the age of 15 days all of the four lambs produced some sero-agglutinins, presumably to the Br.abortus S19 vaccine administered subcutaneously during the first 3 days of life. The effect of the third injection (15th day) cannot be evaluated with certainty. It may have served as a booster dose but conversely it may have suppressed agglutinin production in some degree since the response to the fourth injection (95th day) was poor. The results in Table 28

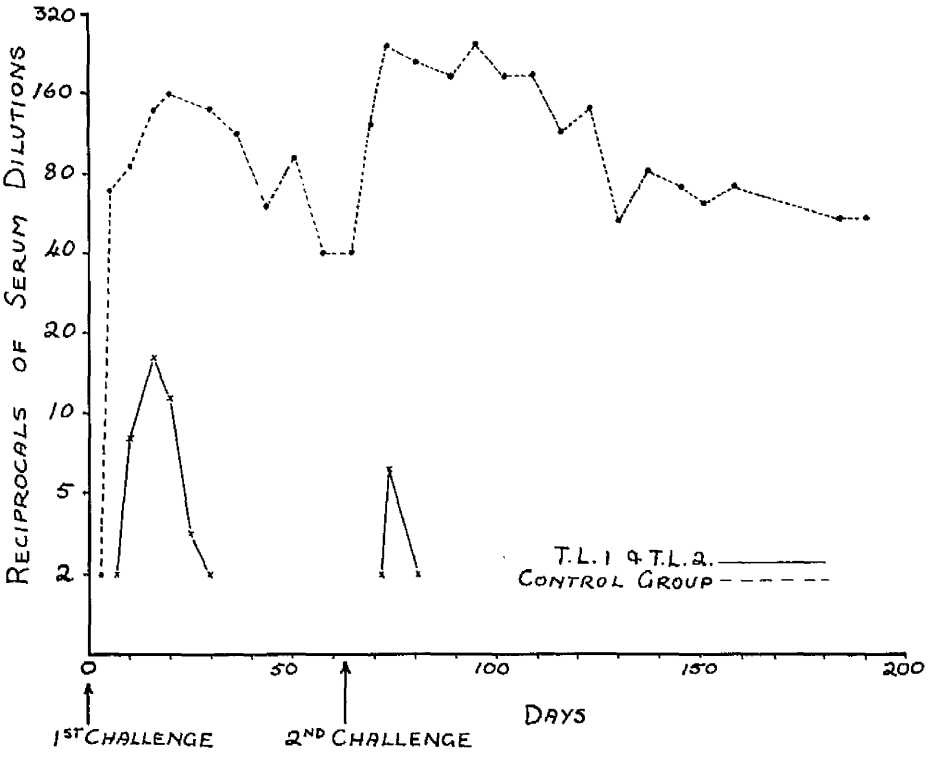


FIGURE 1.

Diagram of average results for the agglutinin titre of sera obtained from the two groups of lambs, TL 1 - TL 2 (x) and C (.), over a period of 196 days following the first challenge (133rd day of life).

TL 2 0.5-1 x 10^6 viable Br.abortus 819 orally for the first (TL 2/5 excluded) 65 days of life.

TL 1 Same as TL 2, in addition 0.5, 2, 2, 2 ml. of Br.abortus 819 standard vaccine suboutaneously on the 1st, 3rd, 15th and 95th day of life respectively.

C No exposure to Brucella until 133 days old.

The two groups were challenged on the 133rd day and 198th day of life by suboutaneous injection of Br.abortus.

indicate that the poor response of lambs in TL 1 to the injection of the 95th day may have been partially at least due to the accumulated effects of the daily oral doses of brucella.

By the time the lambs were 133 days old, TL 1 had had 37 days and TL 2, 67 days free from exposure to brucella. On the 133rd and 189th days of life all three groups (TL 1, TL 2 and C) were challenged with Br.abortus by s/c injections (Table 26).

In the control group all six lambs showed a rapid production of agglutinins after the first challenge and the titre remained high until the second challenge which was followed by a further immediate rise in titre. (Figure 1).

The four animals in TL 1 showed by contrast a markedly inferior response to the first challenge developing only after a lag of 6 - 7 days, reaching a relatively low peak titre (1:17) and falling below 1:2 in about 3 weeks time. One of the animals in this group died shortly before the second challenge. The remaining three showed a similar response to the second challenge as they had done to the first, the antibody response being even poorer on this occasion.

Of the three lambs TL 2, one (TL 2/5) showed no suppression of antibody production, responding to both challenges in much the same way as the lambs in the control group, one (TL 2/4) showed the type of behaviour seen in TL 1 animals and one (TL 2/6) gave a poor response to the first challenge but responded better to the second challenge although even then the agglutinin titre did not reach the level of those shown by the control animals. (Figure 1).

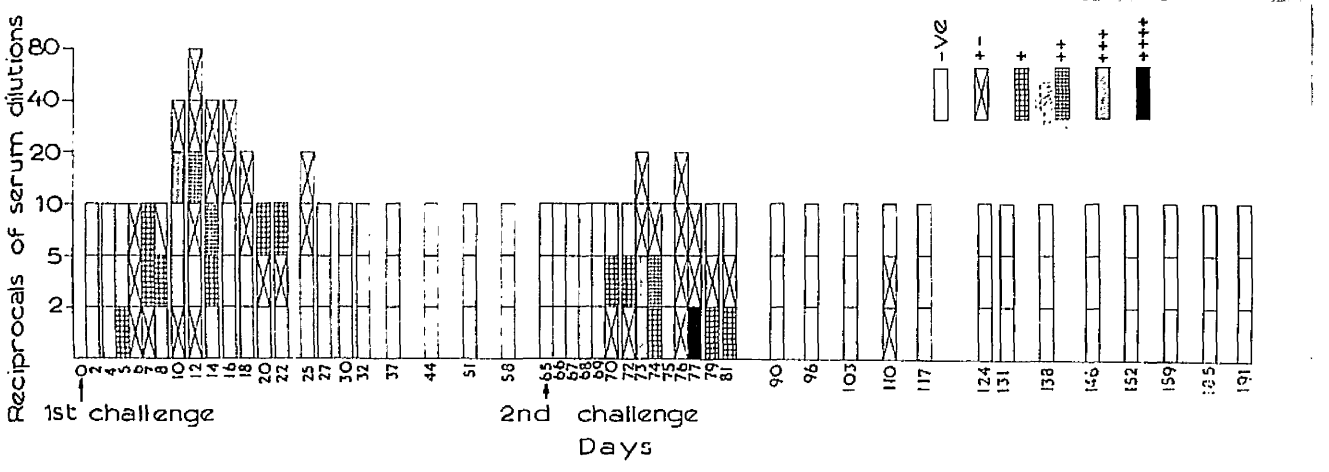


FIGURE 2.

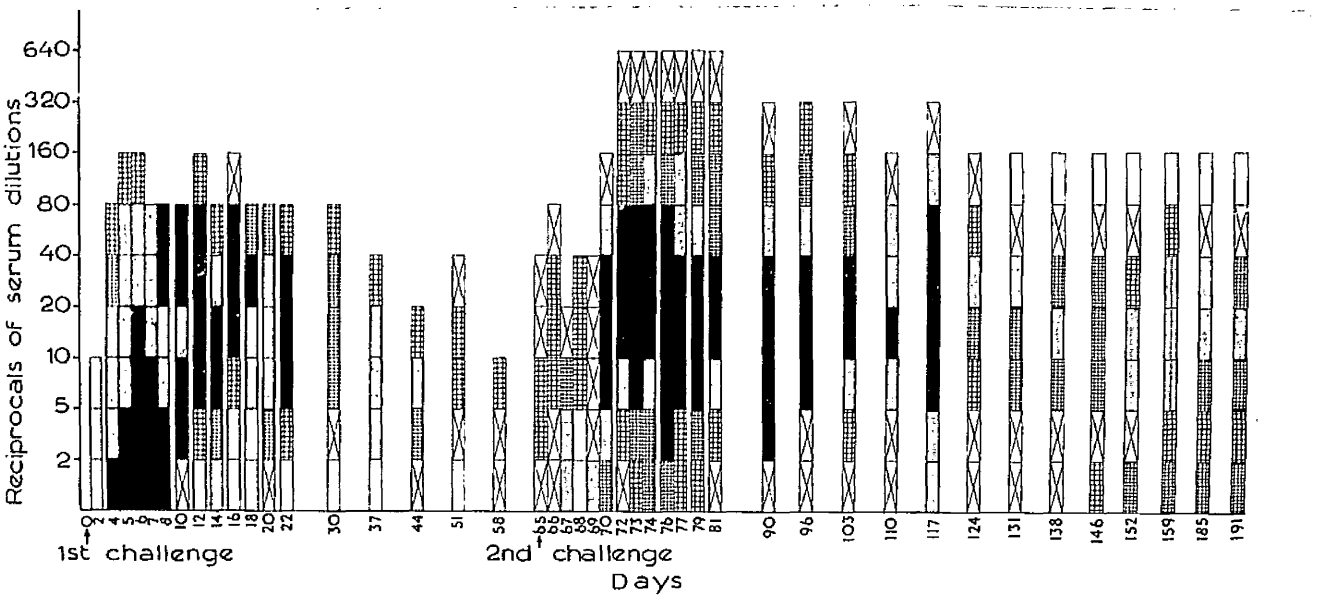


FIGURE 3.

FIGURE 2.

Diagram of the degree of agglutination at different serum dilutions of a lamb (TL 2/4) typical of the test lamb group from the administration of the first challenge (133rd day) until the end of the experiment. TL 2/4 received 0.5-1 x 10^6 viable Br.abortus 819 orally for the first 65 days of life.

-ve	No agglutination at the dilution of the serum.	
±	Traces of agglutination but less than 25% of antigen agglutinated.	
+	25%	of antigen agglutinated.
++	50%	" " "
+++	75%	" " "
++++	100%	" " "

FIGURE 3.

Diagram of the degree of agglutination at different serum dilutions of a lamb (C10) typical of the control group from the administration of first challenge (133rd day) until the end of the experiment. C10 had no experience of Brucella infection until 133 days old. (Symbols as in Fig.2.).

Zoning phenomena and blocking antibodies in the agglutination tests

To consider the serological response of the three groups of lambs (VL 1, VL 2 and C) only in the light of the agglutinin titres would be misleading since there appeared also to be a qualitative difference in the type of antibody produced. As already indicated in 'Methods', the degree of agglutination was recorded at each dilution of all the sera tested throughout the experiment. Figures 2 and 3 show the degree of agglutination at different dilutions for serum of two lambs from the administration of first challenge (153rd day) until the end of the experiment. Figure 2 (VL 2/4) shows behaviour typical of a lamb showing supression of agglutinin production and Figure 3 (lamb C/10) shows typical behaviour of animals in the control group.

In the control group none of the serum samples taken up to the 9th day after the first challenge showed any signs of zoning phenomena. All the test lambs on the other hand showed inhibition of agglutination at the lowest serum dilutions (1:2 - 1:5) and complete agglutination was not seen at any dilution. After the 9th day after the first challenge, sera from the control lambs also exhibited pre-zoning, but complete or almost complete agglutination was present at higher dilutions of these sera.

After the second challenge (198th day) the sera of the control lambs showed pre-zoning and only partial agglutination at higher dilutions for the first 5 days thereafter, although inhibition of agglutination in the first two serum dilutions (1:2 and 1:5) was seen in many cases, complete agglutination almost always occurred at higher dilutions.

Zoning phenomena in the agglutination test suggested the presence of some inhibitory factor, possibly incomplete or blocking antibodies and the agglutination inhibition test showed that blocking antibodies were responsible for most of the prezoning in the agglutination reaction.

Titration of heat-stable blocking antibodies

As pre-zone phenomena was encountered in the agglutination tests of the sera of all the lambs, at some stage of the experiment it became desirable to quantitate these antibodies. Two methods of titration were tried out, the details of techniques are described under the heading 'Serological Techniques' Methods 1 and 2 respectively.

To compare the sensitivity of the two methods a heat-inactivated (56°C for ½ hour) brucella agglutinin free serum sample of a lamb (TL 2/4) was chosen suspected of

TABLE 29

TITRATION OF BLOCKING ANTIBODIES IN OVINE SERUM (USING ONE MINIMAL AGGLUTINATION DOSE OF BRUCELLA ANTIBODIES)

Time of inoculation at 56°C	Reciprocals of the dilutions of TL 2/4 serum in 0.1% formol saline. (each dilution contains 1 m.a.d. of brucella antibodies)						Reciprocals of the dilutions of TL 2/4 serum in 0.1% formol saline.					
(hours)	5	10	20	40	80	160	5	10	20	40	80	160
1	-	-	P	P	-	-	-	-	-	-	-	-
2	-	-	C	C	P	P	-	-	-	-	-	-
3	-	P	C	C	C	C	-	-	-	-	-	-

C = complete agglutination; P = partial agglutination

\- = absence of agglutination

TABLE 30

TITRATION OF BLOCKING ANTIBODIES IN OVINE SERUM (USING THE OVINE SERUM AS DILUENT FOR A BRUCELLA AGGLUTINATING SERUM)

Diluting agent for No.340 reference serum	Time of inoculation at 56°C (hours)	Reciprocals of the dilutions of No.340 ref. serum									
		5	10	20	40	80	160	320	640	1280	2560
Saline	¼	-	P	C	C	C	C	-	-	-	-
	3	C	C	C	C	C	C	C	-	-	-
TL 2/4	¼	P	P	-	-	-	-	-	-	-	-
	3	C	C	C	P	-	-	-	-	-	-
Brucella infection free sheep	¼	C	C	C	C	C	P	P	-	-	-
	3	C	C	C	C	C	C	C	C	P	-

C = complete agglutination P = partial agglutination - = absence of agglutination

containing blocking antibodies. The results of titration of blocking antibodies according to Method 1 are shown in Table 29. In the course of this test one minimal agglutinating dose (m.a.d.) of brucella antibodies is added to each dilution of the serum tested for blocking antibodies. For control TL 2/4 serum was set up for agglutination to show the absence of agglutinins in this serum.

Thus TL 2/4 serum was capable of inhibiting one m.a.d. of brucella antibodies agglutinating the antigens at 1:5 dilution. Agglutination inhibition could not be the result of antibody excess for the quantity of agglutinins was the same at each dilution of TL 2/4 serum which itself was completely free of brucella agglutinins.

The results of titration of blocking antibodies in TL 2/4 serum according to Method 2 are shown in Table 30.

Reference serum No.340 was diluted in TL 2/4 serum. For control the reference serum was also diluted in 0.1% formol saline and in the serum of a sheep free of brucella infection.

It will be seen in Table 30 that this latter method (2) of titrating blocking antibodies in the ovine serum gave

a greater numerical value to the quantity of blocking antibodies than given by the first method. This observation was confirmed by testing numerous sera of both test and control lambs respectively. Therefore Method 2 was adopted for the titration of blocking antibodies throughout the investigation.

Heat sensitive (h.s.s.) blocking factor in ovine sera, its effect on agglutinating systems

In the course of carrying out agglutination inhibition reactions reference serum No. 340, on one occasion, was diluted in a normal heat untreated serum of a sheep which had never been infected with Brucella. The result was unexpected for no agglutination of any dilution of the reference brucella antiserum took place.

The test was repeated the following day using the same normal serum as well as sera of 9 other sheep completely free of brucella agglutinins. One half of each serum was inactivated ($56^{o}C$ for $\frac{1}{2}$ hour) whereas the second half was left without heat treatment. They were all used as diluting agents for the brucella antiserum (No.340). The agglutination test was carried out in the water bath at $56^{o}C$ incubated for 3 hours. On examination of the test tubes no agglutination was detectable in any tube where the diluting agent was the non-heated sera, whereas agglutination was unaffected (1 case) or enhanced by the heat inactivated sera.

Experiments aiming to establish the heat inactivation point of the inhibitory factor in normal ovine sera, showed

that it is relatively stable remaining unimpaired at +4°C for 7 days. Exposure of sera to 50°C for 10 minutes or to 56°C for 5 minutes inactivated this inhibitory factor in all the sera tested. Heat treatment at 37°C for 12 hours inactivated most but not all the sera. Indeed some of them inhibited agglutination to some extent even after 24 hours of incubation at that temperature.

Other experiments showed:

a., that the h.s.e. blocking factor was not strictly specific to the brucella agglutinating system, for it also caused partial inhibition of an E.coli agglutinating system, but enhanced reaction in a Sal. gallinarum agglutinating system.

b., that the h.s.e. inhibitory factor can be almost completely diluted out at 1:10 dilution of sera

c., that it can be removed from brucella cells with relative ease and the interaction between the h.s.e. blocking factor and bacterial cells does not alter the antigenic quality of brucella in any detectable degree.

Heat stable blocking antibodies and some other factors on the pre-zone phenomena

The role of h.st. blocking antibodies in pre-zone phenomena

Sera taken from lambs in the two test groups up to the 6th or 8th day after the first challenge showed no inhibitory effect when used as diluent for the titration of agglutinins in the reference serum (No.340). Inhibitory activity appeared, however, at the same time as some frank agglutinins became detectable in these sera and persisted long after agglutinins had disappeared. Exposure of sera to 56°C for 30 minutes did not eliminate this kind of blocking effect as it eliminated the h.st. blocking factor from normal ovine sera.

The h.st. inhibitory factor reached its peak activity after the disappearance of agglutinins and, on occasion, was so marked as to reduce the titre of the reference serum from 320 to 1:10 or 1:20.

The pattern of agglutination inhibition was very much the same after the second challenge. No detectable agglutinins appeared during the first 7 days and no inhibitory effect either. Some agglutinin production

was then detectable and the serum became agglutination-inhibitory and remained so for many weeks after the disappearance of agglutinins.

Specificity of the heat stable blocking antibodies

For testing the specificity of the agglutination inhibitory factor of inactivated lambs' sera, an E.coli agglutinating system was used in conjunction with the brucella system. No.340 reference serum and E.coli antiserum were diluted in agglutinin-free test lamb's serum respectively. After the addition of the homologous antigens to each system the test tubes were incubated at 56°C for 3 hours before readings were taken. Table 31 shows the results of such an agglutination inhibition test.

It will be seen in Table 31 that whereas the heat inactivated serum of a test lamb inhibited agglutination of the brucella system in a considerable degree, the same lamb's serum enhanced agglutination of the E.coli system. (The heat inactivated serum of the test lambs did not contain agglutinins for E.coli in detectable quantity). It may be taken therefore that the h.st. inhibitory factor in such lambs' sera does not cover up reacting sites non-specifically by being adsorbed onto any bacterial cells.

TABLE 31

SPECIFICITY OF HEAT STABLE BLOCKING ANTIBODIES IN SERA

OF BRUCELLA INFECTED SHEEP

Diluent of antisera	Time of incubation at 56°C (hours)	Reciprocals of the dilutions of *E.coli* antiserum								Reciprocals of the dilutions of *Br.abortus* (No.340) antiserum						
		10	20	40	80	160	320	640	1280	10	20	40	80	160	320	640
Saline	3	+	+	+	+	+	+	-	-	+	+	+	+	+	+	-
Sheep serum x	3	+	+	+	+	+	+	+	-	+	-	-	-	-	-	

x = Serum from a test lamb containing blocking antibodies

More evidence for the specificity of the h.st. blocking antibodies was obtained by adsorbtion tests. Heat stable blocking antibodies could be adsorbed out of the serum specifically and completely by whole cell brucella suspension.

The role of antigen-antibody proportions in the zoning phenomena

Serum samples of the control group of lambs first exposed to Br.abortus when 133 days old presented a more complex picture of agglutination inhibition than sera of the test lambs. In some of these pre-zoning sera of the control lambs inhibition of agglutination was due to h.st. blocking antibodies, but titres were considerably lower. From some of the other sera of the control lambs the zoning effect could be abolished by changing the concentration of antigen added to the particular serum dilution where the zoning effect was manifested.

The heat inactivated (at 56°C for 30 minutes) serum sample of one of the control lambs (C.9 for example) gave the following reading of agglutination after 24 hours of incubation at 37°C.

Serum dilutions	1:2	1:5	1:10	1:20	1:40	1:80	1:160	1:320
Degree of agglutination	++++	++++	+++	±	+	++++	++++	++

Thus a 1:20 dilution of this serum was distributed into test tubes and to each allotment of serum decreasing quantities of the standard antigen added. The test tubes were incubated at 37°C for 24 hours then readings were taken. Table 32 shows the relative quantities of reagents as well as the results of agglutination.

TABLE 32

THE EFFECT OF ANTIGEN CONCENTRATION ON ZONING PHENOMENA MANIFESTED IN THE SERA OF A CONTROL LAMB

1:20 dilution of serum (C/9)	(ml)	0.5	0.5	0.5	0.5	0.5
Standard antigen suspension	(ml)	0.5	0.4	0.3	0.2	0.1
Saline	(ml)	-	0.1	0.2	0.3	0.4
Degree of agglutination (at 37°C for 24 hours)		-ve	±	±	++	+++

Such manifestation of the zoning phenomena as illustrated in Table 32 were relatively infrequent. They appeared to be the result of less than optimal proportion of antigen-antibody at the particular serum dilution where the zoning effect was manifested.

There was another kind of zoning-effect encountered in the agglutination tests carried out on the sera of control lambs.

When sera of the test lambs were used as diluents for the reference serum (No.340) agglutination inhibition was affected at the highest dilutions of the reference serum. By contrast some of the pre-zoning sera of the control lambs inhibited agglutination at the lowest dilutions of No.340 serum. This kind of inhibition suggested a different mode of action not encountered in any of the sera of the test lambs.

To see whether agglutination inhibition may have been due in such cases to simple excess of antibodies attempts were made to bring it about experimentelly.

Three inactivated sera of the control lambs showing no zoning effect at any dilution were chosen with the following agglutinin titres: (at 37°C for 24 hours).

Serum of No. 11 lamb	$1:20^{+}$
" " No. 12 "	$1:80^{+}$
" " No. 8 "	$1:160^{++}$

TABLE 33

PRE-ZONE PHENOMENA IN THE BRUCELLA AGGLUTINATION SYSTEM

CAUSED BY EXCESS OF ANTIBODIES

Diluting agent for No.328 serum	Time of incubation at 56°C (hours)	Reciprocals of the dilutions of Br.abortus antiserum (No. 328)						
		5	10	20	40	80	160	320
Serum of No.11 lamb	0.5	-	-	-	P	C	C	C
	1	±	±	±	C	C	C	C
	3	C	C	C	C	C	C	C
Serum of No.12 lamb	0.5	-	-	-	P	P	C	C
	1	±	±	±	P	C	C	C
	3	P	P	P	C	C	C	C
Serum of No.8 lamb	0.5	-	-	-	-	±	±	P
	1	-	-	-	±	±	±	C
	3	±	±	P	P	P	P	C

C = complete agglutination P = Partial agglutination ± = Traces of agglutination

- = No agglutination

They were used as diluents for a hyperimmune Br.abortus S19 (No.328) antiserum (rabbit) which itself did not show any degree of zoning effect and had an agglutination titre of 1:5120++. The agglutination inhibition test was carried out as usual, readings of the progress of agglutination were taken periodically, the results are shown in Table 33.

It will be seen in Table 33 that agglutination invariably commenced at the highest dilutions of No.328 antiserum and as the concentration of antibodies increased in the lower serum dilutions the degree of agglutination became progressively diminished. Serum of No.8. lamb with the highest agglutinin titre among the three diluent sera caused almost complete inhibition of agglutination at the lowest dilutions of No.328 brucella antiserum. As there had been no sign of zoning effect in either the diluting or in the diluted sera in the saline agglutination tests, agglutination inhibition was presumably due to excess of antibodies.

This conclusion was confirmed by retesting some of the sera of control lambs which inhibited agglutination at the lowest dilution of the reference serum (No.340) in the

agglutination inhibition tests. In these re-tests the reference serum was replaced by the hyperimmune brucella antiserum (No.328) which resulted in a more extensive prozone effect than those caused by the reference (No.340) serum.

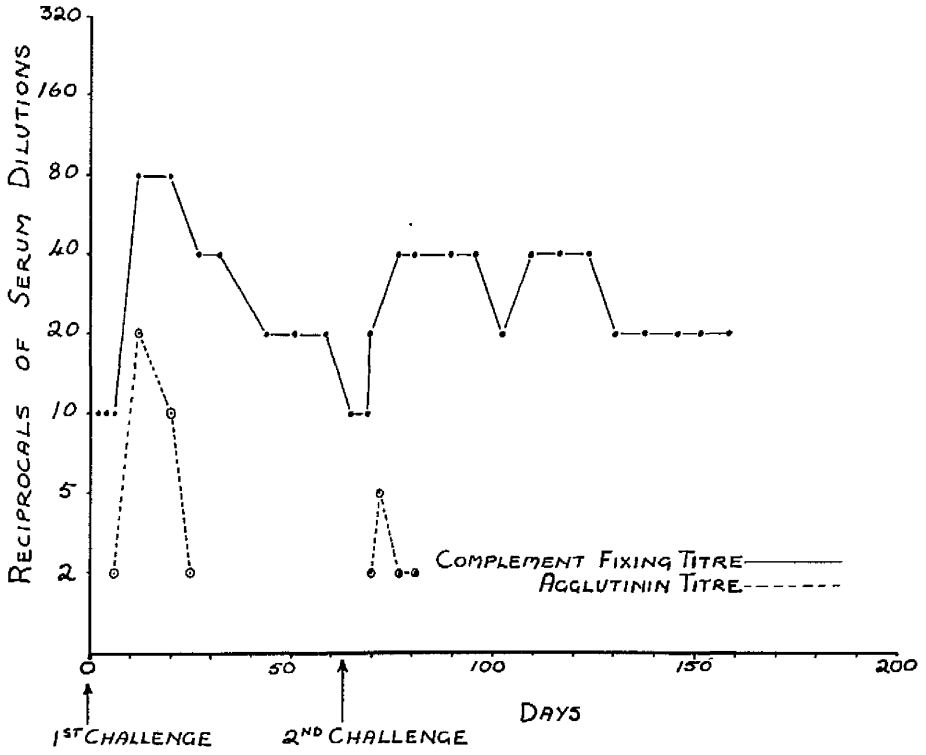


FIGURE 4.

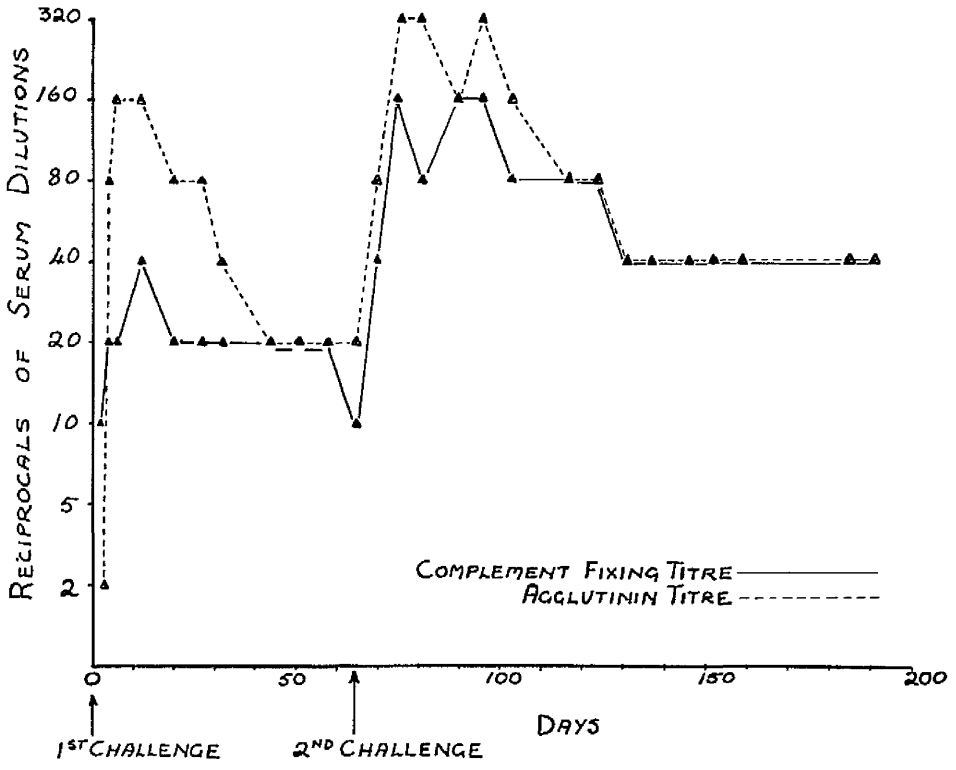


FIGURE 5.

FIGURE 4.

Titre of complement-fixing (•) and the agglutinating (o) antibodies for a typical lamb of the test group (TL 2/4: exposed to 0.5-1 x 10^6 viable Br.abortus S19 daily by the oral route during the first 65 days of life) challenged (first) on the 133rd day of life and (second) on the 198th day of life by Br.abortus.

FIGURE 5.

Titre of complement-fixing (▲) and the agglutinating (△) antibodies for a typical lamb of the control group (C10: no exposure to Brucella until 133 days old) challenged (first) on the 133rd and (second) on the 198th day of life by Br.abortus.

Complement fixing antibodies

The c.f. test also enables antibodies to be demonstrated in the sera of test lambs devoid of agglutinins. Serum samples of the test lambs (excluding TL 2/5 and TL 1/7) as well as samples from all the control lambs were examined for c.f. antibodies from the administration of the first challenge (133 days) until the end of the experiment.

Figure 4 shows the titre of complement fixing and the agglutinating antibodies for a typical animal of the test group (TL 2/4) from the first challenge until the end of the experiment and Figure 5 shows similar results for a typical control lamb (C/10).

All the lamb sera - even those from control animals taken weeks before exposure to brucella - gave fixation of complement in the presence of brucella antigen up to a serum dilution of 1:10. Absorption of serum with whole cell brucella suspensions may decrease the complement fixing activity but does not completely eliminate it.

Serum diluted 1:2 with saline had some anti-complementary activity alone as also had the antigen suspension. These slight non-specific effects did not

interfere with the test at serum dilutions exceeding 1:10. As seen in Figures 4 and 5, there was no significant difference in the complement fixing titres of the sera of test and control lambs. The complement fixing titres of the sera of control lambs ran parallel to their agglutinin titres which perhaps suggests that complement fixation may have been effected by the agglutinating antibodies.

The complement fixing titre of the test lambs' sera rose above the 1:10 non-specific level at the time of the appearance of agglutinins, but remained at a high level at the time when agglutinins were no longer detectable. It came down to the 1:10 level just before the administration of the second challenge (198th day). The same pattern was repeated after the second challenge with brucella.

Complement fixing antibodies were absorbed from sera of test and control lambs by whole cell brucella suspensions bringing their titres down to the 1:10 (non-specific) level.

TABLE 34

SKIN SENSITIVITY TEST USING A SOLUBLE BRUCELLA ANTIGEN PREPARATION ON BRUCELLA INFECTED AND NON-INFECTED LAMBS

Soluble Brucella antigen intracutaneously (ml.)	Time after injection (hours)	Test lamb groups 1 and 2		Control group		Non-infected group	
		Extent of oedema ($cm.^2$)	Thickness of skin at the site of injection (mm.)	Extent of oedema ($cm.^2$)	Thickness of skin at the site of injection (mm.)	Extent of oedema ($cm.^2$)	Thickness of skin at the site of injection (mm.)
0.1	0.0	-ve	3	-ve	3.5	-ve	3.2
0.1	0.5	4.89	8	1.10	4.1	0.22	3.8
0.1	1	5.30	12.7	2.10	5.2	1.76	4.5
0.1	2	10.99	12	3.23	7.3	2.26	7
0.1	3	17.58	8	4.36	5.8	3.79	5.5
0.1	4	19.62	7	4.89	5.0	3.14	5.5
0.1	24	Trace	8	Trace	4.7	-ve	4.5
0.1	48	Trace	6.5	-ve	3.5	-ve	3.5
0.1	72	-ve	3	-ve	3.5	-ve	3.2

Values in the table are averages taken of result obtained from the appropriate groups. TL 1 and TL 2 contained six lambs, the control group consisted of six lambs, and there were two non-infected animals.

Skin sensitivity test

The tests were performed 5 days after the administration of the first challenge dose of brucella (133 days). In the sera of the test lambs (TL1 andTL 2) no agglutinins were detectable at that stage, the c.f. titre was negative (1:10) and the sera had no inhibitory effect on the brucella agglutinating system.

The agglutinin titre of the control lambs was by that time between 1:20 and 1:80 and there was a low c.f. titre. Two lambs with no previous brucella infection were included in the skin sensitivity test for control purposes. The results are summarised in Table 34.

The skin reactions observed were of the immediate type showing swelling, erythema and oedema. Changes appeared from 10 to 20 minutes after injection of the antigen. Injection of 0.1 ml. amounts of thiomersalte saline (1/10,000) gave only a mild reaction, the extent of the oedematous area and the thickness of skin not quite reaching the intensity of reactions of Brucella-negative lambs to the soluble antigen. The reactions of the control group were hardly different from those of the Brucella-free lambs, despite the fact that

circulating antibodies were present in the sera of the control lambs. The reaction of the test lambs (TL 1 and TL 2) both with regard to the extent of oedematous swelling the the thickness of the skin was markedly greater than that of the control animals or of the uninfected lambs.

DISCUSSION

Although the experiments described here were carried out on a small number of animals, the results indicate that long exposure of very young lambs to viable Br.abortus S19 brings about a marked change in their serological response to the same and closely related antigens when these are encountered later in life. The route of primary exposure to antigen appears to be important. Lambs exposed to infection only by the oral route showed very little agglutinin production during the period of exposure and varied in the extent to which they exhibited a changed ability to respond to later challenges with the same antigen. By contrast animals receiving antigen parenterally in addition to oral exposure gave a vigorous antibody response and a uniform and more marked interference with the pattern of response on later challenges. This more pronounced interference, however, may not be attributed entirely to the route of exposure, since this group of lambs (TL 1) received a higher total quantity of the antigens. These results are in agreement with those of Buxton (1954) and of Smith and Bridges (1950).

Antibody production in lambs showing suppression of antibody formation differed from that seen in the control

animals in several ways: there was a marked increase in the lag period after challenge before agglutinins appeared in the circulation (a finding which agrees with that of Owen, 1956; who observed a similar lag phase after injection of human red blood cells into rabbits and chicks that had had neonatal experience of the same antigen); the maximum titres reached were very much lower and agglutinins persisted for a shorter length of time. Perhaps the most striking differences were connected with the actual types of antibody produced. Test animals showed an increased proportion of non-agglutinating antibodies which appeared coincidentally with agglutinins but persisted long after these had disappeared. These non-agglutinating antibodies were responsible for 'zoning' in agglutination test with test lamb sera and could be detected by agglutination inhibition.

The presence of some blocking antibodies in the sera of control lambs is worthy of attention. These antibodies do not appear to be identical with the c.f. antibodies since a high c.f. titre did not imply agglutination inhibition while serum with a low c.f. titre could exhibit inhibition. The biological basis of the production of blocking antibodies is a matter of speculation, but studies regarding the intracellular survival and growth of brucella may be relevent here.

Holland and Pickett (1956) and Fomales-Lebron and Stinebring (1957) have demonstrated the ability of brucella to multiply within monocytes and other tissue cells under cell culture conditions. Holland et al. (1958) using normal rat, guinea-pig and mouse monocytes obtained data to indicate that smooth strains of Br.abortus, suis and melitensis are capable of excessive intracellular multiplication and such extensive multiplication of brucella within the antibody producing cells may be of importance in the formation of incomplete antibodies.

The underlying causes of zoning phenomena, as manifested in the sera of the control lambs, however, were somewhat more complex than that in the sera of the test lambs. Pre-zone phenomena was brought about by an excess of agglutinins in the sera of the control lambs at low serum dilutions or less frequently by unfavourable antigen-antibody proportions at dilutions which were preceded by complete agglutination of the brucella antigens.

Agglutination inhibition may be caused in both test and control lambs' sera by a heat sensitive blocking factor which was present not only in the sera of the experimental animals but also in the sera of 10 other sheep which were not

infected with brucella and had no agglutinins to these organisms. This blocking factor can be inactivated at 50°C for 10 minutes or at 56°C for 5 minutes. The agglutination inhibitory power of the heat sensitive blocking factor can be almost completely abolished by diluting such sera 1:10 in physiological saline, but it remains unimpaired for at least 7 days at +4°C. The adherence of the blocking factor to brucella cells is relatively weak and if washed off the cells, the antigenic properties of the washed cells remain unaltered.

Complement-fixing antibodies were detected in both test and control animals' sera in roughly similar amounts after challenge. Since it has been established that the brucella agglutinin response in lambs may be seriously depressed as a result of experience of brucella antigen early in life, it is suggested that the complement fixation test may have important practical value in the detection of brucella infections.

The skin sensitivity test carried out 5 days after the first challenge doses of brucella revealed an immediate type of hypersensitivity of the test lambs to the intradermal injection of soluble brucella antigens. No attempt was made

to effect the passive transfer of hypersensitivity to other animals since, at that time, no circulating antibodies could be detected by the agglutination, complement-fixation and agglutination-inhibition reactions. The control lambs showed no marked hypersensitivity when tested at the same stage in the experiment, though they showed both circulating agglutinins and complement-fixing antibodies. It appears, therefore, that the hypersensitivity of test lambs was not due to circulating antibodies.

Several workers have noted decreased production of certain antibodies whilst others have been unaffected after attempts at induction of immunological tolerance.

Wolf, Tempelis, Mieller and Reibel (1957) studying the precipitin production of chicks and the effect of the injection of large quantities of bovine serum albumin (BSA) on the precipitin productions remarked: "We cannot at this time explain why the disappearance rate of antigen from the circulation does not seem to change in chicks made unresponsive or poorly responsive as a result of antigen injections at hatching. Since the rapid disappearance (3rd phase) of antigen from the circulation has been associated with antibody appearance, it is possible that the chicken is producing non-precipitating antibodies" .

Smith et al. (1958) describing the response of rabbits injected neonatally with BSA to the same antigen later in life noted that of those rabbits that received just the threshold amount of BSA (10 mg.) to produce tolerance, three produced antibodies on the third challenge at 380 days. These rabbits had cleared antigen rapidly (half-life, 1.9; 2.6; 4 days) after the 194th day of challenge in contrast to their initial challenge, but failed to produce detectable circulating antibodies (precipitins).

In this connection, the observations of Billingham, Brent and Medawar (1956) may be of importance; they noted that in chicks that had lived in parabiosis, the disappearance of red cell chimaerisms and the concurrent development of intolerance towards an established skin graft occurred in chicks that still continued to display a considerable degree of inhibition of the development of agglutinating antibodies upon immunization.

Hasek's (1956) observation of suppressed precipitin formation in a hen that had lost chimaerism with turkey cells is a similar case in point.

Owen (1956) describing the effect on subsequent challenge of the injection of human red cells into newborn animals of different species found a marked change in the types of antibodies produced as compared with the controls. This change in the types of antibody population was expressed in sharp zones in the saline agglutination tests of serum samples from the neonatally injected chick. Such sera would agglutinate the appropriate red cells at low dilutions, which would be followed by dilutions where no agglutination would be apparent, to be followed by marked agglutinations at even higher dilutions of the serum. Still higher dilutions of the serum would give negative reactions. He found that this zoning effect was due to 'blocking' antibodies, the amount of which was high relative to that of the complete antibodies in the neonatally treated birds. Owen (1957) suggests that the apparent depression of agglutinin production may partly derive from another channel of immune response, namely, the development of incomplete rather than complete antibodies.

Buxton (1954) studying the effect of Salmonella pullorum infection in pre-natal and post-natal birds and their response to the same antigen on subsequent challenge, noted in all of his experiments the appearance of non-agglutinating antibodies

before agglutinin production. These non-agglutinating antibodies often caused 'zoning' in the agglutination test, and had a higher titre than the agglutinins. In interpreting his findings he suggests that, for the production of complete antibodies (agglutinins), the antibody-producing cells of the bird must be mature and that the incomplete antibodies are probably the products of the immature antibody-producing mechanism which cannot yet form complete antibodies. This interpretation of the presence of incomplete antibodies is in accordance with the suggestion of Burnet and Fenner (1948) who believed that the type of antibody produced might differ according to the type and stage of specification of the cells responsible for its production.

Such an interpretation of the presence of incomplete antibodies cannot be applied to those found in the sera of lambs neonatally injected with brucella. These lambs were capable of agglutinin production at the age of 15 days without evidence of the presence of incomplete antibodies, which only became apparent on subsequent challenges at the age of 133 days and thereafter. The presence of incomplete antibodies is well known in the brucella agglutinating system.

Glenchur, Zinneman and Hall (1961) experimenting on the significance of blocking antibodies in experimental brucellosis found a direct correlation between the quantity of heat-killed brucella administered to young adult rabbits and the appearance and extent of blocking antibodies produced.

Non-agglutinating antibodies have been demonstrated after immunization with several other antigens as diverse in type as insulin (Lowell and Franklin, 1949), diphtheria toxoid (Kuhns, 1955) and Shigella (Morgan and Schutze, 1946). The common feature of all of these experiments is the repeated and/or heavy antigenic stimulation used.

The most outstanding feature of the experiments described here is the marked shift in the type of antibodies produced in the post-natally infected lambs compared to the controls. A form of split immunological tolerance may be advanced as a possible explanation of the production of incomplete antibodies together with the marked suppression of agglutinin formation. To some of the major agglutinogenic components of the bacterial cells the tolerance may be complete but suppressed in different degrees to the lesser ones giving an overall impression of partial tolerance.

Split tolerance may further account for the high level of incomplete antibodies if we assume that the antigen components giving rise to the incomplete antibodies are minor components of the bacterial cells to which no tolerance or indeed sensitization has developed.

This hypothesis is, however, contradicted to some extent by the finding that these incomplete antibodies can completely inhibit antigen-antibody reactions taking place between complete antibodies and their appropriate antigens. This inhibition may be effected in two different ways: the first mode of inhibition would be due to the fact that the antigens responsible for the stimulation of incomplete antibody production are so situated on the bacterial cells that, by reacting with the incomplete antibodies, they would block access of complete antibody to the cell, thus preventing agglutination non-specifically. Alternatively, the blocking antibodies may have the same specificity as the agglutinins, that is to say, they are incompletely formed or monovalent 'agglutinins'. These observations and those of such workers as Wolfe et al. (1957), Smith and Bridges (1958), Billingham et al. (1956), Hasek (1956), Owen (1956) and Buxton (1954), regarding the presence of some incomplete forms of antibodies at a time of total or

partial suppression of the formation of more complete forms might be explained by postulating a gradual change in the process of antibody formation resulting in the replacement of complete forms by incomplete, preceding and/or following the development of complete immunological tolerance. This hypothesis, however, must be essentially tentative until the gradual degradation of the more complete antibodies into incomplete forms preceding the complete suppression of antibody formation against a single antigen is conclusively demonstrated.

D. THE LONG TERM EFFECTS OF BRUCELLA INFECTION OF THE BOVINE NEONATE

The results of the experiments on the brucella infection of young lambs in respect to their serological responsiveness to re-infection, were sufficient to stimulate similar investigations in other species. At the beginning of 1963 the question of accommodation of large experimental animals and the financial support of new experiments were both solved, so that it became possible to examine the long term effects of brucella infection of the bovine neonate.

The objectives of these experiments are as follows:

1. The examination of the effect of brucella infection of young calves in terms of resistance to re-infection at the normal vaccination age of 7 months.

2. To examine the serological capacities of such calves to respond to re-infection.

3. To re-examine the possibility of calves becoming permanently infected as a result of exposure to brucella at 7 months of age.

As some aspects of these experiments are essentially long-term in nature (at least 5 years in duration) it is not possible to include results at this stage beyond the first 12 months of the investigation.

MATERIALS AND METHODS

Animals

Ten heifer calves of dairy breed were used for the experiments. They were born to cows with negative (< 1:10) sero-agglutinin titres to brucella.

The calves had one feed of colostrum and were then separated from their dams. They were divided into two groups (6 and 4 respectively) and within the group the calves were accommodated individually or by the pair in calf pens during the first 3 months of life while bottle fed.

Between the age of three to eleven months all ten of them shared the same pasture and cattle court, after which the 4 control calves were separated from the rest and housed in isolation.

Antigens

For the agglutination, complement fixation (c.f.) and Coombs' antiglobulin tests the antigen used was that issued by the Ministry of Agriculture, Fisheries and Food, Central Veterinary Laboratories, Weybridge, for the Standard tube agglutination test for bovine brucellosis.

Preparation of brucella suspensions for the infection of calves

The virulent organism chosen for this purpose was the reference strain of Brucella abortus (544). It was grown on serum-dextrose agar (Brinley-Morgan, 1960) for three days at 37°C in the presence of 10% CO_2. Growth was harvested in sterile Ringer's Solution. The total number of organisms per ml. suspension was established by means of the opacity tubes. (Burroughs Wellcome & Co. England). The number of viable cells per ml. of such suspension was determined by viability counts.

Fresh suspension of brucella was prepared each day and the required number of organisms added to the milk ration of calves and warmed to 37°C. Viability counts were carried out on every 3rd inoculum used for the contamination of brucella-free milk. For the injection of calves at 7 months of age the organism used and the method of preparation of inoculum was identical to that employed for the oral infection of calves.

Sera

Blood samples of calves were collected via the jugular vein. The first sample was taken a few hours after the ingestion of colostrum but before the first exposure to brucella infection. Thereafter samples were collected from 1 to 14 day intervals as required and the sera separated were stored at -20°C without the addition of any preservatives.

The anti-bovine globulin serum for the Coombs' test was raised in a rabbit. After a course of 4 injections of BGG (1.5 cc each) in Freund's adjuvant, the rabbit was bled repeatedly at short intervals, the sera pooled and its precipitin titre adjusted with pre-inoculation rabbit serum to a titre of 1:4000. This pooled anti-BGG serum (No.910) was used for the Coombs' anti-globulin test throughout the investigation described here.

Serological techniques

The agglutination and c.f. tests were carried out the same way as described in Part 2 A under 'Serological techniques'. Agglutinin titres of 1:10, 1:20,

etc., with sera tested indicate approximately 20, 40, etc. units of antibody per ml. respectively. The agglutinin titre of a serum was taken as the highest dilution of the serum in which 50% of the brucella antigen was agglutinated. The c.f. titre of the serum was read at 50% fixation of complement. The sensitivity of the c.f. test is such as to give 50% fixation of complement at 1:80 dilution of the International Standard Anti-Brucella abortus Serum.

The antiglobulin test of Coombs' was performed as follows: Standard agglutination test on the heat inactivated (56°C for 30 minutes) calf sera were carried out. After over-night incubation at 37°C the agglutination reaction was read and those dilutions of serum which caused any macroscopic agglutination were discarded. The four consecutive dilutions of serum following the last one in which agglutination took place was used for the anti-globulin test. The test tubes containing the antigen - serum dilution mixtures were centrifuged at about 4 - 5000 Gs. for 20 minutes then the supernatant fluid poured off and the sedimented cells re-suspended in 0.6 ml. physiological saline by agitation with a Pasteur pipette. The suspension

was centrifuged again and the washing of cells was repeated three times in all. After the last washing the cells were re-suspended in 0.6 ml. physiological saline and into each test tube 1 drop (approximately 0.03 ml.) of anti-BGG serum (No.918) was added. After overnight incubation at 37°C the results were read. The titre was taken as the highest dilution of the serum where 50% or more of the brucella cells were agglutinated by the anti-BGG serum.

For negative control the bovine serum used was completely free of brucella agglutinins, it was also negative to the anti-globulin test. The positive control serum had a titre of 1:20000 when examined by Coombs' anti-globulin test. (Agglutination titre from 1:2 to 1:80 -ve, from 1:160 to 1:640 +ve).

RESULTS

Schedule of bleeding and exposure of the test and control calves to brucella

In the course of the experiment each of the 6 test calves received 1 x 10^{12} Brucella abortus 544 (viability 57% ± 4%) in their milk ration daily for the first 15 days of life beginning at ∠24 hours of age. The 4 controls were meanwhile fed on brucella-free milk. After the elapse of 15 days the feeding of brucella contaminated milk was discontinued and from then on they were not exposed to brucella infection until they reached 7 months of age. At 7 months of age (± 1 week) each of the 10 calves was injected with 6 x 10^{10} Brucella abortus 544 subcutaneously. Immediately prior to injection they were bled and blood samples were collected from them all at two day intervals for 3 weeks. During the following 6 weeks blood samples were taken once a week and thereafter at fortnightly intervals.

Serum agglutination test

The results of agglutination tests carried out on the sera of the 10 calves are shown in diagramatic

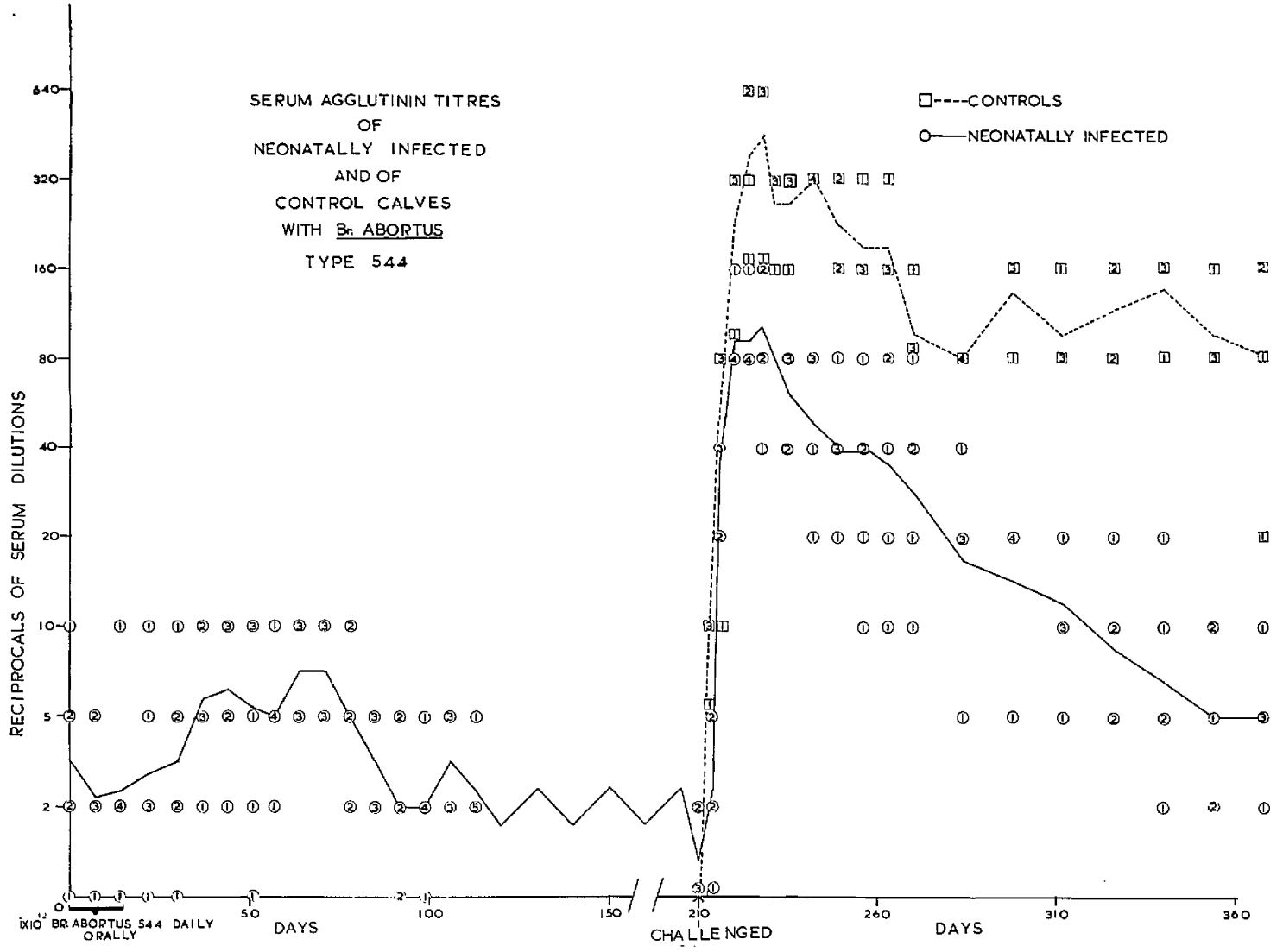

SERUM AGGLUTININ TITRES
OF
NEONATALLY INFECTED
AND OF
CONTROL CALVES
WITH Br. ABORTUS
TYPE 544
□----CONTROLS
O——NEONATALLY INFECTED
RECIPROCALS OF SERUM DILUTIONS
640
320
160
80
40
20
10
5
2
0
1X10⁻² BR. ABORTUS 544 DAILY ORALLY
50
100
150
210
260
310
360
DAYS
CHALLENGED
DAYS

FIGURE 6.

FIGURE 6.

Serum agglutinin titres of neonatally infected and of control calves with Br.abortus type 544 from the first day of life until 12 months of age. The figures in the circles and squares show the number of calves having a particular titre at a particular time. The position of the curves was calculated to represent average results of agglutinin titres.

form in Figure 6, from the first day of life until 12 months of age. The figures in the circles and squares show the number of calves having a particular titre at a particular time. As there were some differences in titres in both groups of calves the position of a curve was calculated for test and control calves respectively, representing average results of agglutinin titres.

The points of interest arising from Figure 6 may be summarised as follows:

1. The small amount of passively transferred brucella agglutinins disappeared from the sera of calves within a short period of time, but the active production of agglutinins did not commence until 30 to 40 days of age. Agglutinin production started, in other words, 15 days or even later after the last exposure to brucella infection.

2. The titre of actively produced agglutinins was very low in comparison to the agglutinin titre of calves at vaccination age (6 - 8 months).

3. Agglutinin titres dropped to 1:2 serum dilution or even less than that 12 weeks after their first appearance and remained at this level in the case of 5 out of 6 calves until they were injected with brucella.

4. The 6th calf (IF/9) in the neonatally infected group developed a titre of 1:10, after having dropped to $\angle$ 1:2, during the last week prior to the brucella injection. Her responses to this injection as measured by the three serological tests (agglutination, c.f. and Coombs' anti-globulin test) were strictly comparable to that of the control calves. (For that reason the results of sero-logical tests on the sera of this calf were excluded from all the diagrams after the administration of the challenge dose of brucella).

5. The other 5 neonatally infected calves reacted to the challenge dose of brucella without the prolonged lag-phase observed with the agglutinin production of neo-natally infected lambs.

6. The average sero-agglutinin titre of the five test calves fell approximately two serum dilutions short of that of the control calves.

7. Serum agglutinin titre of the test calves came under the significant level (1:20+) within approximately 3 months after infection, whereas titre of the control calves remained positive even 5 months after exposure to infection.

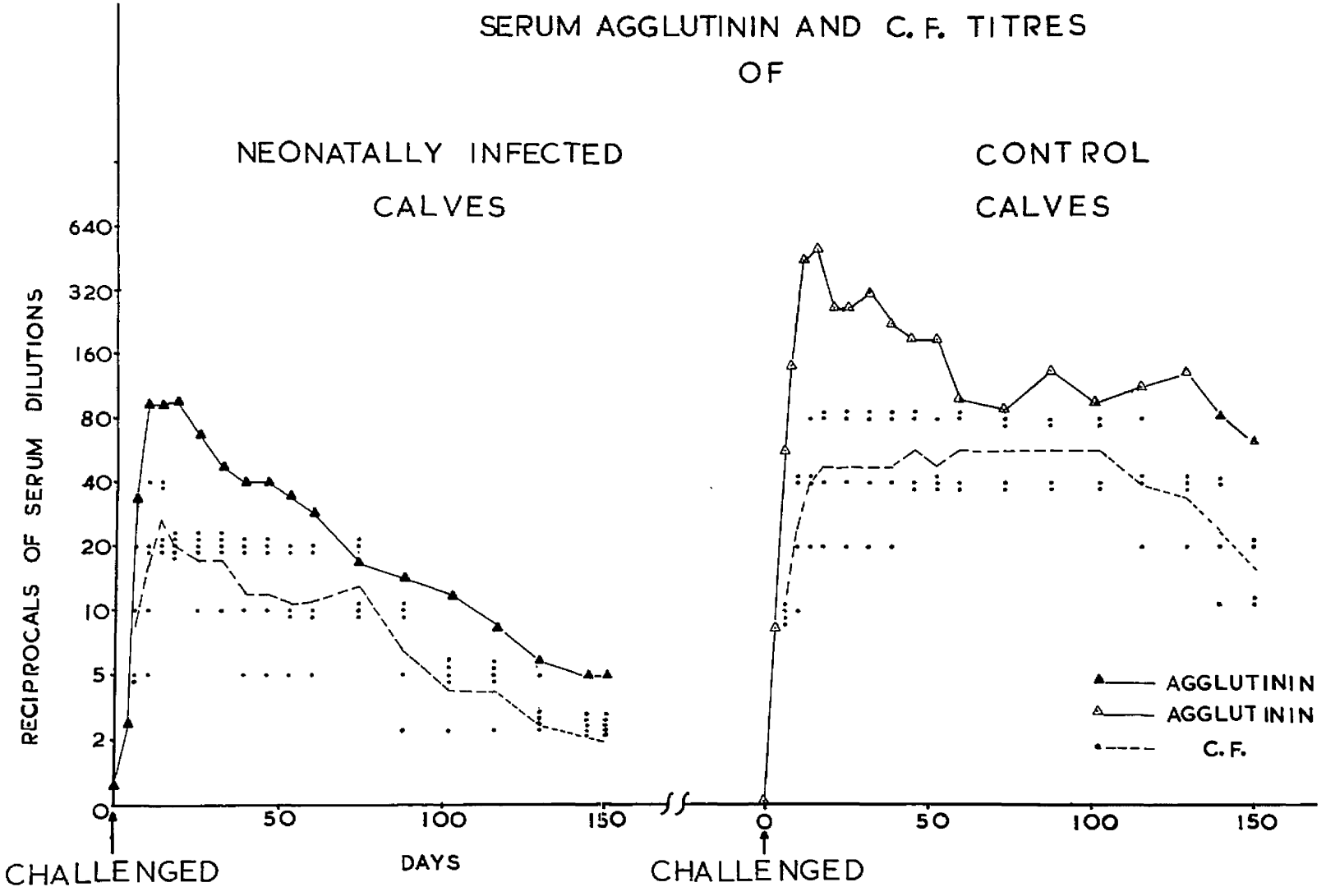

SERUM AGGLUTININ AND C. F. TITRES
OF
NEONATALLY INFECTED
CALVES
CONTROL
CALVES
RECIPROCALS OF SERUM DILUTIONS
640
320
160
80
40
20
10
5
2
0
50
100
150
DAYS
CHALLENGED
0
50
100
150
CHALLENGED
AGGLUTININ
AGGLUTININ
C. F.

FIGURE 7.

FIGURE 7.

Brucella sero-agglutinin and complement fixing titres of 5 neonatally infected (1×10^{12} Br. abortus 544 daily by the oral route during first 15 days of life) and of 4 control calves to a challenge dose of viable Br.abortus 544 administered subcutaneously at 7 months of age.

Average agglutinin titre of neonatally infected (▲ - ▲) and of control calves (△ - △). The black dots in diagram show c.f. titres of the individuals and the position of curves (---) was calculated to represent average results of c.f. titres.

Complement fixation test

The results of c.f. tests are presented in Figure 7, for both neonatally infected as well as for the control calves parallel to their mean agglutinin titres. The results are given as from the day of injection of the challenge dose of brucella (at 7 months of age). All the sera of the 10 calves collected during the first 7 months of life were negative ($< 1:5$) to the c.f. test. The black dots in the diagram show c.f. titres of the individuals.

The points of interest arising from the results of complement fixation are as follows:

1. Complement fixing antibodies were not detectable in the sera of any of the 10 calves during the first 7 months of life despite the fact that all of the 6 neonatally infected calves produced some agglutinins as a result of oral exposure to brucella.

2. Complement fixing antibodies appeared at the same time in the sera of both test and control calves after the injection of the challenge dose of brucella.

3. The peak values of c.f. titres - unlike the agglutinin titres - are comparable in both groups of calves.

4. The c.f. titres of the test calves came under non-significant level (< 1:5) approximately 3 months after infection, whereas the c.f. titres of the control calves are positive at a significant level(1:5 or higher) even 5 months after exposure to virulent brucella.

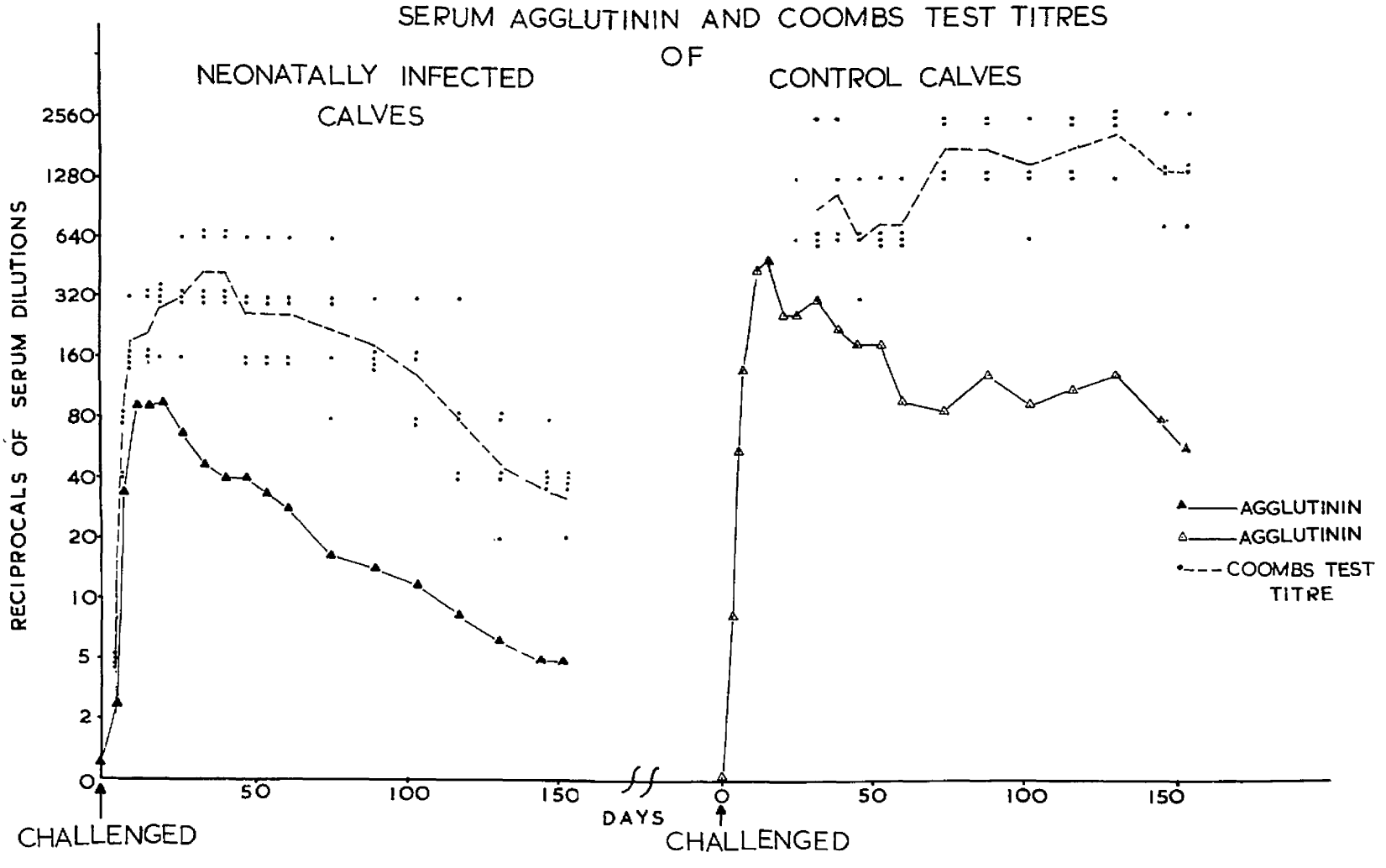

SERUM AGGLUTININ AND COOMBS TEST TITRES
OF
NEONATALLY INFECTED CALVES
CONTROL CALVES
RECIPROCALS OF SERUM DILUTIONS
2560
1280
640
320
160
80
40
20
10
5
2
0
50
100
150
0
50
100
150
DAYS
CHALLENGED
CHALLENGED
AGGLUTININ
AGGLUTININ
COOMBS TEST TITRE

FIGURE 8.

FIGURE 8.

Brucella sero-agglutinins and Coomb's test titres of 5 neonatally infected (1×10^{12} Br.abortus 544 daily by the oral route during first 15 days of life) and of 4 control calves to a challenge dose of viable Br.abortus 544 administered subcutaneously at 7 months of age.

Average agglutinin titre of neonatally infected (▲ - ▲) and of control calves (△ - △). The black dots in diagram show anti-globulin titres of the individuals and the position of curves (•--•) was calculated to represent average results of anti-globulin titres.

Anti-globulin test

Coombs' anti-globulin test also detected the presence of antibodies in the sera of both test and control calves. Figure 8 represents in diagramatic form titres obtained by the Coombs' test from the day of injection of brucella until 12 months of age.

Mean agglutinin titres of the test and control calves are shown respectively parallel to the anti-globulin titres.

The following observations may be made on the results of the anti-globulin tests:

1. Sera of the control calves collected during the first 7 months of life were negative to the anti-globulin test, but sera of the test calves gave positive reactions. Antibodies could be detected for the first time in the sera of the test calves from 2 to 5 weeks after the appearance of actively produced agglutinins. Their maximum titre exceeded that of the agglutinins by two folds of serum dilution and came to negative by the time the calves received the challenge dose of brucella.

2. In the sera of the test calves the Coombs' test detected antibodies 4 days after the injection of brucella, whereas no such antibodies were detectable in the sera of control calves until 3 to 4 weeks after infection, at a time when both agglutination and c.f. tests showed that those calves were brucella infected.

3. The highest values of incomplete antibody titres were measured in the sera of both groups of calves approximately 5 weeks after their first appearance.

4. The mean values of incomplete antibody titres of the test calves fell approximately two serum dilutions short of that of the control calves.

5. The greatest differences between the agglutinin and incomplete antibody titres of test and control calves amounted to 3 to 4 folds of serum dilution.

6. The values of the anti-globulin titres of the control calves are considerably higher than that of the test calves 5 months after brucella infection.

DISCUSSION

Although the results of the investigation presented here are incomplete, nevertheless, certain observations may be made with reasonable certainty.

It was shown in Part 2, Section A, of this thesis that the exposure of very young lambs to brucella seriously interfered with their agglutinin production when re-exposed to the same organisms in early adult life. Very young calves used in this experiment were exposed to large doses of virulent brucella. Under natural conditions it is unlikely that calves would be exposed to heavier infection via the ingestion of colostrum and milk (Stableforth et al. 1959).

The active production of agglutinins by the test calves well after the last oral exposure to brucella indicated that the organisms invaded the tissues of these calves and reached the antibody producing sites therein. The delayed serological response to the primary infection supports the findings of Pierce (1962) who has shown that although calves as young as 7 - 14 days begin synthesising globulin, their ability of responding to antigenic stimuli is still limited. The newly born calf can respond to rinderpest vaccine but not to the

injection of Brucella abortus or Salmonella dublin antigens (Pierce, 1962). The almost complete disappearance of agglutinins from the sera 10 - 12 weeks after the last oral exposure would suggest the diminution or complete lack of infection in the case of five out of the six calves, for after re-infection at 7 months of age they were all capable of producing agglutinins well in excess of that produced just prior to re-infection. Unlike the neonatally infected lambs these calves reacted to the challenge dose of brucella without a prolonged lag-phase before the appearance of agglutinins in the sera. Nor was there any sign of the pre-zone phenomena so often encountered in the agglutination test carried out on lambs' sera. Nevertheless, their capacity of producing agglutinins was distinctly suppressed in comparison to the agglutinin production of the control calves which were exposed to brucella infection for the first time.

This last observation is at some variance with that of Kerr (1956) who found no impairment of the immune response of calves injected with Strain 19 vaccine during the first weeks of life. The reasons for this may be manyfold including the age at which calves were exposed to infection, the virulence of bacteria used and the total quantity of

organisms applied for infection.

Despite the suppression of agglutinin production the test calves could be detected as brucella infected ones, after having been re-exposed to these organisms at 7 months of age, by the use of the agglutination test. The question of what would have happened had the challenge dose of brucella been much higher must remain unanswered, for the number of animals in the group did not permit the use of different challenge doses.

Perhaps the most interesting feature of the agglutinin response of the test calves was the comparatively rapid fall of agglutinin titres. Approximately three months after massive infection the titres fell to a level ($< 1:20$) insignificant even for non-vaccinated cattle. The agglutination titre of all the control calves remained highly significant at least for 5 months after infection. It was shown in Part 1 (Table 14) of this thesis that even after Strain 19 vaccination of calves in a brucella-free environment it was not before the elapse of six months after vaccination that the agglutinin titres fell to under 1:40 serum dilution. The case of the 6th calf (I?/9) in the test group has to be discussed on its own. This was the calf which had a rising titre before the injection of brucella and after the injection

her serological responses were comparable to that of the control calves. It is perhaps significant that this calf suffered from scours from the 2nd day until the 12th day of life. It is likely that a proportion of brucella ingested was excreted before they could invade the tissues of the bowel. Such an event, if applicable, may account for the different serological behaviour of this calf from that of the rest in the same group.

Considering the serological responses of the test calves as measured by the c.f. test it is notable that none of the sera tested were positive at a serum dilution of 1:5 during the first 7 months of life. The reason for this is not clear, but it may be stated that the agglutination test - at this stage - was a more sensitive method of diagnosing exposure to brucella infection than was the c.f. test.

After the injection of the challenge dose of brucella the serological responses of the test calves measured by the c.f. test were virtually identical to those of the control calves in regard to the time of appearance of these antibodies and the value of their titres in the sera. The essential difference between the two groups of calves concerned the rate of decline of c.f. antibodies. The titre

of the test calves fell under 1:5 serum dilution at about the same time as did the agglutinin titres. The sensitivity of the test was such that a titre higher than 1:5 serum dilution may be considered as indicating brucella infection in non-vaccinated cattle. McKinnon (personal communication, 1963) examining the sera of a substantial number of cattle of known status of vaccination and of infection found that non-vaccinated cattle whose sera show 1/48th or 1/24th of the complement fixing antibody activity of the International Standard Serum should be considered as suspicious and positive respectively.

Bürki (1961) reported that 1/24th of the complement fixing antibody activity of the International Standard Serum should be considered as suspicious and anything $<$ 1/24th as positive for calfhood vaccinated cattle. Alton and Jones (1963) suggested to regard any degree of reaction as suspicious and a positive reaction at a dilution of 1:10 (1/10th of the activity of the International Standard Serum) or higher as indicating brucella infection.

The c.f. titre of all the controls remained positive at a significant level 5 months after exposure to infection. It is worth noting that the complement fixing antibody content

of the sera of the control calves reached a significant level at the same time when did the sero-agglutinins. There are differing opinions as to whether the agglutinins or c.f. antibodies appear earlier following infection. Carpenter and Boak (1930); Wise and Craig (1942); Jones (1958) and Okazaki (1961) suggested that c.f. antibodies may precede agglutinins. Rice et al. (1952); Waveren (1960) on the other hand stated that agglutinins preceded c.f. antibodies. The results of the agglutination and c.f. tests carried out on the sera of the control calves following infection are in support of the latter view although the titre of agglutinins did not reach a significant level (1:20++) before the appearance of c.f. antibodies.

The significance of antibodies in the bovine sera measured by the antiglobulin test of Coombs' is not clearly established. According to the observations of Renoux et al. (1957) the Coombs' test gives excellent results in the diagnosis of brucella infection of goats. Waveren (1960) considers a titre more than two folds serum dilution in excess of that of the agglutinins in the sera of cattle indicative of field infection.

Test calves during the first 7 months of life produced incomplete antibodies detectable by Coombs' test but their titre was lower than those considered significant by Waveren. After infection at 7 months of age, sera of the test calves became positive to the anti-globulin test within a few days following infection whereas sera of the control calves did not react to this test for weeks. It would appear therefore that early appearance of antibodies reacting to the Coombs' test signifies previous exposure to brucella. The titre of both agglutinating and complement fixing antibodies reached diagnostically significant levels in the sera of control calves much sooner than did the titre of incomplete antibodies. However, these titres did not decline even 5 months after the infection of the control calves, whereas, they diminished together with the agglutinins in the sera of the test calves to low levels exceeding the agglutinin titres by 1 to 2 folds of serum dilution at the end of the 5th month following infection.

Although this investigation is yet to be completed, it is clear from the evidence so far accumulated that the degree of exposure to brucella applied to very young calves was not sufficient to suppress their immune response completely.

When they were re-exposed to the same organisms as sero-logically mature animals the fact of exposure was detectable for a moderately short time by all the three serological tests applied. But did they become truly free of infection when the titre of their humoral antibodies indicated them as such. If they did, their resistance to infection must have derived from the neonatal exposure, for all the control calves receiving the same infectious dose of brucella remained positive reactors to all the three serological tests applied for at least 5 months following experimental infection. The final answer to some of the outstanding questions must await until such times when all the calves become pregnant and the course and outcome of their pregnancies as well as the bacteriological tests which become possible at that time, may furnish the evidence to answer these questions.

SUMMARY AND CONCLUSIONS OF PART 2.

In the foregoing part of this thesis an investigation was described aiming to establish the effects of brucella infection of the ovine and bovine neonata on their serological responsiveness on re-exposure to the same organisms in adult life. The results may be summarised as follows:

Section A

1. Oral exposure of very young lambs to Brucella abortus S19 induces only a very mild agglutinin response to the ingested organisms. In contrast, parenteral as well as oral exposure of lambs to the same organisms brings about a vigorous serological response manifested as early as 15 days of age.

2. Repeated parenteral exposure of young adult lambs to Brucella abortus:

a. which had never been exposed to this organism, causes a rapid production of agglutinins after the first exposure, agglutinin titres remaining high until the second challenge which was followed by a further immediate rise of agglutinin titre.

b. These lambs which had been infected as neonata by the oral as well as the parenteral routes, showed by contrast a markedly inferior response to the first challenge, developing only after a lag phase, reaching a low peak titre which diminished at a rapid rate. After the second challenge, the serological response was even poorer.

c. Those lambs which had been infected as neonata only by the oral route varied in the extent to which they exhibited a changed ability to produce agglutinins to later challenges with Brucella abortus.

3. Animals showing tolerance to brucella exhibited an increased proportion of non-agglutinating "blocking" antibodies, which appeared co-incidentally with agglutinins but persisted long after these had disappeared. These "blocking" antibodies were responsible for most of the zoning phenomena in the agglutination test.

4. "Blocking" antibodies were also found in the sera of lambs showing no tolerance although in much smaller quantities. Zoning phenomena in these sera was often caused by an excess of antibody or unfavourable antigen-antibody proportions.

5. A heat-sensitive agglutination inhibitory factor was found in all the ovine sera examined, which was not specific to brucella but could interfere with the agglutination test of brucella.

6. Complement fixing antibodies were detected in the sera of both tolerant and non-tolerant lambs in roughly similar amounts.

7. Tolerant lambs exhibited a marked immediate type of hypersensitivity on the intradermal injection of a soluble extract of brucella, at a time when no humoral antibodies could be detected. Non-tolerant lambs with circulating antibodies showed only moderate hypersensitivity to the injection of the same soluble antigen.

Since it has been established that brucella agglutinin response in lambs may be seriously impaired as a result of experience of brucella antigen early in life, it is suggested that the complement fixation test may have important practical value in the detection of brucella infection. Furthermore, it is felt that the results of Part 2, Section A necessitates a similar investigation using cattle as the subject of the experiment.

Section B

Although the investigation in Part 2, Section B is yet to be completed, nevertheless, certain observations may be made with reasonable certainty.

1. Oral exposure of very young calves to large doses of virulent Brucella abortus, over a period of 15 days, stimulates the formation of some agglutinins which become detectable for the first time at approximately 30-40 days of age, and them remains detectable for at least three months.

2. Re-exposure of such calves to the same organisms at 7 months of age stimulate serological response without a prolonged lag-phase, but the average sero-agglutinin titres are lower and persist for a much shorter time than the titre of control calves of the same age, experiencing brucella infection for the first time.

3. Complement fixing antibodies were not detectable in the sera of the neonatally infected calves during the first 7 months of life, but appear after re-infection, at the same time, and in comparable quantities, to the complement fixing antibody content of the sera of control

calves. The c.f. antibodies persisted in the sera of the neonatally infected calves for a much shorter time than in the sera of the controls.

4. The anti-globulin test of Coombs' detected antibodies in the sera of the neonatally infected calves prior to, and after re-exposure to brucella. The maximum titre exceeded that of the agglutinins by 3 - 4 folds of serum dilution following re-exposure. In the sera of the control calves the Coombs' test detected antibodies for the first time 3 - 4 weeks after injection, reaching numerically higher values but comparable in proportion to that of the neonatally infected calves.

From the evidence so far accumulated it is clear that the degree of exposure to brucella applied to very young calves wasn't sufficient to inhibit their immune response completely. On re-exposure to the same organisms as serologically mature animals, the fact of exposure is detectable for a moderately short time. Whether or not they are free of infection when the titre of the humoral antibody indicates them as such, remains to be established.

PART 3

ANTIGENIC ANALYSIS OF STRAINS OF *BRUCELLA ABORTUS*

INTRODUCTION

The anomalies in the serological diagnosis of bovine brucellosis - introduced by over-age vaccination of cattle with Brucella abortus Strain 19 - are well known to those concerned with this problem. As a result of vaccination over the age of 9 months in the sera of an appreciable proportion of cattle, vaccinal agglutinins are maintained - often for years - at such a high titre which is indistinguishable from titres due to natural infection.

It was shown in Part 1 of this thesis (Table 18) that approximately 54% of adult vaccinated cows, free of natural infection, exhibited serum agglutinin titres at one time or another in their lives (after these titres fell to their lowest level following vaccination) which were in excess of that accepted as indicating natural infection by the W.H.O. Expert Committee on Brucellosis (W.H.O. techn. Rep. Ser. (1958) 148). At the same time ≥ 13% of cattle with proven brucella infection had, on occasions while infected, titres lower than that accepted as indicating natural infection.

Numerous attempts have been made to devise serological methods which would differentiate between residual

titres due to vaccination and superimposed natural infection of vaccinated cattle. These methods included the agglutination test carried out on whole milk or its derivatives or on vaginal mucus. Unfortunately, none of these methods were found to be of real value in the eradication work (Part 1) when they were applied to the samples of the so-called borderline cases in diagnosis. By the time agglutinins in the milk or vaginal mucus became detectable, the serum agglutinin titre by itself was sufficient to show the fact of infection.

The value of the complement fixation test (c.f. test) in the diagnosis of brucella infection of cattle has been variously assessed. In the pre- S19 vaccination era Hadley and Beach (1912) did not find the agglutination test reliable enough but reported good correlation between the results of the c.f. test and the clinical history of the animal. Boerner and Stubbs (1924) and Zeissig and Mansfield (1930) found that the c.f. test differentiated cattle as brucella reactor and non-reactor more clearly than did the agglutination test. Rice et al. (1952) found that the complement fixing antibodies disappeared from the sera of vaccinated cattle sooner than did the agglutinins. Other workers (Yneskovets, 1956; Wisniowski, 1957; and Kocowicz et al. 1960) have also suggested that the combined use of c.f. and agglutination

tests on sera from vaccinated cattle would help to differentiate titres due to vaccination from that due to natural infection. In Holland the c.f. test has been widely used on all bovine sera reacting to the agglutination test. (Schaaf et al. 1959; Waveren, 1960). Although it is fairly well established that the c.f. titre declines more rapidly in vaccinated non-infected cattle than does the agglutinin titre, the time required for the disappearance of c.f. antibodies from the sera is a limiting factor. Wisniocki (1957) and Kocowicz et al (1960) reported that up to 10 months may elapse before the sera of adult vaccinated cattle becomes free of c.f. antibodies thus limiting the applicability of the test during this time.

Another method for the differentiation of vaccinal from infection titres of Brucella abortus was first reported by Dick, Venzke and York (1947). They found that animals with a vaccinal titre responded to an injection of Brucella abortus S19 by a rise in the sero-agglutinin titre within a maximum period of 17 days whereas known infected animals did not. This observation was later confirmed by Venzke (1948) and Barner et al. (1953).

Elder and Rodabough (1951) and Elder et al. (1956) however, found that this technique was inaccurate with some animals or gave inconclusive results.

The acidified plate antigen first described by Rose and Roepke (1957) was modified by Rodabough and Elder (1961) and applied to differentiate between vaccinal titres and those due to natural infection. They considered the results obtained by this technique as promising but emphasised the need for further evaluation before final conclusions may be drawn about its value.

None of the diagnostic methods enumerated were based on a systematic search for possible antigenic differences which may exist between the vaccinal strain of brucella and those causing natural infection. An antigenic component totally absent from S19 but shared by all the virulent field strains of Brucella abortus would be of real value. By testing the serum for the homologous antibody against this hypothetical antigen its presence would indicate natural infection, alternatively, its absence would show freedom of such infection.

Prior to the introduction of the agar gel diffusion technique (Ouchterlony, 1948) to the antigenic analysis of

the genus Brucella various relevant pieces of information had accumulated. It is beyond the scope of this introduction to enumerate them all thus only a few shall be mentioned.

Evans (1918) showed by means of cross-absorbtion tests that Brucella abortus and melitensis had more then one agglutinogenic substance and that these substances were present in the cells of these two bacteria in different proportions. Feusieur and Meyer (1920) described four distinct serological types of brucella. In 1932 Wilson and Miles evolved their well known diagram showing the presence in all smoth strains of brucella two antigens: A and M varying in proportion according to whether Brucella abortus, melitensis or suis is involved. Their postulation was borne out in that it made it possible to prepare by cross absorbtion monospecific Brucella abortus and melitensis antisera. Hersey et al. (1935) observed that from an albuminoid fraction of brucella a cleavage product may be obtained - what they called S substance - which was precipitinogenic and was shared by all the three species of brucella. Higginbotham and Heathman (1936) extracted precipitating substances from the three species, some of these appeared to be serologically distinct.

Paterson, Pirie and Stableforth (1947) reported that Brucella abortus and melitensis contained antigens of broadly similar nature which can be separated from the bacterial cell in relatively pure state.

Renoux and Mahaffei (1955) proposed a detailed antigen scheme of the genus Brucella according to which the smooth strains of the three species contain A, M, Z and R in different distributions. Rough strains contain only R with or without Z.

The varied results obtained in these earlier investigations led Olitski (1959) to re-examine this subject by the method perfected by Ouchterlony (1948). Preliminary experiments of Olitzki and Sulitzeanu (1957) showed that an extract obtained by ultrasonic effect from Brucella suis, when applied against its homologous antiserum, exhibited at least 6 precipitin lines in agar gel. When serum, prepared with the aid of Freund's adjuvant, was used three additional precipitin lines could be detected. By the application of adsorbtion technique they found that some of the precipitin were formed against surface components, others against antigens more deeply situated in the bacterial cell. Bruce and Jones (1958) prepared a trichloroacetic acid extract

of Brucella melitensis which gave rise to from one to three precipitin lines when used in agar gel against rabbit, goat and cattle sera which had been infected either with Brucella abortus or melitensis. No diffusable antigen could be obtained from Brucella abortus or suis by the same method which was effective in the case of Brucella melitensis. Further studies of Olitzki and Sulizeanu (1958) characterised the different soluble antigenic components of Brucella suis by chemical and physical means and determined optimal conditions under which the maximum number of precipitin lines in agar gel may be formed.

Carrare et al. (1958) also studied the antigenic structure of the three classical species of brucella. They concluded that antigens obtained by grinding or ultrasonic action are identical, furthermore, that these endo-antigens are common to all three species of brucella. However, a glucidic-lipidic-polypeptidic fraction - which can be extracted by trichloroacetic acid - is only shared by Brucella abortus and melitensis.

Olitzki (1959) examined the soluble antigenic components of the three species of brucella. Quantitative titration of the antigens of each of the three species was

carried out in an attempt to determine whether any difference existed in the antigenic structure of Brucella abortus, melitensis and suis. He concluded that each of the three species possessed at least six soluble antigens. These antigens differed in their relative concentration in the bacterial extracts of different origin and in their ability to stimulate antibody formation. No antigen specific for a single species was demonstrated.

Most of the studies pertaining to this subject used in their antigenic analysis of brucella, antisera prepared in rabbits. The antigenicity of a substance, however, may not be the same even in two different individuals of the same species let alone in two species as far apart as rodents and bovine are.

The objective of this study was to extract the water soluble antigens of various biotypes of Brucella abortus in order to subject them to comparative analysis (qualitative and quantitative). The aim of the analysis was the detection of any antigenic difference which may exist between the various biotypes (found in Great Britain) which may constitute a basis for a diagnostic test capable of differentiating vaccinal titres from those caused by superimposed natural infection of cattle.

EXPERIMENT No.1

COMPARISON OF SOLUBLE ANTIGENS OF BRUCELLA ABORTUS S19 PREPARED BY VARIOUS METHODS

Among the various techniques employed for the preparation of soluble antigens of brucella the most efficient appeared to be those of Olitzki and Sulitzeanu (1958). These techniques included - among others - the application of cold acetone to bacterial suspensions which were subsequently dried in-vacuo over Ca Cl_2 and secondly, the exposure of this acetone dried preparation to sonic action in a 9 Ko Raytheon magnetostriction oscillator. The preparations obtained by these techniques contained at least 6 soluble antigens of brucella. In order to see whether slight modifications of these two methods would alter the soluble antigen yield of brucella the following experiment was performed.

MATERIALS AND METHODS

Antigen for serum preparation

The antigen was prepared from Brucella abortus S19 vaccine (Ministry of Agriculture Fisheries and Food, Central Veterinary Laboratory, Weybridge, Surrey). 40 ml. of the

vaccine was centrifuged in a MSE refrigerated centrifuge at 12000 gs. for 60 minutes. The sedimented cells were washed in physiological saline and suspended in 20 times their own volume of cold acetone (-20°C) and left to stand overnight. The following morning the suspension was centrifuged (approximately 2000 gs. for 10 minutes) and the resulting sediment re-exposed to the same volume of cold acetone for an additional 3 - 5 hours. After centrifugation the supernatant was poured off and the bacterial cells dried in a dessicator in-vacuo over $CaCl_2$.

Preparation of immune serum

Two rabbits were immunised by intra-muscular injections. The acetone-dried bacteria was suspended in 40 ml. of physiological saline and injected in 2 ml. quantities in the course of 10 weekly injections, thus each rabbit received a total of 20 ml. of suspension. Between injections the antigen was kept at -20°C. Seven days after the last injection the rabbits were bled and to the pooled sera merthiolate was added in a final concentration of 1/10000 and kept frozen at -20°C.

Antigens for agar gel precipitation

Brucella abortus S19 was grown on serum dextrose agar (Brinley Morgan, 1960) in Roux flasks. The inoculum

was examined for S - R phase variation by the oblique light technique of Henry (1933). Colonies in the S phase of growth were used as inoculum. After seven days incubation at 37°C the growth in the Roux flasks was washed off with 0.1% formol saline and after a few hours of standing at room temperature the suspension was filtered through a thick pad of non-adsorbent cotton wool to get rid of any particulate agar which may have been present. The suspension was centrifuged at +10°C for 60 minutes at 12000 gs. The sedimented cells were washed in physiological saline and after centrifugation the packed cells were distributed in 2 ml. quantities and treated by one of the following methods:

1. Acetone-dried as described above yielding 305 mg. of dry antigen which was suspended in 3 ml. of merthiolate-saline (1:5000; pH 9.6). The crude suspension was broken up in Griffith's tube and the homogenate was either used as such or incubated in water bath at 50°C for one hour.

2. Homogenate prepared as in 1., from 600 mg. of acetone-dried cells and divided into 1 ml. quantities. Each 1 ml. lot in turn was exposed to ultrasonic action in an M.S.E. Mullard 60 KV. (M.S.E., London) ultrasonic disintegrator for the following length of time:

5 minutes

10 minutes

15 minutes

22 minutes

30 minutes

During the time of ultrasonic exposure the vessel containing the cell suspension was immersed in iced water.

3. Two ml. of the packed cells were suspended in 3 ml. of merthiolate-saline (1:5000; pH 9.6) and divided into five lots of 1 ml. quantities then exposed to ultrasonic action as in 2. As a result of this procedure the Brucella abortus S19 content of each antigenic preparation was the same per unit-volume of the preparation.

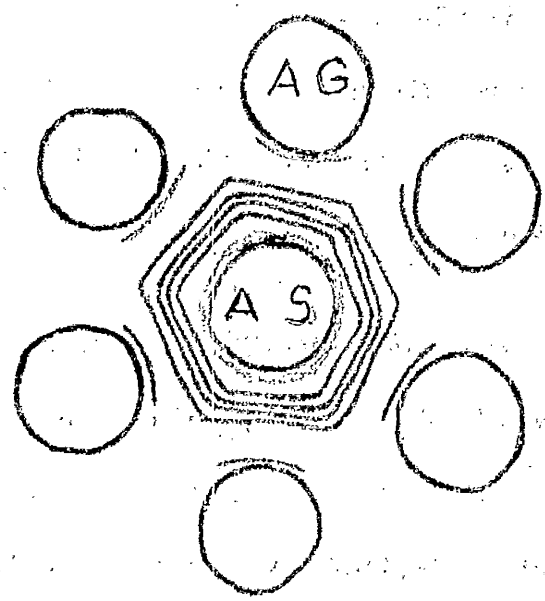
Agar gel for precipitation tests

As it was shown by Olitzki (1959) that at lower than physiological salt concentrations sharper precipitin lines could be obtained, the following quantities of salts were added to 0.9 litres of 1% oxoid ion agar No.2 : 0.353 g. $Na\ H_2\ PO_4$, 0.639 g. $Na_2\ H\ PO_4$ and 0.172 g. Na Cl.

After 10 minutes of autoclaving at 121°C, 100 ml. of merthiolate solution was added to the molten agar to give

a final concentration of 1:10000. Filtration of agar through a thick pad of non-adsorbent cotton wool followed and the water clear agar was distributed into 100 ml. medical flasks. The final pH of the agar gel was 8.3 - 8.4.

The experiments were carried out in Petri dishes containing 20 ml. of agar. The reservoirs for the antigens and sera were made with the aid of No.4. cork borer giving large enough wells to take 0.1 ml. of the reagents. Around a central well six peripheric wells were cut over a template which was placed under the Petri dish. The distance between the edges of the central and peripheric wells was 5 mm. The plates were incubated at 37°C for 7 days in plastic bags closed with rubber bands. Cotton wool moistened with water served to prevent drying.



Graphic picture of precipitation-in-agar-gel of Br.abortus S19 homologous system.

Central reservoir contained 0.1 ml. of Br.abortus S19 antiserum (AS).

Each peripheric reservoir was charged with 10 mg. of acetone-dried Br.abortus S19 antigen dissolved in merthiolate-saline (AG).

RESULTS

In this series of tests (Experiment No.1) 0.1 ml. of undiluted immune serum was placed in the central well and 0.1 ml. of the undiluted antigens in the peripheric reservoirs.

When the acetone-dried antigenic preparation was employed in this arrangement against its homologous antiserum five distinct and one broad diffuse precipitin lines developed. The graphic picture of these lines is shown opposite. Heating of this preparation at 50°C for one hour improved the intensity of the precipitin line nearest the source of antigens to a considerable degree.

When the acetone-dried ultrasonic treated preparations were employed in the peripheric wells the pattern of precipitin lines between the antiserum containing well and those charged with antigen exposed to ultrasonic action from 5 - 15 minutes was identical to that shown in the graphic picture. Ultrasonic treatment beyond 15 minutes resulted in the formation of less clearly defined lines or the annihilation of at least one of the precipitin lines.

Antigens prepared by ultrasonic effect alone gave identical results to those obtained from acetone-dried cells provided ultrasonic exposure lasted from 10 - 15 minutes. Five minutes of exposure did not release the antigen responsible for the formation of precipitin line nearest to the source of antigens. Ultrasonic exposure beyond 20 minutes gave similar results to those obtained with acetone-dried sonic treated cells when exposure time exceeded 15 minutes.

EXPERIMENT NO. 2.

COMPARISON OF VARIATIONS IN THE AGAR GEL DIFFUSION TEST: THEIR EFFECT ON THE BRUCELLA PRECIPITATING SYSTEM

In order to establish optimal conditions for the agar gel precipitation reaction as applied to brucella and to assure constant reproduction of results a series of tests was performed.

MATERIALS AND METHODS

Antigens and immune serum used were those employed in Experiment No.1.

Agar gel for precipitation test: variations in the composition of agar gel included the use of:

Oxoid agar No.3 Oxoid ion agar No.2	concentrations varying between from 0.5 to 2%
Concentration of NaCl	between from 0.- to 1.5%
pH of agar gel	between from pH 6.5 to pH 9

In addition:

Agar gel plates were incubated at 20^{0} and 37^{0}C respectively for up to 10 days.

Various distances between wells containing antigen and antiserum were employed.

Different arrangement of wells in relation to each other were tried.

RESULTS

The application of oxoid agar No.3. and ion agar No.2. at identical concentration in the agar gel showed that the latter one gave a firmer and more transparent gel. Concentrations of ion agar No.2. over 1.5% slowed down the formation of precipitin lines and at 2% concentration the formation of one of the precipitin lines (nearest the source of antigen) was almost completely prevented. Agar concentration $< 1\%$ in the gel made it increasing fragile to handle.

The effect of NaCl concentration in the agar gel may be summarised as follows:

between 0.1 - 0.3% concentration of NaCl no difference in the formation of precipitin lines was observed. If no salt (NaCl) was added to the gel at all the appearance of the precipitin lines was delayed. At concentrations in excess of 0.3% the precipitin lines became increasingly

diffuse and at 1.5% concentration of NaCl the adjacent lines became confluent, others failed to be formed. The effect of pH changes in the agar gel between pH 7 and pH 9 was inconsequential, but decreasing pH values of the agar in the acid region resulted in gradual prevention of precipitate formation.

Incubation temperatures of 20° and 37°C respectively did not influence the quality of precipitate formed. However, an indirect correlation was observed between incubation time and temperature. Six days incubation of the plates at 37°C was always sufficient for the complete development of all the precipitin lines, whereas 7 to 9 days were required for the same end at an incubation temperature of 20°C.

Variation of distances between wells containing antigen and immune serum respectively had a profound effect on the overall picture of precipitin lines formed. In these tests 0.1 ml. of immune serum was placed in the central well and 0.1 ml. of antigen in the peripheric wells. Each of the three antigenic preparations used in Experiment No.1 were applied in turn in this manner. The distance between the central and peripheric reservoirs was gradually increased beginning at 3, 4, 5 etc. mm. up to 15 mm.

The results of this treatment may be summarised as follows: at distances <5 mm. between antigen and immune serum containing wells a broad and diffuse precipitin band developed around the reservoir containing the immune serum and the other lines formed were so closely packed that they were practically indistinguishable. The highest number of lines (6 - 7) were obtained at a distance of 6 mm. If the distance was increased beyond 6 mm. some of the precipitin lines, nearest to the source of antigen become fainter or disappeared altogether. At the same time new lines appeared as a result of cleavage of some of the existing single lines. The broad and diffuse precipitin band around the central well also developed into well defined individual lines.

The use of three different antigenic preparations in identical manner showed that they were interchangeable. Thus the results of this series of tests clearly indicated that no single distance between the antigen and antiserum containing wells was adequate for the formation of all the potential precipitin lines.

To examine the effect of the number of peripheric reservoirs around a single centrally situated well on the development of precipitin lines, agar gel plates were

prepared in the following way. Around the central well peripheric holes were cut, increasing in numbers gradually from 4 to 8, ensuring equal distances between adjacent wells. The central well was charged with immune serum and every alternate peripheric well received the antigen, while those in between were filled with saline. The results showed that the application of more than 4 peripheric wells in this manner would interfere with quantitative titrations, for sufficient antigen diffused from the antigen-charged wells to form precipitin lines in front of such wells which contained no antigen at all.

Thus the choice of distribution pattern of reservoirs and of the distances separating them were found to be critical. The cutting of identical gels with a suitable degree of accuracy proved to be time consuming and tedious using cork borer and template of the required pattern.

The use of permanently assembled cutters would not have provided the flexibility of arrangement of reservoirs required. To satisfy this need of flexibility, an adjustable cutter was designed. The reservoir cutters were cork borer tubes made from chromium plated nickel, and the

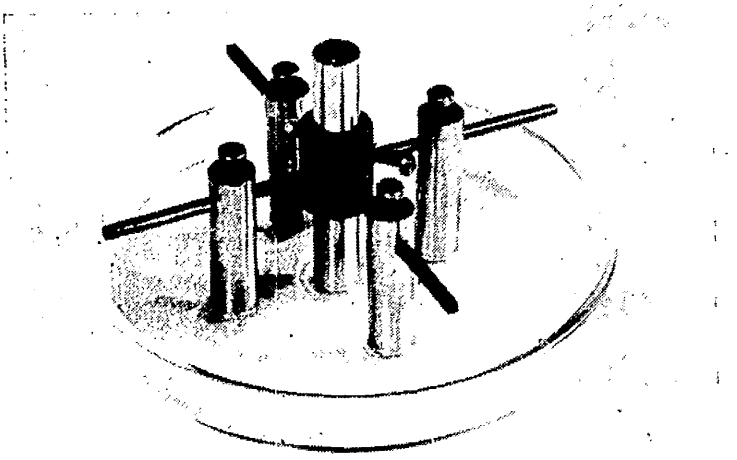


FIGURE 9 - Photograph of an adjustable cutter as used with agar gel contained in a standard Petri dish of 3½ inches diameter.

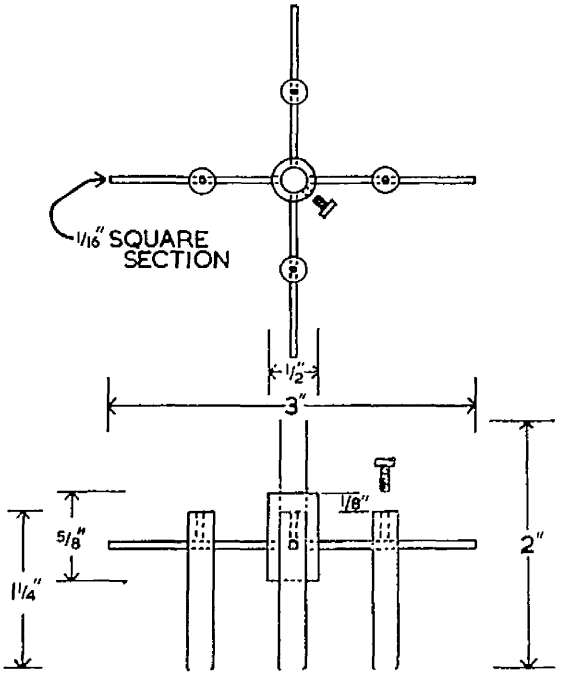
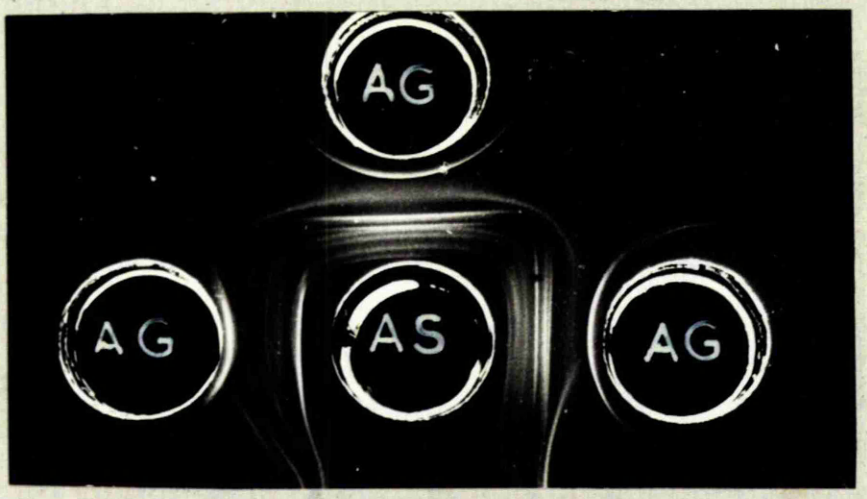


FIGURE 10 - Diagram of adjustable agar gel cutter showing dimensions of model designed for use with gels contained in standard Petri dishes of 3½ inches diameter.

rest of the instrument was made from brass. The simple design (illustrated Figures 9 and 10) can be modified by changing the number of side arms or the size of cork borers to satisfy special requirements. By punching one pattern rotating or transporting the cutter and punching a second time, a wide variety of wall arrangements is possible. It was found in practice that there are few arrangements which cannot be duplicated easily and accurately with this instrument.

FIGURE 11



AS = 0.1 ml. of Br.abortus S19 antiserum

AG = 10, 7 and 5 mgr. of acetone dried Br.abortus S19 antigen suspended in 0.1 ml. saline, respectively.

FIGURE 12

AG = 10 mg. of acetone-dried Br.abortus S19 antigen suspended in 0.1 ml. saline.

AS = Dilutions of Br.abortus S19 antiserum in 0.1 ml. quantities.

In [redacted] the experiments performed so far the immune serum was placed into the central reservoir (Figure 11). The result of placing the immune serum around a centrally situated source of antigen is shown in Figure 12.

It will be appreciated from Figures 11 and 12 that the central location of the immune serum in the agar gel diffusion plates resulted in more and better defined precipitin lines. Dilution of the peripherically situated antiserum did not improve the definition of any of the precipitin lines (Figure 12).

EXPERIMENT NO. 3.

COMPARISON OF THE EFFICIENCY OF LIVE BRUCELLA ABORTUS S19 AND ITS ACETONE-DRIED DERIVATIVE AS IMMUNISING AGENTS IN RABBITS

It was found in Experiment No.1. that the preparation of a potent immune serum against acetone-dried Br.abortus S19 was a lengthy procedure requiring 10 weeks of immunisation. To examine the efficiency of live S19 organisms as immunising agent as well as to obtain information whether identical quantities of live organisms to that of acetone killed ones may be tolerated by rabbits Experiment No.3. was performed.

MATERIALS AND METHODS

Antigens for serum preparation

Brucella abortus S19 was grown on Serum-Dextrose agar. The growth of 5 days old cultures was washed off with Ringers solution and the total number of organisms per ml. of suspension was established by means of the Burroughs Wellcome opacity tubes. (Burroughs Wellcome & Co. London). Batches of suspension containing 15×10^{10}

organisms were acetone-dried as described in Experiment No.1. Each batch of acetone-dried cells was dissolved in 10 ml. sterile saline to serve as immunising agent for each rabbit in Group No.1.

Rabbits in group No.2. were immunised with the same total number of Brucella Abortus S19 as used in Group No.1. but these organisms were harvested from slopes just prior to use.

Preparation of immune sera

Two groups of adult rabbits, each containing 4 animals, were used. Each rabbit in Group No.1. received the equivalent of 15×10^{10} acetone-dried Brucella abortus S19 in saline suspension in the course of 10 equal size intramuscular injections administered at weekly intervals. Rabbits in group No.2. received viable suspension of S19 in identical dosage and manner to those in group 1. Rabbits were bled just prior to each injection during the first 9 weeks of immunisation. After the 10th injection the rabbits were bled every 2nd day, beginning on the 4th and ending on the 14th day after injection.

Agar gel diffusion test

Agar gel for the immuno-diffusion tests was prepared as described in Experiment No.1. The punching of reservoirs in the agar was carried out with the aid of the adjustable cutter using 6 mm. distances between wells containing antigen and antiserum respectively. The potency of the sera obtained was tested against 10 mg. of acetone-dried cells contained in 0.1 ml. merthiolate-saline. After 6 days or incubation at 37°C the results were read.

RESULTS

The apparent good health of rabbits during and after immunisation showed that the use of 15×10^{10} Brucella abortus S19 injected intramuscularly was not a sufficiently heavy dosage to cause clinical illness.

The results of agar gel diffusion tests show that live suspension of S19 was a more efficient immunising agent than its acetone-dried derivative. Highly potent immune sera were obtained from rabbits in group 2. six weeks after the first injection of brucella. Sera of similar potency was not obtained from rabbits in group No.1. before the 9th or 10th week after the commencement

of immunisation. The test of sera, collected at short intervals after the last injection, show that the most potent precipitating sera are obtained from blood taken from between 6 to 12 days after injection.

DISCUSSION

Brucella abortus 519 was treated in various ways in an attempt to compare the antigenicity of the various preparations to those of Olitzki and Sulitzenna (1938). The exposure of acetone-dried cells to ultrasonic action did not detectably improve the antigen yield compared to that of acetone-dried cells dissolved in merthiolate-saline and heated at 50°C for one hour. Indeed, prolonged exposure to ultrasonic waves resulted in gradual destruction of the antigenicity of some of the components. No loss of antigenic components of Brucella abortus 519 was noted as a result of cold acetone treatment of bacteria, for the preparation obtained by ultrasonic disruption alone yielded no extra antigen.

Of the three methods employed for the preparation of soluble antigens of brucella it was the acetone drying which was the easiest to perform, furthermore, it was the simplest to quantitate and the antigen could be kept in dry powder form, without any signs of deterioration, until shortly before use.

Thus in all the agar gel diffusion tests to be presented the acetone dried cells of brucella dissolved in merthiolate-saline (1:5000 pH 9.6) and heated at 50°C

for 1 hour, were used as the antigen unless otherwise stated.

Tests aiming to establish optimal conditions for the precipitation reaction of brucella in agar gel showed that oxoid ion agar No.2. at a concentration of 1% in the gel at pH 8.4 was the most suitable. The concentration of NaCl in the gel was of importance. The best results were obtained with a concentration of NaCl between 0.1 and 0.3%, and thus an 0.2% concentration was adopted in later work. The incubation of plates at 37°C for 6 days proved to be adequate for the development of all the precipitin lines.

Experiments on the distribution of patterns of reservoirs and the distances separating them showed that the best results may be obtained by placing the immune serum into the centre of no more than 4 peripheric wells which should be 6 mm. away from the central reservoir. The adjustable agar gel cutter devised proved to be a suitable means of cutting duplicate gels with a high degree of accuracy.

Immunisation experiments on rabbits using live Brucella abortus S19 and its acetone-dried derivative in

identical quantities indicated that the live S19 preparation was a more efficient immunising agent. As a result of 6 intramuscular injections (each containing 15 x 10^9 cells) of viable suspensions of brucella, administered at one week intervals, highly potent immune sera were obtained from blood collected from the 6th - the 12th day after the last injection.

EXPERIMENT NO. 4.

QUANTITATIVE TITRATION OF THE SOLUBLE ANTIGENIC COMPONENTS OF *BRUCELLA ABORTUS* BIOTYPES FOUND IN GREAT BRITAIN

Before a comparative study of the precipitin content of immune sera of various origin could be undertaken, it was felt desirable to become thoroughly familiar with the quantitative aspects of the soluble antigenic components of the various strains of brucella to be examined. Of the 9 biotypes of *Brucella abortus* recognised by the Sub-Committee on Toxonomy of Brucella (Eighth International Congress for Microbiology, Montreal, August 1962), 6 have been found to occur in Great Britain. The results of typing of 1000 isolates (sent in from Great Britain to the Central Veterinary Laboratory, Weybridge in 1961-62, private communication) showed the following frequency of occurrence of the 6 biotypes:

Brucella abortus	type 1 (544)		76.6%
do	do	type 2 (Wilson)	3.8%
do	do	type 3	0.1%
do	do	type 4 (*abortus* biochemically *melitensis* serologically)	2.0%
do	do	type 5 (so called British *melitensis*)	15.9%
do	do	type 9	1.6%

four of these biotypes - accounting for 98.3% of the virulent strains found in Great Britain - as well as S19 were available for this study.

It was noted in the course of the preliminary experiments (Experiments No.1 to No.3) that the number of precipitin lines formed in agar gel was dependent on the distance separating the source of antigen from that of the antibody. This was interpreted as an indication that no single distance was satisfactory for the development of optimal proportions for every antigen-antibody system present in the soluble antigenic extract of Brucella abortus S19 and its homologous antiserum. It was hoped that by systematic dilution of one of the reagents (antigen) while leaving undiluted the other (antiserum) optimal proportions may be brought about for each precipitin system thus revealing all those present. It was also hoped that by the application of decreasing quantities of the soluble antigen preparation of strains of brucella against an undiluted highly potent immune serum the relative quantities of the different antigenic components of the various biotypes of brucella may be established. To make the results of titration of antigens of various origin comparable it was essential to use only a single kind of hyperimmune serum.

Unfortunately, at this stage of the work no such serum of bovine origin was obtainable, thus rabbit serum was used.

MATERIALS AND METHODS

Bacteria

Brucella abortus		Type 1
do	do	Type 2
do	do	Type 4
do	do	Type 5
do	do	S 19

were obtained by the courtesy of Dr. A.W. Stableforth (Central Veterinary Laboratories, Weybridge). They were maintained on serum dextrose agar (Brinley Morgan, 1960) throughout this investigation.

Antigens for agar gel precipitation

The 5 biotypes of brucella were grown on serum dextrose agar for 7 days, the cells harvested were washed in physiological saline then acetone-dried as described in Experiment No.1. One hundred mg. of acetone-dried cells were suspended per ml. of merthiolate-saline (conc. of merthiolate 1:5000, pH: 9.6 - 9.9) and heated at 50°C for 1 hour.

From this stock preparation of antigen various dilutions were made using merthiolate-saline as diluent to obtain graded quantities of acetone-dried cells per 0.1 ml. of the preparation used per reservoir.

Immune serum

was prepared as for Experiment No.3. against live suspension of Brucella abortus S19. Those sera which proved to be the most potent in the agar gel diffusion tests against the homologous antigen were pooled and merthiolate added in 1:10000 concentration. The pooled sera was divided into 1 ml. quantities and kept frozen at - 20°C until used.

Agar gel diffusion test

The agar gel was prepared the same way as described in Experiment No.1. but using an 0.2% concentration of NaCl. The tests were performed in Petri dishes containing 20 ml. of agar gel. The cutting of duplicate plates was carried out with the aid of the adjustable cutter using a 6 mm. distance between reservoirs containing antigen and antiserum respectively.

The two centrally situated wells contained 0.1 ml. of undiluted immune serum, while the peripheric wells contained graded quantities of antigen of various origin.

Plates were incubated for 6 days at 37°C in polythene bags. Cotton wool moistened with water served to prevent drying. After 6 days of incubation the agar gel was washed in saline and photographed.

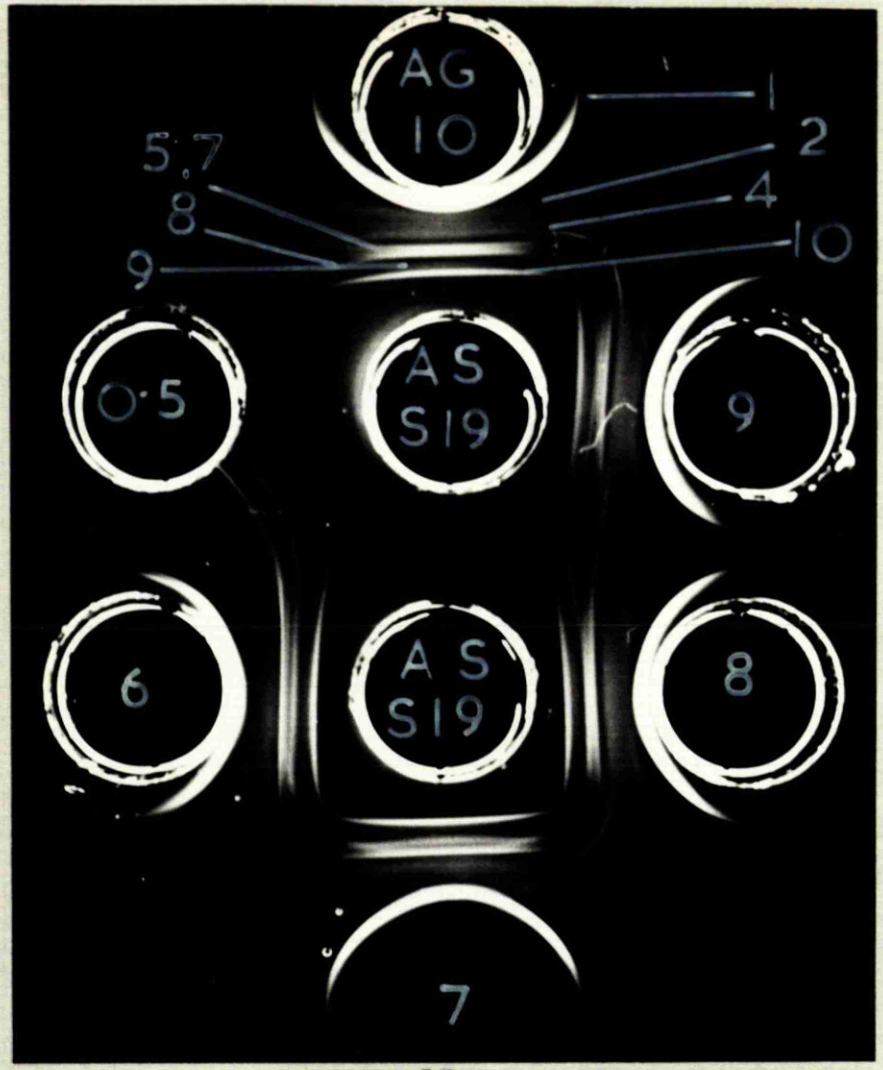


FIGURE 13.

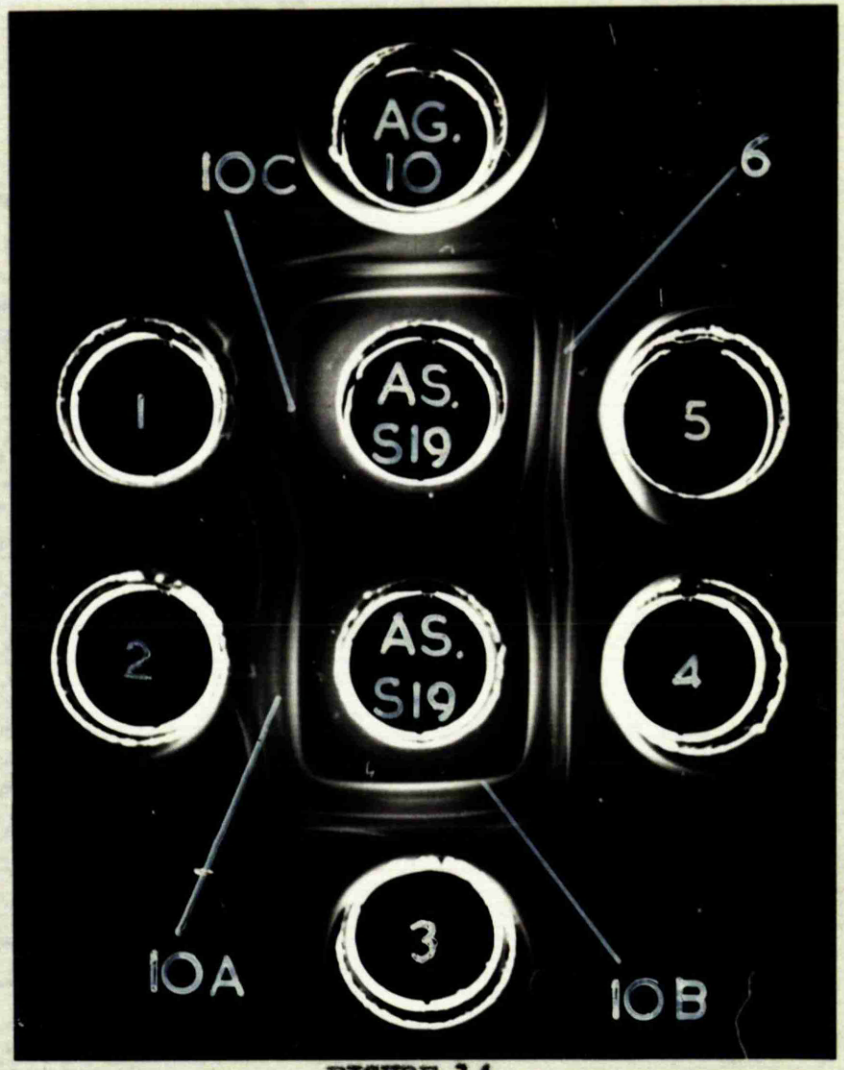


FIGURE 14.

FIGURE 15.

FIGURES 13 - 15

Quantitative titration of the soluble antigenic fractions of Brucella abortus S19.

The central wells contained 0.1 ml. of undiluted Brucella abortus S19 hyperimmune serum. The peripheric reservoirs were charged with the homologous antigen prepared from acetone-dried cells. The Arabic figures superimposed on the peripheric wells show mg. of acetone-dried cells applied in that particular reservoir in 0.1 ml. merthiolate-saline. Arrows and figures denote particular precipitate lines.

RESULTS

The results of titration of the soluble antigenic components of Brucella abortus S19 are shown in Figures 13 - 15. The highest quantity of antigen applied per reservoir in these plates in terms of acetone-dried cells was 10 mg. Increasing the quantity of antigen up to 30 mg. per reservoir yielded only one extra precipitin line at 15 mg. (line No.3.) but any increase over 10 mg. of antigen per well in fact spoiled the overall precipitate pattern. For easier identification of the precipitin lines they were given Arabic numbers beginning with the one nearest to the source of antigen and designated No.1, No.2, etc., up to No.11. The Arabic figures superimposed on the peripheric wells show the mg. of acetone-dried cells applied in that particular reservoir. It will be seen in Figure 13 that at 10 mg. of antigen all but 3 of the 11 precipitin systems may be observed. Of those 3 missing, line No.3. needs a minimum of 15 mg. of acetone-dried cells, No.6. can only be seen at 5 and 4 mg. of antigen respectively (Figure 14) whereas No.11 can only be observed at from 0.25 to 0.06 mg. of antigen (Figure 15). At is was impossible to identify some of the precipitin lines, using decreasing quantities of antigen, some means of identification had to be devised.

This requirement was met by the use of the top half of one of the three agar gel plates (Figure 14) in a standard manner. Thus the 3 uppermost peripheric wells were charged with 10, 5 and 1 mg. of antigen respectively, for it was found that at these quantities of antigen all but 3 (Nos. 3, 8 and 11) of the precipitin lines would be formed.

In the order of disappearance of precipitin lines, resulting from the application of decreasing quantities of antigens, the next two to diminish after No.3 are lines No.2 and No.8. which are last seen at 6 mg. of antigen (Figure 13). Line No.4 and 6 are last seen at 5 mg. (Figure 14) whereas Nos. 5, 7 and 9 may be last observed at 3 mg. of antigen (Figure 14).

Line No.10 is a complex of 3 precipitin lines, No. 10A can only be seen at 3 and 2 mgs. (Figure 14), whereas 10B and 10C appear as two separate lines at from 1 to 0.5 mg. of antigen (Figures 14 - 15).

Line No.11 require the smallest quantity of acetone-dried cells for it is only seen at from 0.25 to 0.06 mgs. (Figure 15) of antigen.

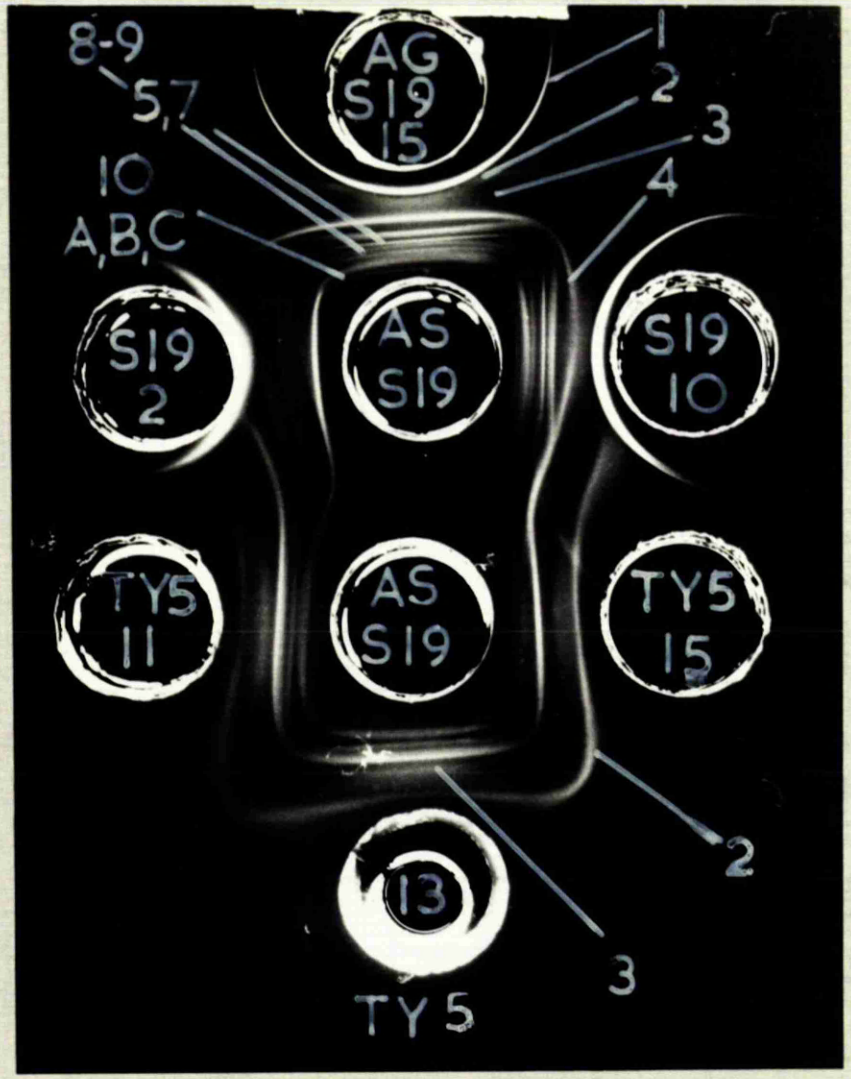


FIGURE 16.

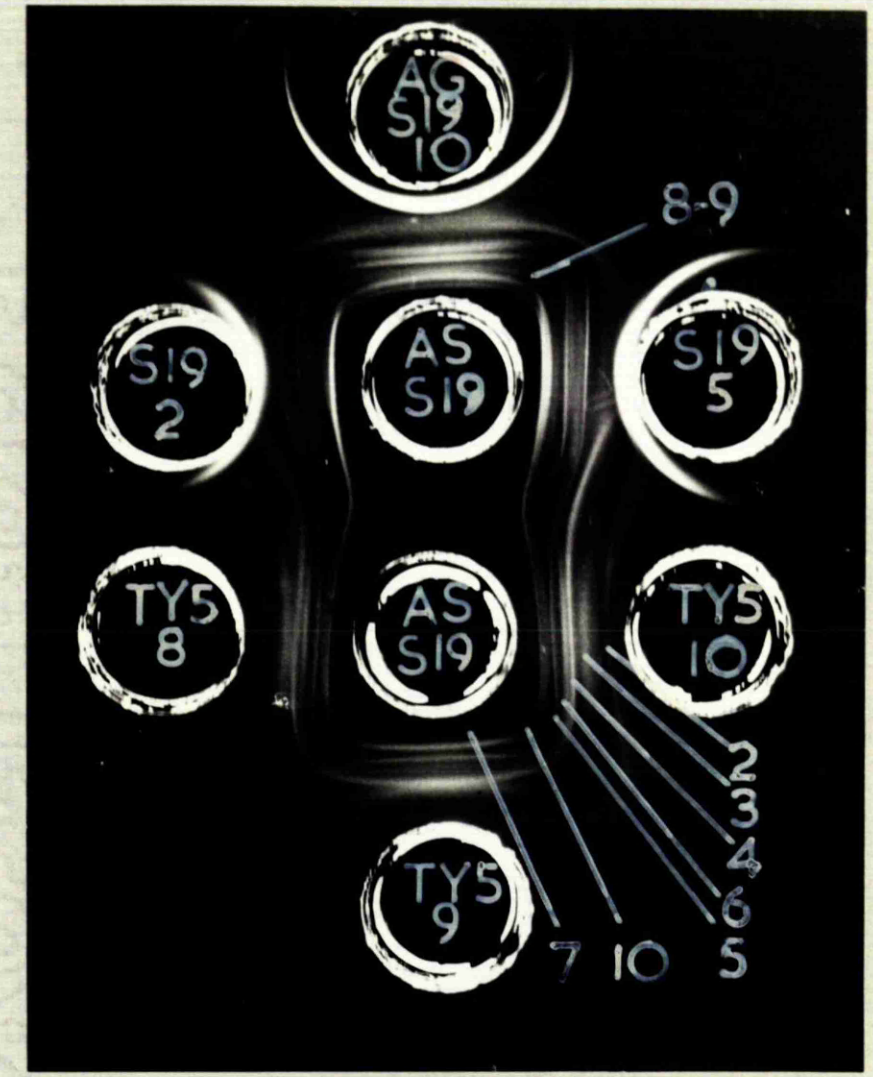


FIGURE 17.

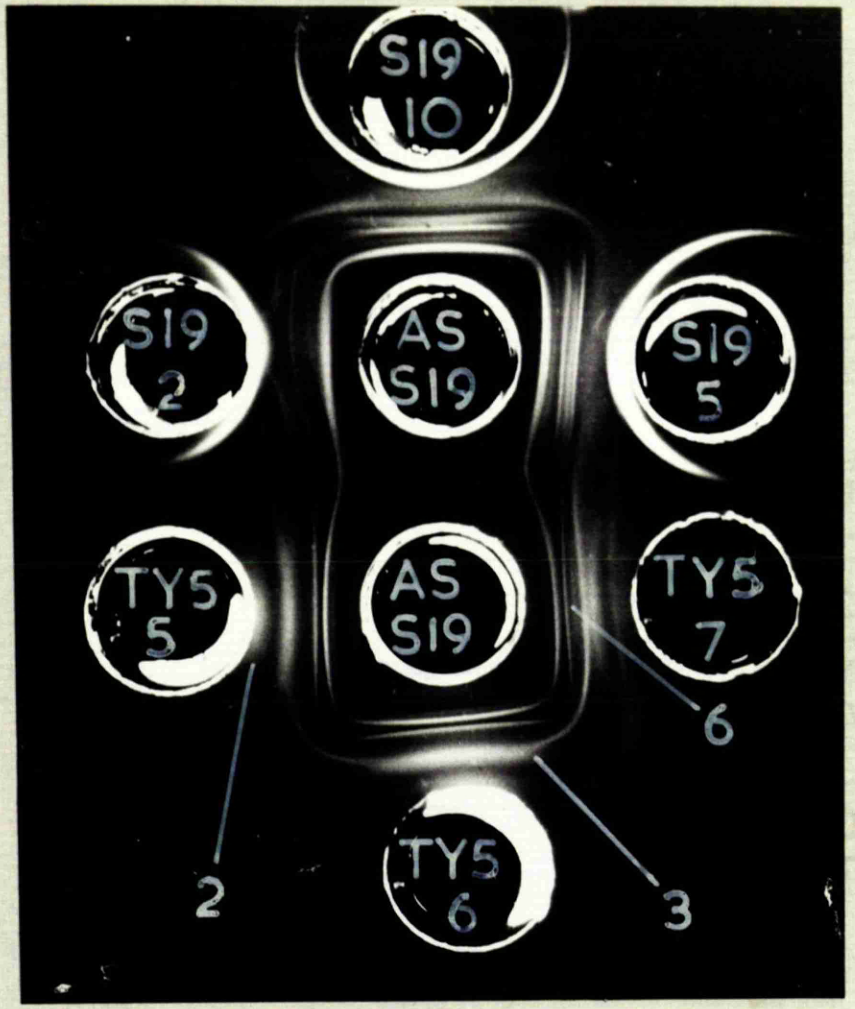


FIGURE 18.

FIGURE 19.

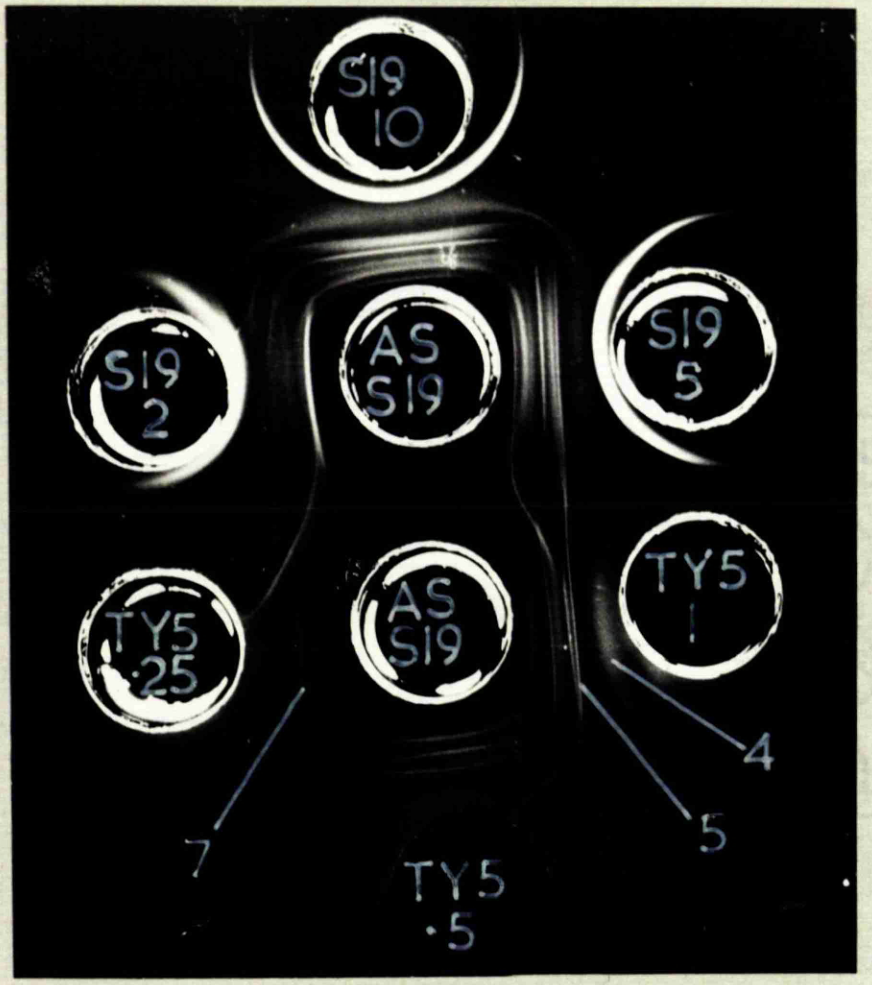


FIGURE 20.

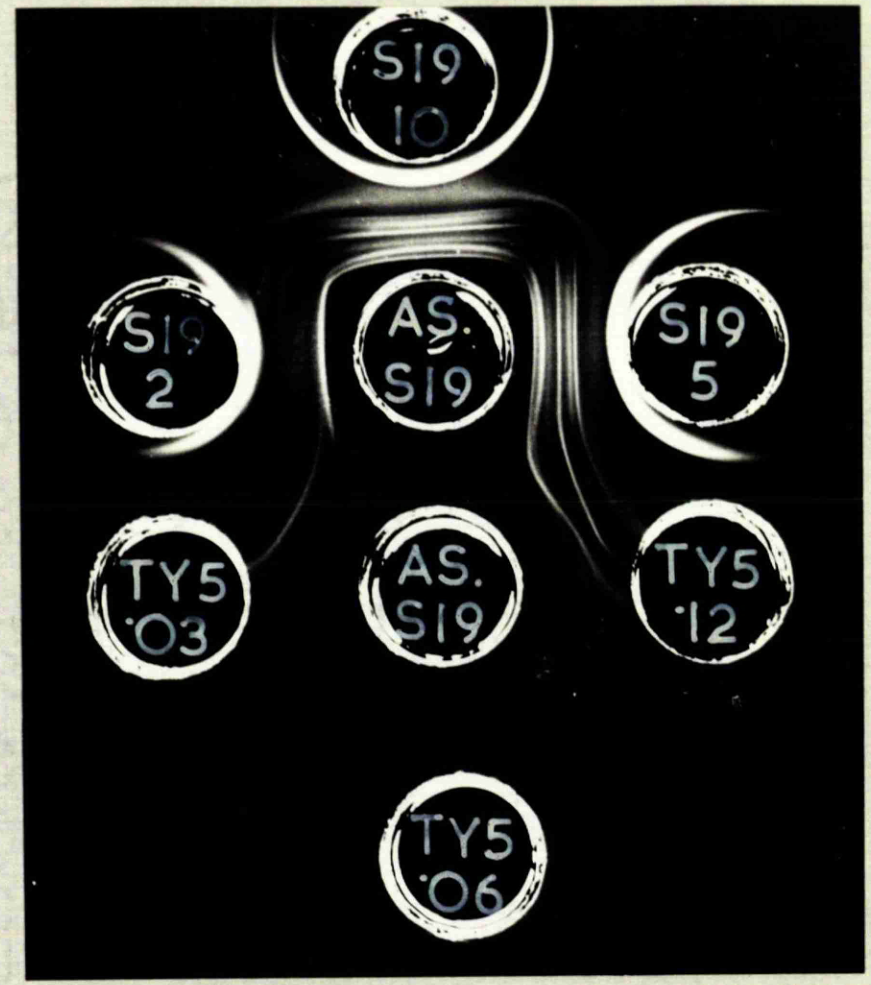


FIGURE 21.

FIGURES 16 - 21

Quantitative titration of the soluble antigen fractions of Brucella abortus type 5.

Central wells contain 0.1 ml. of undiluted Brucella abortus 819 hyperimmune serum. The uppermost 3 peripheric wells contain graded quantities of the homologous antigen serving as controls, whereas the lowermost 3 pheripheric wells contain decreasing quantities of acetone-dried cells of Brucella abortus type 5 in 0.1 ml. merthiolate-saline. Arabic figures superimposed on peripheric wells show mg. of acetone-dried cells applied per reservoir. Arrows and figures denote particular precipitate lines.

Perhaps the most difficult titration of the precipitinogenic antigens of brucella tested was that of Brucella abortus type 5, therefore, the results of this titration are fully illustrated (Figures 16 - 21). To facilitate identification of the various precipitin lines of Brucella abortus type 5, the top half of each plate was used in a standard manner. Thus the three uppermost peripheric wells were charged with 10, 5 and 2 mg. of Brucella abortus 519 antigen respectively, whereas the lower three wells received decreasing quantities of Brucella abortus type 5 antigen as indicated by the Arabic figures superimposed on these reservoirs. The central wells were charged with 0.1 ml. of the same 519 antiserum which was used for the titration of 519 antigens.

It will be noted that No.1 precipitin line cannot be observed in any of these plates. Increasing the quantity of antigen up to 30 mg. per reservoir did not bring forth any new precipitin lines, although line No.2. was best seen at 15 mg. of antigen (Figure 16).

In Figure 17 seven of the ten precipitin systems of Brucella abortus type 5 may be observed. Of these missing No. 8 and No.9 can never be seen as individual lines but they may be confluent with No.7 and No.10 precipitin lines respectively.

TABLE 35

QUANTITATIVE PRECIPITATION OF THE SOLUBLE ANTIGENS OF 5 BIOTYPES OF BR.ABORTUS

Antiserum	Antigen		Minimal amount of bacterial substance (u.gr.) required to form precipitin line											
	Species	Biotype	1	2	3	4	5	6	7	8	9	10		11
Br.abortus S19	Brucella abortus	S19	2000	6000	15000	4000	3000	4000	3000	6000	3000	2000°;	500°;	500° 60
do	do	1	5000	-	-	11000	3000	7000	7000	2000	2000	3000;	2000;	250 250
do	do	2	5000	-	-	13000	2000x	2000x	2000x	2000x	250	2000;	1000	-
do	do	4	-	9000	500	6000	500	4000	7000	-	-	250;	250	-
do	do	5	-	5000	6000	1000	1000	7000	250	-	-	120;	500	

x = appears as a single line ° components in the complex of No.10 precipitin line.

On decreasing the quantity of antigen the first of the lines to fade out is No.6 (at 7 mg. Figure 18) followed by:

No.3 (at 6 mg. Figure 18)

No.2 (at 5 mg. Figure 18)

Nos. 4 & 5 (at 1 mg. Figure 20)

No.7 (at 0.25 mg. Figure 20)

Line No.10 is again a complex of at least 2 precipitin lines. Between from 10 to 4 mg. of antigen it appears as a single line, (Figures 16 - 19) but at 3 mg. a new line appears corresponding to No.10A in the S19 system. The identity of the 2nd of these 2 lines to No.10B or 10C could not be ascertained (Figures 19-20).

The titration of the soluble antigens of Br.abortus type 1, 2 and 4 was carried out in exactly the same way as that applied to Br.abortus type 5. The results of titrations of the soluble antigens of the 5 biotypes of Br.abortus are summarised in Table 35.

It will be seen in Table 35. that all 3 biotypes of Br.abortus (S19, Ty.1 and Ty.2), which agglutinate in Br.abortus monospecific antiserum, shared the antigenic component required for the formation of No.1 precipitin line, whereas Type 4 and Type 5, which agglutinate in Br.melitensis

monospecific antiserum, did not yield this component even at 30 mgs. of acetone-dried cells applied per reservoir.

Soluble antigen No.2 and No.3. were not detectable in Br.abortus Type 1, and Type 2; but they were present in varying quantities in the other 3 biotypes.

Soluble antigens No.4 - No.7 were shared by all but one of the biotypes examined, differing only in their relative quantities present. The exception, Br.abortus Type 2, never exhibited precipitin lines from No.5. to No.8. as individual lines but as a single strong line, last seen at 2 mg. of antigen. Similarly, precipitin lines No.8 and No.9 were never given as individual lines by types 4 and 5, thus their relative quantities in the antigen preparation could not be ascertained.

In the complex making up No.10 precipitin line the titration of the 3 individual antigen components was only possible in case of S19 and biotype 1, for the other 3 types of Br.abortus only exhibited two out of three precipitin lines, the exact identity of which could not be established.

The antigen component contributing to the formation of No.11 precipitin line was only detectable in Br.abortus S19 and Type 1 respectively.

DISCUSSION

In the quantitative titration of the soluble antigenic components of the 5 biotypes of Brucella abortus, 13 precipitinogenic antigens in all were revealed by the agar gel double diffusion technique. In studies of this nature the analytic reagent (antiserum in this instance) must be highly potent and homologous to only one of the related organisms under study if the results of titration of antigens of various origin are to be comparable. In order to prevent the drawing of false conclusions about the relative proportion of soluble antigens present in the different biotypes of Br.abortus, pooled sera of several rabbits immunised with S19 was used. For it has been found (Williams and Grabar, 1955, Jennings, 1959; Crowle, 1960) that no two different individuals even within the same species vaccinated with the same antigen under identical conditions would give identical antibody responses. Crowle (1961) found that some of the animals, immunised with a single antigen in identical way, failed to produce antibody to the antigen, others responded with the production of much antibody to this same antigenic material.

In the course of the titration of antigens of various origin what appeared to be the only qualitative difference

was the soluble fraction of Brucella abortus S19 contributing to the formation of No.1 precipitin line. It was shared in comparable quantities by the 3 biotypes which have been both biochemically as well as seroligically typical Br.abortus. This finding is at some variance with that of Olitzki (1959) who failed to show No.1 antigen component in two virulent strains of Br.abortus (Strains: 2308 and Ru.) whereas he found it to be always present in S19.

The two other strains of Br.abortus (biotypes 4 and 5) examined in this experiment did not yield No.1 antigen. These two strains, when examined in monospecific sera of brucella, agglutinated only with Br.melitensis monospecific serum although in every other respect they were Br.abortus.

Apart from the No.1 antigen fraction of brucella, there were 5 other soluble antigen fractions (Nos. 2, 3, 8, 9, 11), which were present in the S19 homologous system but failed to form distinct precipitin lines when acetone-dried antigen preparation of one or the other strain of Br.abortus was applied against S19 antiserum. Although these antigen components did not form distinct precipitin bands the absence of such bands may not be taken as definite evidence of the

total lack of those particular soluble antigens in that preparation. It is possible that despite the application of comparatively large quantities of antigen (up to 30 mg./well) the quantities of these particular fractions were not sufficient to bring about optimal proportions with their respective antibodies and thus form individual precipitin lines. Alternatively, some of these antigenic determinants may have been carried on a single particle in the antigenic preparation of the virulent strain, whereas in the S19 preparation those determinants were carried by separate particles. Thus if in the S19 preparation, antigen determinants A and B are carried on two separate particles, whereas in the antigen preparation of one of the virulent strains these same two determinants, A and B, are carried on a single particle (AB) then the reaction of antigens A and B of S19 and antigens AB of the virulent strain with S19 antiserum would result in the formation of two distinct lines of precipitate in the homologous and only in one line in the heterologous system.

The 3 precipitin systems referred to as No.10A, B and C respectively, were peculiar in that over a wide range of antigen quantities applied per reservoir they appeared

as a single band and only split up to form distinct lines when a comparatively small quantity of the antigen was used. This would suggest that the antigens involved are closely related at least in as much as they all have similar very narrow range of optimal proportions. The conclusion that they represented ? genuine precipitin systems was supported by the fact that they appeared at much the same time during the time of incubation.

Although the precipitin lines formed between S19 antiserum and the heterologous strains of brucella were in perfect fusion with the precipitin lines of the S19 homologous systems, to conclude complete identity of antigens taking part in the formation of such continuous precipitin lines may be erroneous. It has to be remembered that antibody in S19 antiserum would react with closely related antigen of a heterologous organism even if only some of the determinant groups on the related antigen molecules were identical. Thus antigens of various origins although listed under the same Arabic number in Table 35 this enumeration does not necessarily imply complete antigenic identity of such antigens. It also follows of the conditions of these double diffusion tests that any extra antigen which may have been present in heterologous organisms would not have been revealed by S19 antiserum.

EXPERIMENT NO. 5.

COMPARATIVE STUDIES ON THE PRECIPITATE PATTERN OF VARIOUS BIOTYPES OF BRUCELLA ABORTUS

Agar gel diffusion tests, performed in the course of Experiment No.4., revealed only such soluble antigenic components of virulent biotypes of Br.abortus which were shared with the vaccine strain. This followed from the use of S19 antiserum as the analytic reagent.

In order to detect any qualitatively different component of the virulent biotypes of Br.abortus which may be antigenic in cattle, it is essential to examine each of them with its homologous bovine antiserum by the aid of the agar gel diffusion technique and compare the precipitin lines therin to that of a S19 homologous system.

MATERIALS AND METHODS

Antigens for agar gel precipitation

These were prepared in identical ways to those used in Experiment No.4. using 100 mg. of the appropriate acetone-dried preparation of brucella per 1 ml. of merthiolate-saline.

Antigens for the immunisation of cattle

The 5 biotypes of Brucella abortus (S19, Ty.1, 2; Ty. 4 and 5) were grown on serum dextrose agar for 5 days, then harvested in saline. To assess the total number of organisms per ml. suspension, opacity counts were carried out by means of the Burroughs Wellcome opacity tubes.

Immune sera

These were prepared in cattle using groups of two heifers (1½ to 2 years old) for the preparation of antisera gainst each of the 4 virulent biotypes of brucella, whereas a group of 4 animals was used for S19 antiserum preparation. Each of the 12 animals received a total of approximately 35 x 10^{10} organisms (total count) of the appropriate biotype, in the course of 6 subcutaneous injections administered at approximately one month intervals. The animals were bled from 5 to 14 days intervals after each injection and the sera obtained were examined by the agar gel diffusion technique for their precipitating potency. Part of the most potent immune sera of animals immunised with the same biotype of brucella were pooled and kept frozen until used.

Agar gel diffusion test

Agar gel for the diffusion tests was prepared the same way as that used in Experiment No.4. applying the same

pattern of distribution of reservoirs at the same distance in relation to each other. The exact way of application of the various immune sera and antigens shall be described in Results. From the various undiluted immune sera 0.1 ml. was used per reservoir and the graduated quantities of antigen applied were contained in 0.1 ml. volume of the preparation. The plates were incubated for 6 days at 37°C then washed in saline and photographed.

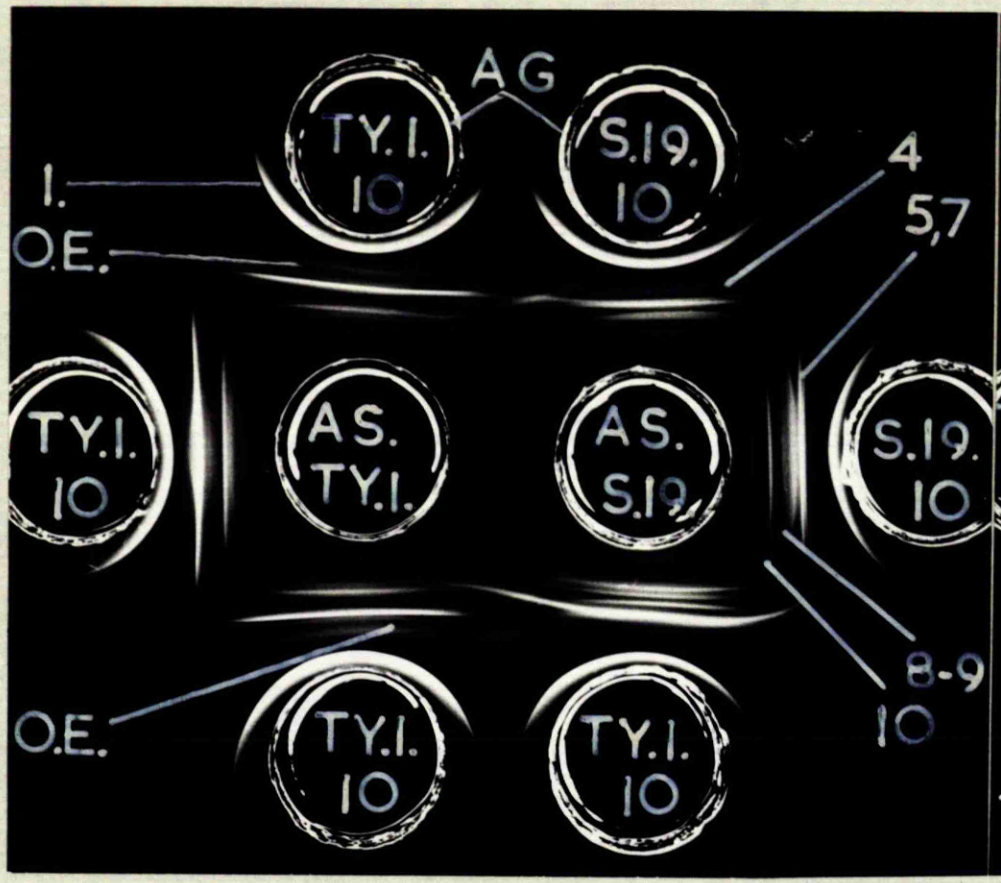


FIGURE 22. Comparative analysis of the precipitate systems of S19 and type 1 Brucella abortus.

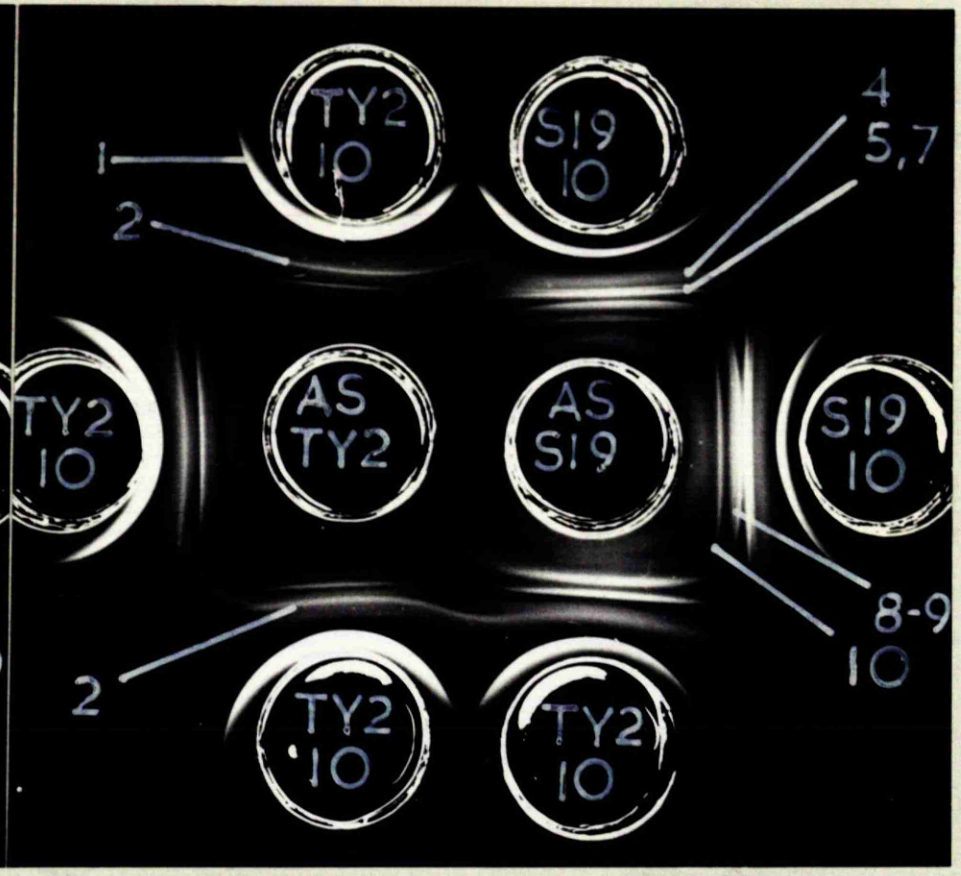


FIGURE 23. Comparative analysis of the precipitate systems of S19 and type 2 Brucella abortus.

Central reservoirs contained 0.1 ml. of undiluted immune serum (bovine) whereas the peripheric ones were charged with graded quantities of antigen (10 mg.) as indicated. Arrows and figures denote particular precipitate lines.

RESULTS

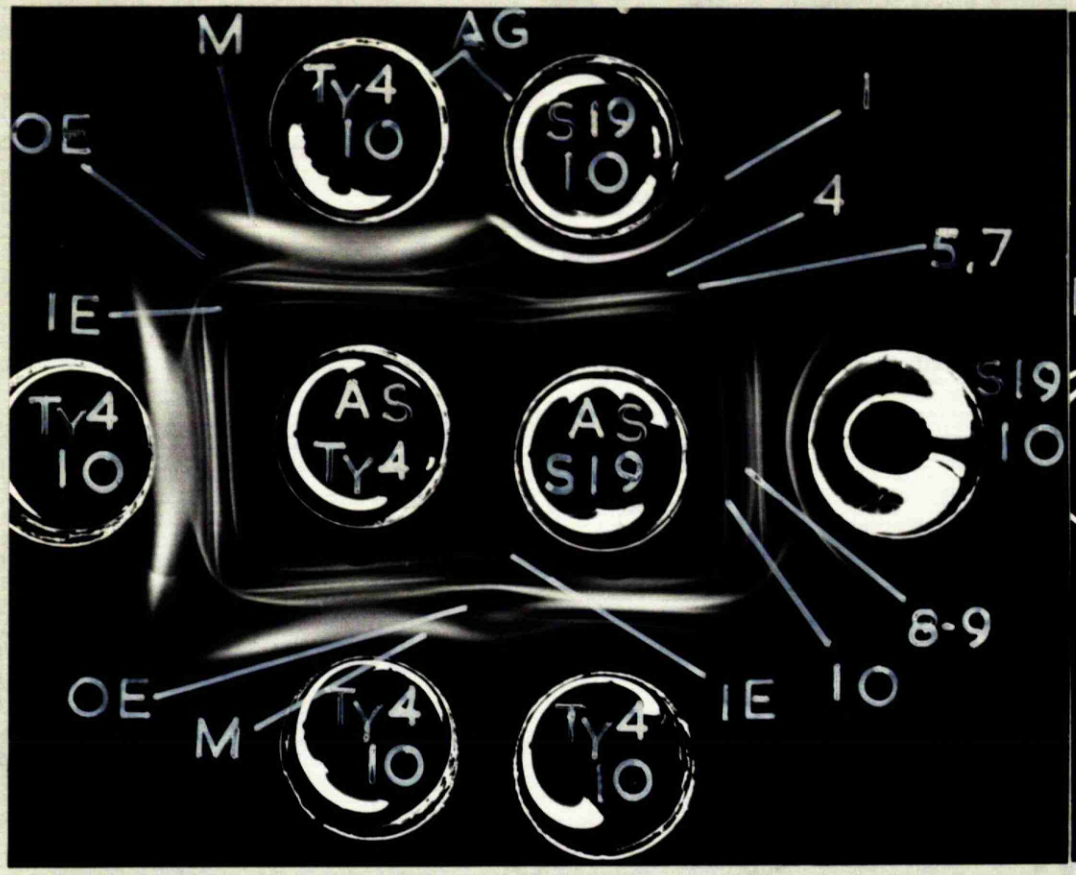
The results of the comparative studies on the precipitin systems of the 5 biotypes of Br.abortus are illustrated in Figures 22 - 28.

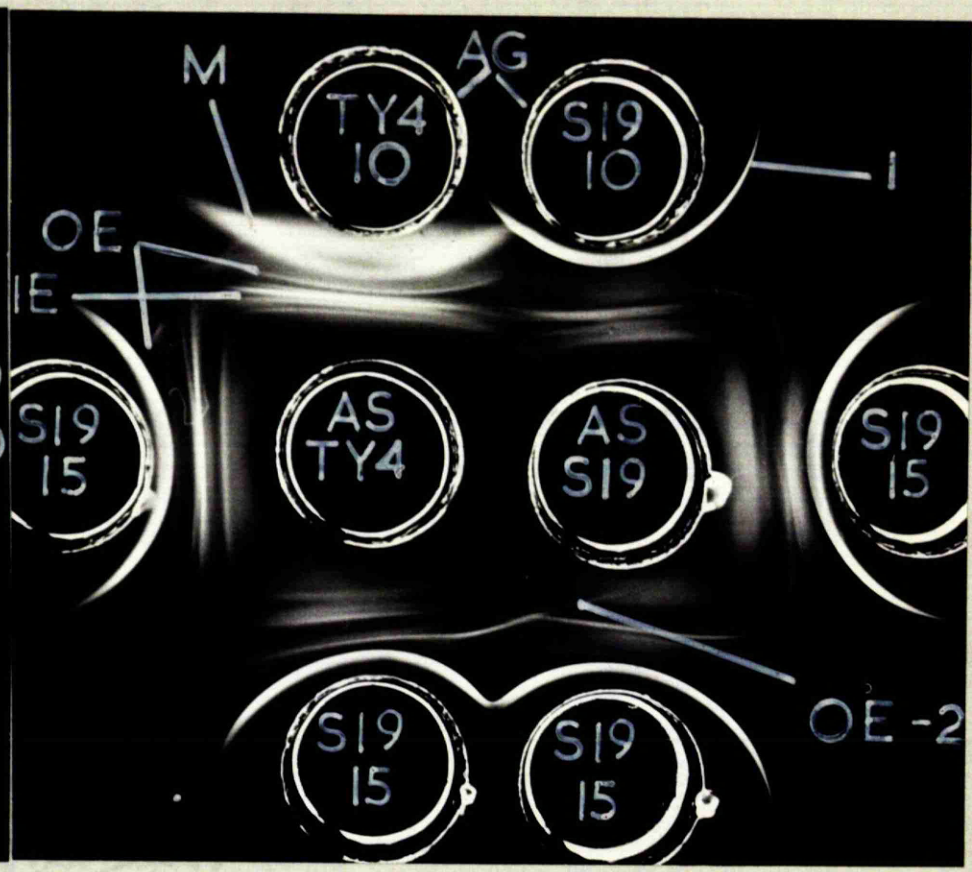
In Figure 22 the precipitate pattern of Br.abortus type 1 is compared to that of the vaccine strain (S19 Br.abortus). It will be seen that the application of 10 mg. of type 1 antigen against its homologous antiserum results in a precipitin line, marked OE, which has no counterpart in the S19 homologous system. (Upper half of Figure 22). The inner end of line OE points to the source of S19 antigen thus indicating either the relative shortage or the total absence of this antigen component therein. To examine whether S19 antiserum contained antibody capable of forming precipitin line OE, with the antigen preparation of type 1 Brucella abortus, 10 mg. of this antigen was applied against S19 antiserum side by side to the type 1 homologous system (lower half of Figure 22). The result shows a somewhat hazy precipitin line between the reservoirs containing S19 antiserum and type 1 antigen respectively, which is the direct continuation of line OE of the adjacent type 1 homologous system.

In addition to precipitin line OE, Figure 22 also shows some other differences between the precipitin bands of the 2 brucella systems in homologous position. From the type 1 homologous system the precipitin lines from No.8 to No.10 are absent. However, this difference did not prove to be a qualitative one either, for the application of 10 mg. of type 1 antigen against S19 antiserum revealed the corresponding antigens therein (see lower right quarter of Figure 22).

Figure 23 shows the comparison of precipitate pattern of Brucella abortus S19 and type 2 systems. The overall precipitate pattern of the type 2 homologous system is rather poor. There is no apparent extra precipitin band in this system. The S19 homologous system on the other hand shows a number of precipitin lines (from No.6 to No.10) not represented in the type 2 Br.abortus homologous system.

This difference, however, proved to be due to the relative shortage of antibody in type 2 antiserum, for the application of 10 mg. of type 2 antigen against S19 antiserum revealed antigens in type 2 preparation corresponding to the missing precipitin lines (lower right quarter of Figure 23).





FIGURES 24 - 25.

Comparative analysis of the precipitate systems of S19 and type 4 Brucella abortus.

Central reservoirs contained 0.1 ml. of undiluted immune serum (bovine) whereas the peripheric ones were charged with graded quantities of antigen as indicated. Arrows and figures denote particular precipitate lines.

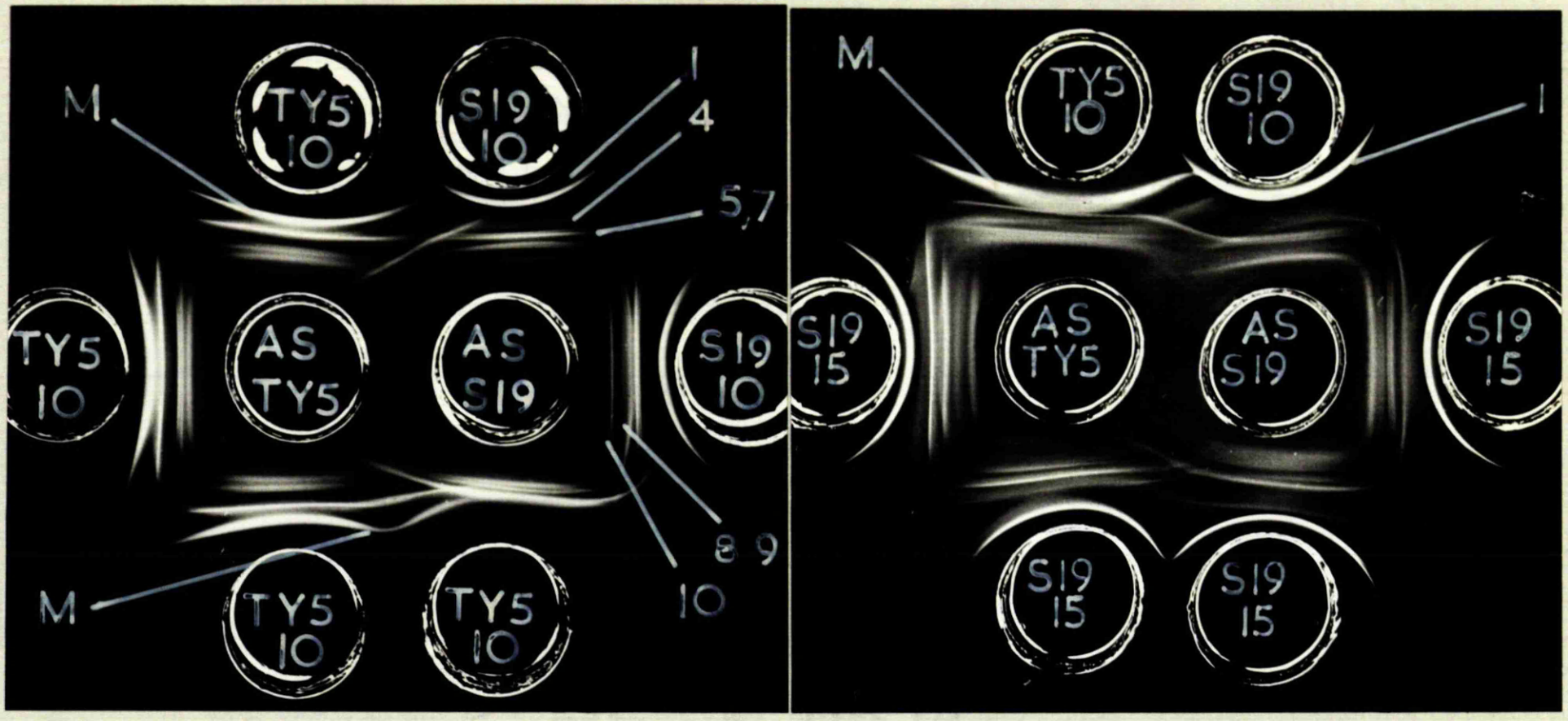
Figures 24 - 25 show the comparison of precipitate pattern of Brucella abortus S19 and type 4 systems. The first point of interest arising from this figure is the emergence of a new precipitin line (M) not seen at the quantitative titration of the soluble antigens of type 4 organisms against S19 antiserum.

Testing the acetone-dried preparation of type 4 Brucella abortus with its homologous antiserum the broad and somewhat diffuse precipitin line M gives a reaction of partial identity with No.1 precipitin line of the adjacent S19 homologous system (upper half of Figure 24).

Figure 25 gives a better picture of the intersection between those two precipitin lines showing a well marked spur formation by line No.1. To examine S19 antiserum for the presence of antibody specific to antigen M, 10 mg. of the acetone-dried preparation of type 4 organisms was applied against S19 antiserum in adjacent position to type 4 homologous system (lower half of Figure 24). The results show the broad precipitin band M tapering off and becoming confluent with another line (OE) between the reservoirs containing type 4 antigen and S19 antiserum respectively. The experiment was repeated several times but the results were always the same, lines M and OE of the

type 4 homologous system being represented by a single well defined line in the S19 antiserum type 4 antigen system. As no conclusive evidence could be obtained this way about the presence of antibody in S19 antiserum capable of reacting with antigen M of type 4 organisms, a different approach had to be applied. In this experiment S19 organisms were tested for antigen M by the use of type 4 antiserum. The lower half of Figure 25 shows the results of such experiment. It will be seen that the interaction between type 4 antiserum and S19 antigen resulted in No.1 precipitin band which gave a reaction of identity with precipitin line No.1 of the adjacent S19 homologous system.

There were two other precipitin bands (OE and IE) present in the type 4 but not in the S19 homologous system. (See upper half of Figure 24). The tip of line OE points towards the source of S19 antigen thus suggesting the shortage of antigen OE therein. It has already been pointed out that the application of this antigen component in the form of type 4 antigen preparation did not give a decisive answer about the presence of the corresponding antibody in S19 antiserum (see lower half of Figure 24), for precipitin bands OE and M showed up as a single line in this situation which may or may not have been a compound line.



FIGURES 26 - 27.

Comparative analysis of the precipitate systems of S19 and type 5 Brucella abortus.

Central reservoirs contained 0.1. of of undiluted immune serum (bovine) whereas the peripheric ones were charged with graded quantities of antigen as indicated. Arrows and figures denote particular precipitate lines.

Thus the alternative method of attempting to demonstrate antigen OE in S19 antigenic preparation was resorted to. The upper left quarter of Figure 25 shows the result of such an experiment. It will be observed that precipitin band OE of the type 4 homologous system shows a pattern of partial intersection with another line of the heterologous system. (Both line OE and the intersection are pointed out by a pair of arrows in the top left corner of Figure 25). Subsidiary tests showed that the intersected line corresponded to precipitin band No.2 of the S19 homologous system. Somewhat similar considerations apply to precipitin line IE of the type 4 homologous system. It is absent from the S19 homologous system. It cannot be demonstrated by applying type 4 antigen against S19 antiserum (see lower right quarter of Figure 24) nor can it be shown when type 4 antiserum is applied against S19 antigen. (See top right quarter of Figure 25).

Figure 26 and 27 show the comparison of precipitate patterns of Brucella abortus S19 and type 5 systems. It will be recalled that type 5 of Brucella abortus was the other organism which did not yield No.1 antigen component when tested with S19 antiserum. When its homologous

antiserum was applied against it a new precipitate line developed. (Marked M in Figures 26 - 27). Line M, just like that of the type 4 homologous system, gave a reaction of partial identity with No.1 precipitin line of Br.abortus S19, the latter one showing marked spur formation (see upper half of Figure 27).

In order to examine whether S19 antiserum contained antibody capable of reacting with antigen M, 10 mg. of type 5 antigen was applied against S19 antiserum in adjacent position to type 5 homologous system. (See lower half of Figure 26). It will be seen that precipitin line M of the type 5 system joins another line (probably line No.4) in a manner suggesting partial identity to the intersected line. The application of S19 antigen against type 5 antiserum resulted in the formation of the sickle shaped line No.1. (see lower half of Figure 27). Apart from this partial difference between antigen M of type 5 and antigen No.1 of S19 Brucella abortus no other qualitative difference was noted in the precipitate patterns of the two organisms examined.

It has been shown so far that 3 out of the 4 antisera prepared against the virulent biotypes of brucella exhibited one or more precipitin lines withtheir homologous antigens which appeared to be either parially different (lines M and OE)

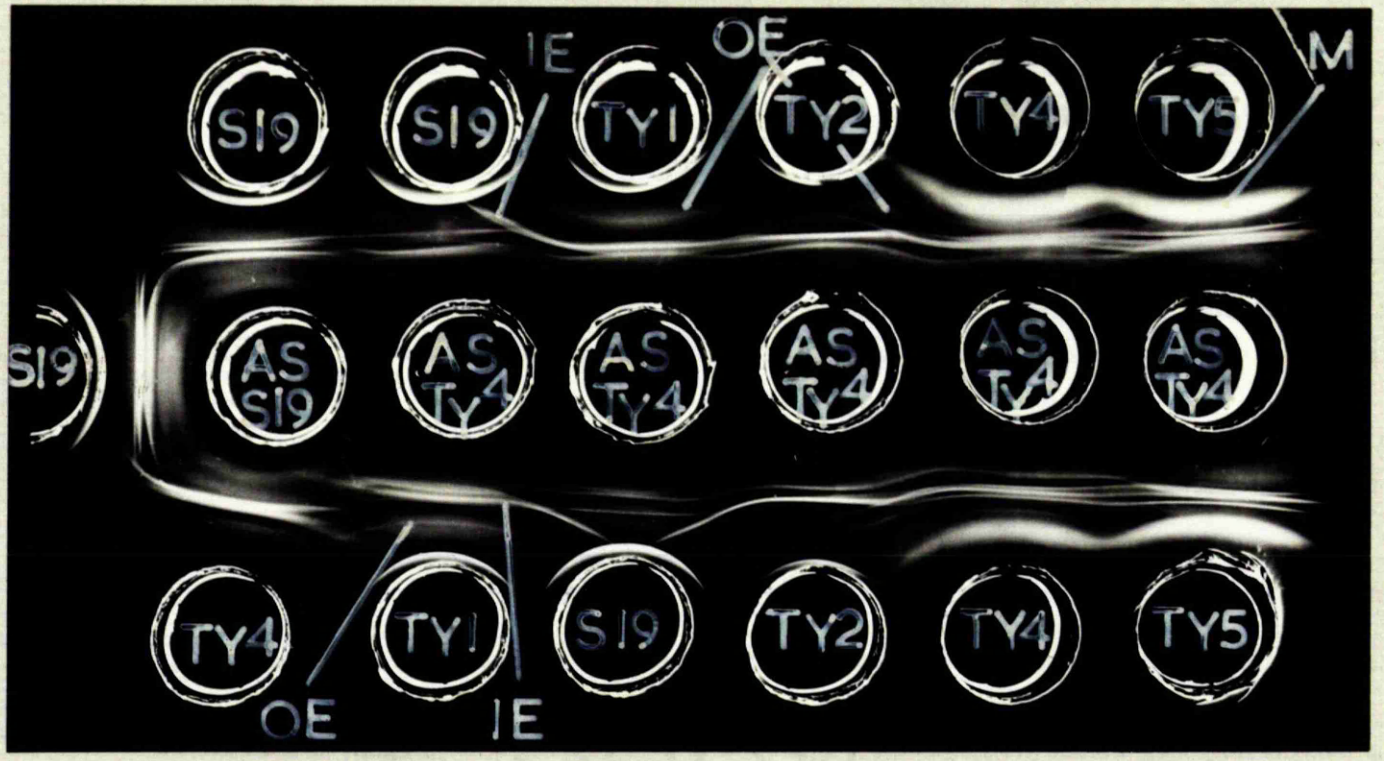


FIGURE 28.

Comparative analysis of the soluble antigens of S19, Type 1, Type 2, Type 4 and Type 5 Brucella abortus.

Central reservoirs contained 0.1 ml. of undiluted type 4 and S19 immune serum (bovine) whereas the peripheric reservoirs were charged with graded quantities (10 mg.) of antigen of various origin as indicated. Figures and capital letters, outside the wells, denote particular precipitate lines.

or totally absent (line IE) in S19 homologous system. It became apparent, however, that some of the antisera (Types 1, 2 and Type 5) used were less potent than others. It was possible therefore that some of the antigenic components present in type 4 but appearing to be absent in the other virulent biotypes of brucellae may not have been demonstrated due to the shortage of the corresponding antibodies in their respective homologous antiserum rather than the absence of such antigens.

To examine this possibility Brucella abortus type 4 antiserum was chosen which was known to contain the maximum number of precipitins not present in S19 antiserum. The comprehensive results of such an analysis are shown in Figure 28. In this experiment 10 mg. of antigen of each biotype of brucella was applied against type 4 antiserum. In addition, S19 homologous system is shown in the top left corner of Figure 28, whereas in its lower left corner type 4 antigen was used against S19 antiserum. It will be seen that the two biotypes of brucella agglutinating only with Brucella melitensis monospecific serum share precipitin line M. It will also be observed that types 1 and 4 of Brucella abortus share precipitin line OE. But the most

interesting feature of this figure is the precipitate line running along in front of all the reservoirs containing antigens of virulent brucellae but stops short before reaching the S19 containing reservoir (the arrow marked IE points out this line in the upper half of Figure 28). The tip of the precipitin band IE points to the source of S19 antigen indicating the shortage of the total absence of the corresponding antigen component therein. The lower left corner of Figure 28 serves to demonstrate the absence of antibody in S19 antiserum corresponding to precipitin band IE. The left tip of this slightly S shaped precipitin line (IE) points towards the source of S19 antiserum indicating the shortage or absence of antibody corresponding to the line, whereas its right tip is directed towards the source of S19 antigen just like in the upper half of that figure. It appears therefore that the 4 virulent biotypes of Br.abortus share an antigen component not present in the vaccine strain, nor can the corresponding antibody to this extra antigen be demonstrated in antisera prepared against S19 Br.abortus.

DISCUSSION

The results obtained in these comparative studies on the precipitate patterns of the various Brucella abortus systems cannot directly be compared to those obtained by Olitzki (1959) for the antisera he used were prepared in rabbits whereas those used in these studies were of bovine origin. It is a well established fact that the antigenicity of a substance may not be the same in two different individuals within the same species (Williams and Grabar, 1955; Jennings, 1959) and even less so in two different species (Crowle, 1961). Therefore, Olitzki's failure of showing any qualitative differences in the antigenic structure of the 3 brucella species may be attributed either to the use of rabbit sera or to the fact that in his analytic sera only 6 precipitating antibodies could be detected with regularity. (Pooled antiserum prepared against S19 Br.abortus in rabbits and used in Experiment No.4 contained 13 different antibody species.)

Hyperimmunisation of animals, however, is not without drawbacks especially if such hyperimmune sera are used in the qualitative analysis of closely related antigenic substances. Crowle (1961) pointed out that

mild stimulation of the antibody producing mechanism induces it to form antibodies which would cross-react little or not at all with very similar antigens, whereas hyperimmunisation tends to induce the production of a range of antibodies which may cross-react even with distantly related antigens. Sera used in these studies may be considered as hyperimmune, thus the loss of strict specificity may well apply to the antibody content of the bovine sera used. The loss of strict specificity of antibody would result in minimising differences between two related but not identical antigens, but would not show two identical antigens as different ones. It would therefore follow that the antigenic differences shown between the various biotypes of Brucella abortus represent the minimal rather than the complete extent of antigenic differences.

Consideration of precipitin lines present in the S19, but absent in the homologous systems of the various virulent biotypes of Brucella abortus effects precipitate lines from No.5 to 10 of the type 1 and lines from No.6 to 10 of the type 2 homologous systems.

The application of 10 mg. antigen of the appropriate virulent biotypes (types 1 or 2) against S19 antiserum, however, showed the presence of antigens therein corresponding to the missing precipitin lines. It appears, therefore, that the absence of these precipitin bands in the homologous systems of biotypes 1 and 2 respectively, was due to the relative shortage of antibodies in the homologous antisera of these two biotypes. Apart from these precipitin lines, the S19 homologous system did not exhibit any other bands of precipitate which were not demonstrated in the homologous system of the various virulent strains of Br.abortus. Some of these later systems, however, exhibited precipitin lines which were either totally absent (line IE) or were partially different (lines M and OE) from those in the S19 homologous system. The first of the partially different precipitin lines (M) was found in type 4 and 5 homologous systems of Br.abortus. This line M intersected line No.1 of the S19 homologous system in a manner which resembled the type of intersection described by Ouchterlony (1960) and referred to as "type III reaction" of basic precipitation patterns, occurring in the comparative tests of soluble antigens.

The application of "type III reaction" of Ouchterlony to the type of intersection of line No.1 of S19 and line M

of types 4 or 5 of Brucella abortus homologous systems, necessitates the postulation of at least two antigenically determinant groups carried on a single particle (ab) in S19 which forms line No.1 with its homologous antibodies (A and B). The corresponding particle of biotype 4 or 5 of Brucella abortus would carry only one of these two determinants (b) and its homologous antiserum would contain only one kind of antibody (b) to this particle.

Thus Brucella abortus S19 and type 4 reacting with their homologous antiserum side by side as in the upper half of Figures 24 - 25 exhibit precipitin lines No.1 consisting of ab - AB reagents up to the point of intersection with line M, which itself is composed of b - B reagents. The spur of line No.1 consists of ab - A components for antibody A can diffuse through b - B precipitate (line M) whereas antibody B cannot. The reaction of false identity seen in the lower half of Figure 25 may also be explained if the same assumption is made about the nature of antigens contributing to the formation of line No.1 and M respectively. Thus line No.1 of the S19 homologous system consists of ab - AB reagents as before but fusing this time completely with the adjacent crescent shaped line of the heterologous system which itself is made up of ab - B components.

In this situation no spur formation can develop for precipitates ab - AB and ab - B are impenetrable for both antibody B of type 4 as well as antibodies A and B of S19 antiserum.

The demonstration of antibody B in S19 antiserum capable of forming precipitin lines with antigen b of type 4 or type 5 Brucella abortus (see Figures 24 and 25; S19 antiserum reacting with 10 mg. of type 4 and type 5 antigen respectively) was only partially successful for the resulting precipitin line coincided with other precipitin lines.

Thus no evidence was found in these experiments contrary to the assumption made about the antigenic nature of the soluble fraction of either S19 or type 4 and 5 Brucella abortus contributing to the formation of precipitin line No.1 and M respectively.

The second of the partially different precipitin lines, OS, was observed in type 1 and type 4 homologous systems of Br.abortus. The available information about the antigenic nature of the substance taking part in the formation of line OS allows no more to suggest than that S19 Brucella abortus possesses at least one antigenic

determinant on one of its antigenic particles in common with the antigenic determinants of antigen OE. This postulate is supported by the observed spur formation of line OE of type 4 homologous system with a line of the adjacent S19 antigen - type 4 antiserum system. (See Figure 24 upper left quarter). The presence of antibody in S19 antiserum capable of reacting with antigen OE of type 1 Brucella abortus (lower half of Figure 22) is another indication to show that antigen OE is not altogether foreign to S19 organisms.

The most important difference in precipitin patterns, encountered in these comparative studies, was precipitin line IE of the homologous system of type 4 Br.abortus. Line IE was never observed in S19 homologous system. The corresponding antibody to this line could not be demonstrated in S19 antiserum, nor was it possible to show its antigen in S19 Brucella abortus.

Antisera prepared against the other virulent biotypes of brucella failed to form line IE when their respective homologous antigen was applied against them. However, the application of type 4 antiserum against the antigen preparation of the various virulent biotypes of Br.abortus

resulted in the formation of precipitin band IE, thus showing the presence of the corresponding antigen therein. It appears therefore that the 4 virulent biotypes of Brucella abortus, accounting for over 90% of field infection of cattle in Great Britain, possess at least one antigen which is common to all of them but is absent in the vaccine strain. A word of caution is felt justified at this point and the reader is reminded of the limitations of sensitivity of the precipitation reaction in agar gel in regard of showing very small quantities of either antigen or antibody. Subject to this observation, it would follow that a preparation of antigen IE in relatively pure form should be of great practical value in the diagnosis of superimposed field infection of vaccinated cattle provided that antibody production was a general occurrence to this antigen in bovines. The sera of vaccinated cattle, irrespective of the time of vaccination, should contain no antibody to antigen IE, whereas sera of those infected with either one of the four virulent biotypes examined should possess such antibody.

EXPERIMENT NO. 6.

TITRATION OF PRECIPITATING ANTIBODIES IN BOVINE SERA PREPARED AGAINST THE FIVE BIOTYPES OF BRUCELLA ABORTUS

Before an attempt would be made to isolate antigen IE from the soluble extract of brucella, a series of tests were carried out to determine the titre of the various precipitins present in these sera. The knowledge of the relative quantity of antigens in the antigenic preparation of the various organisms and the titre of corresponding antibodies, should give some indication of their antigenicity. To make the results of titration of the various antisera comparable it was essential to use the same basis of comparison. This was achieved by the use of S19 antigen preparation against which the titrations were carried out. In such cases where this preparation did not contain antigen, or the antigen was known to be partially different against an antibody of a heterologous serum its homologous antigen preparation was used for the titration of that particular antibody.

MATERIALS AND METHODS

Antigens for precipitin titrations

Those were prepared in the same way as those used in Experiment No.4.

Immune sera

employed in these tests were those used in Experiment No.5.

Precipitation in agar gel

For these tests the agar was prepared in the same way as that used in Experiment No.4. applying the same pattern of distribution of reservoirs at the same distance in relation to each other. The exact method of application of the reagents shall be described in 'Results'. From the various serum dilutions 0.1 ml. was applied per reservoir and the graded quantities of antigen used per reservoir were contained in 0.1 ml. volume of the preparation. Plates were incubated for 6 days at 37°C then washed in saline and photographed.

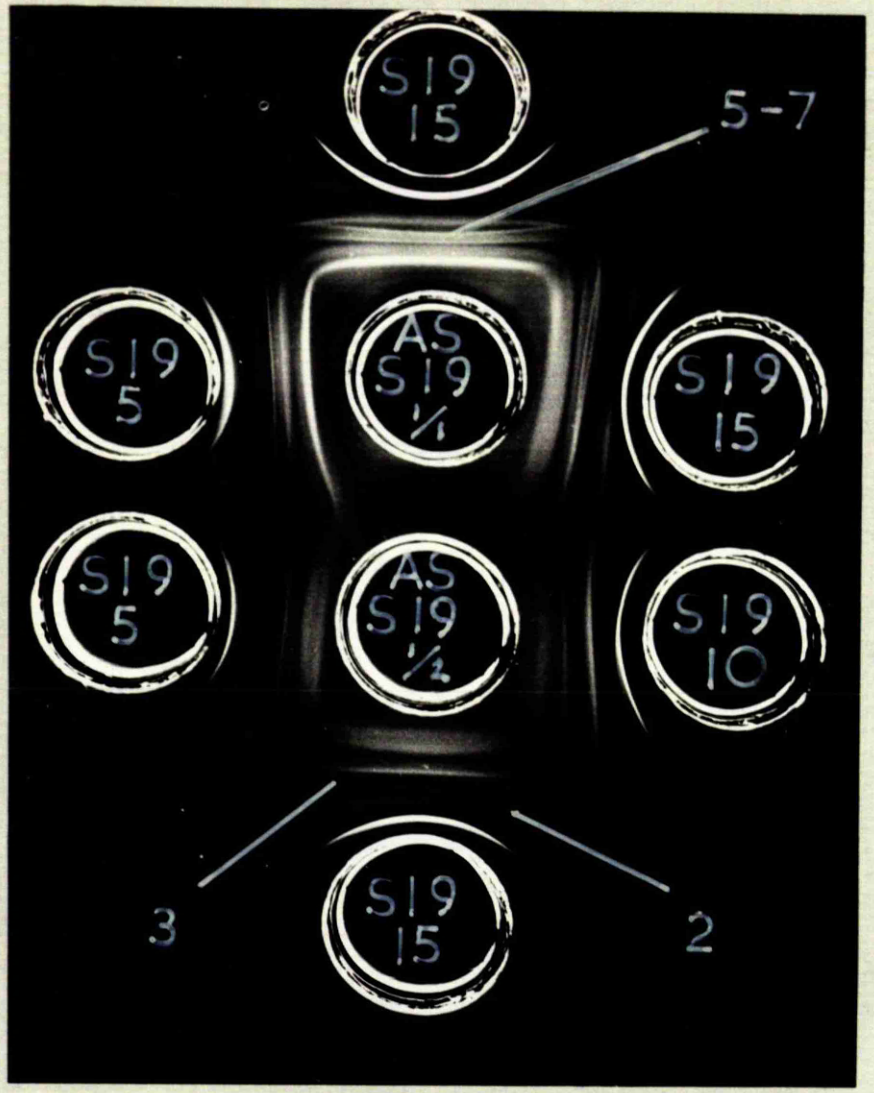


FIGURE 29.

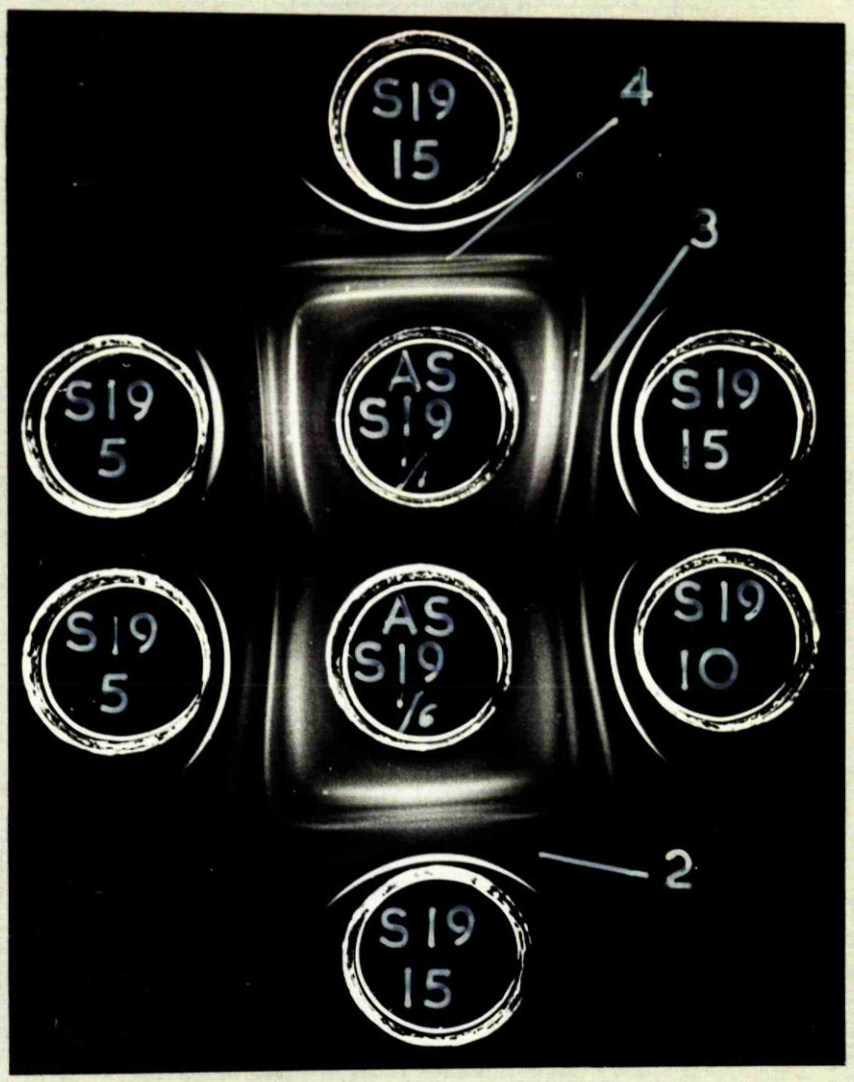


FIGURE 30.

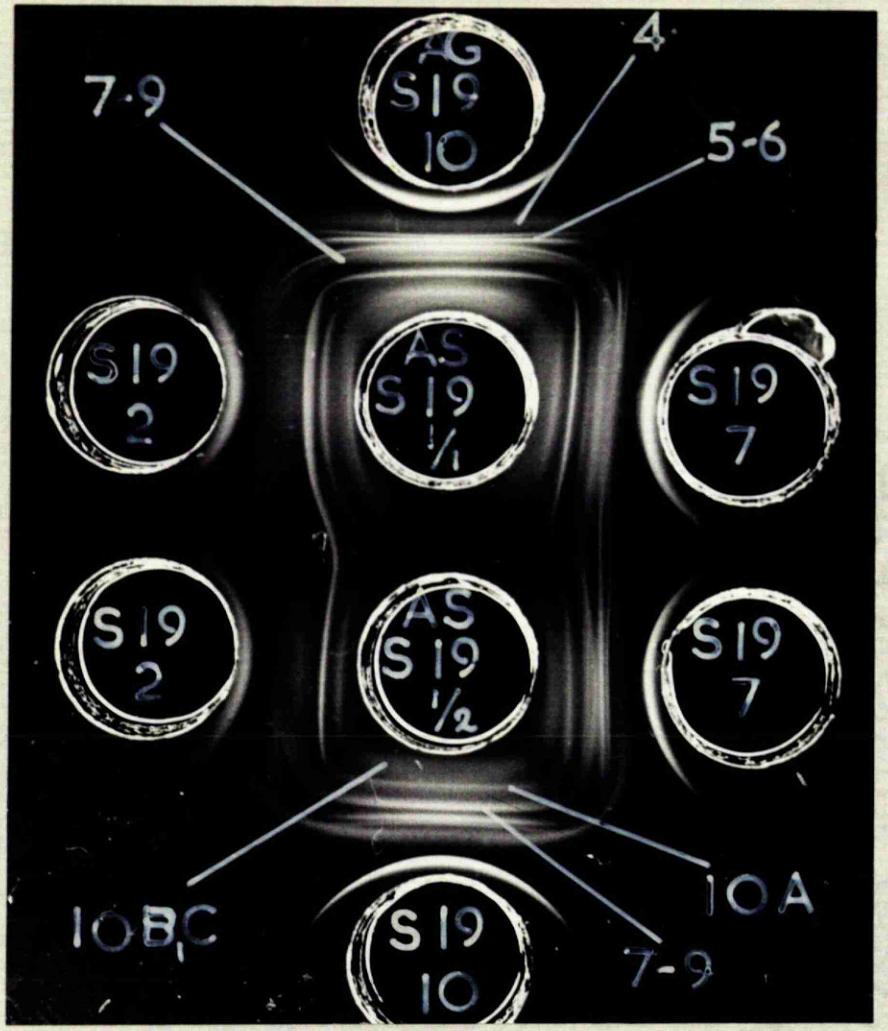


FIGURE 31.

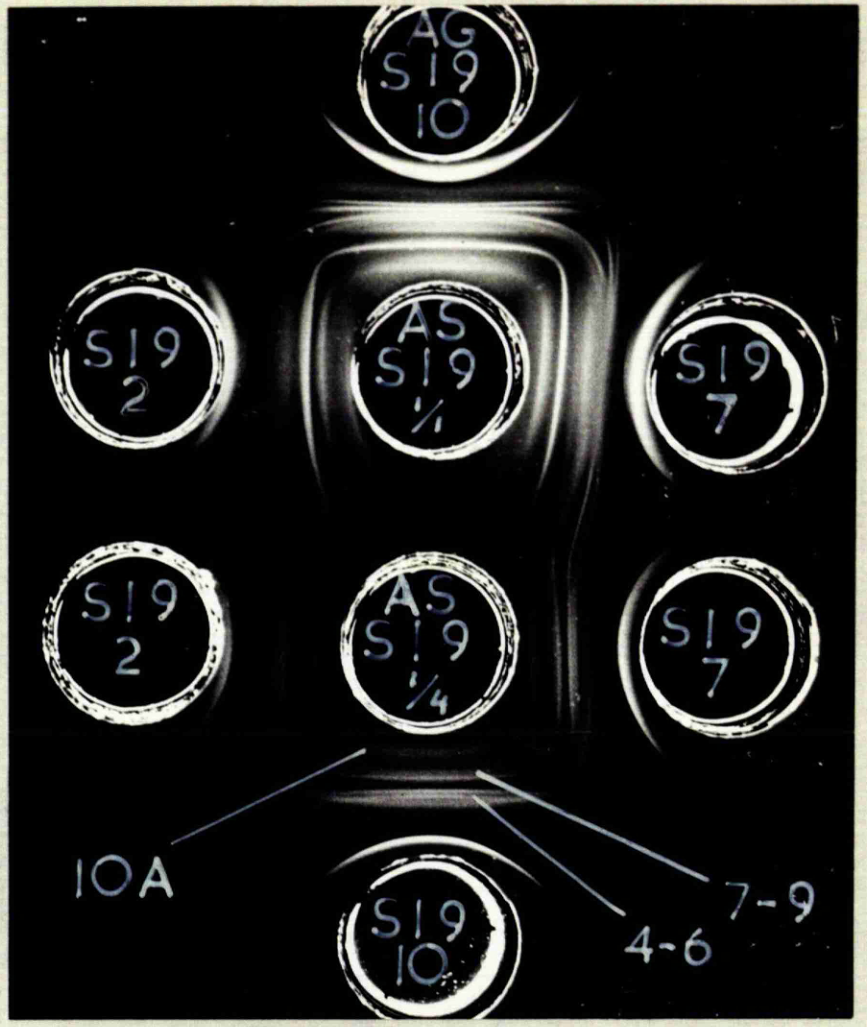


FIGURE 32.

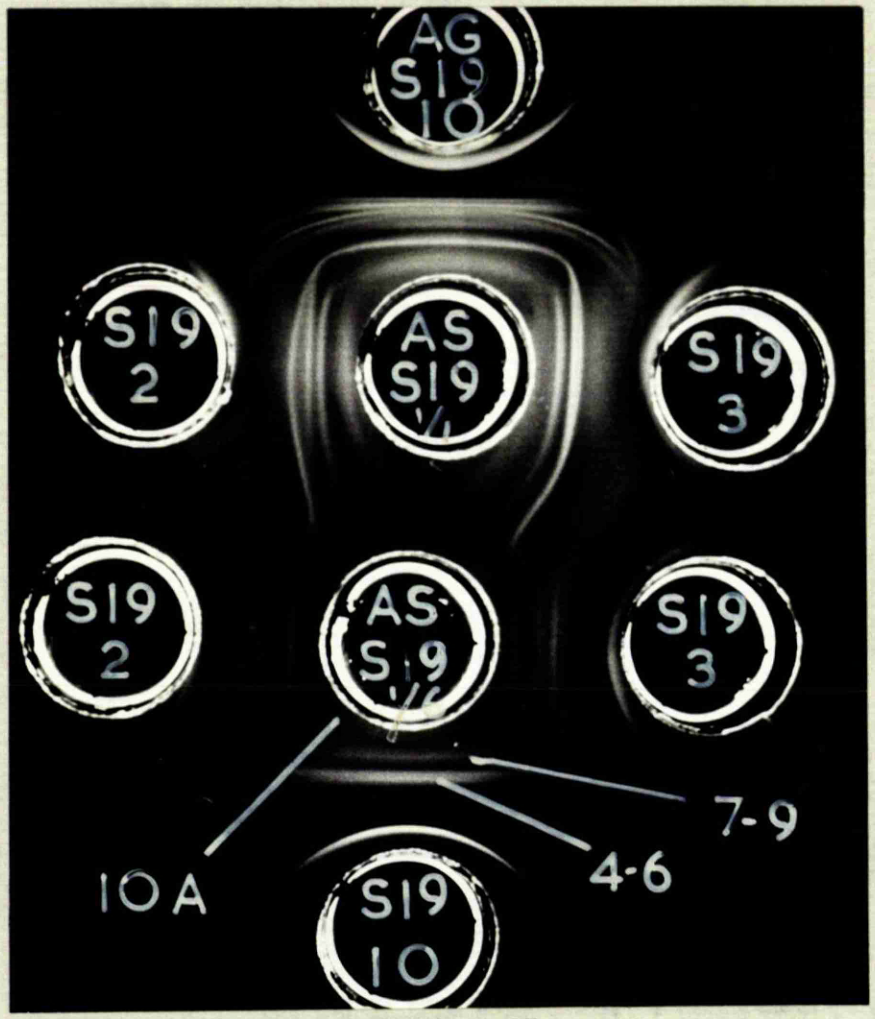


FIGURE 33.

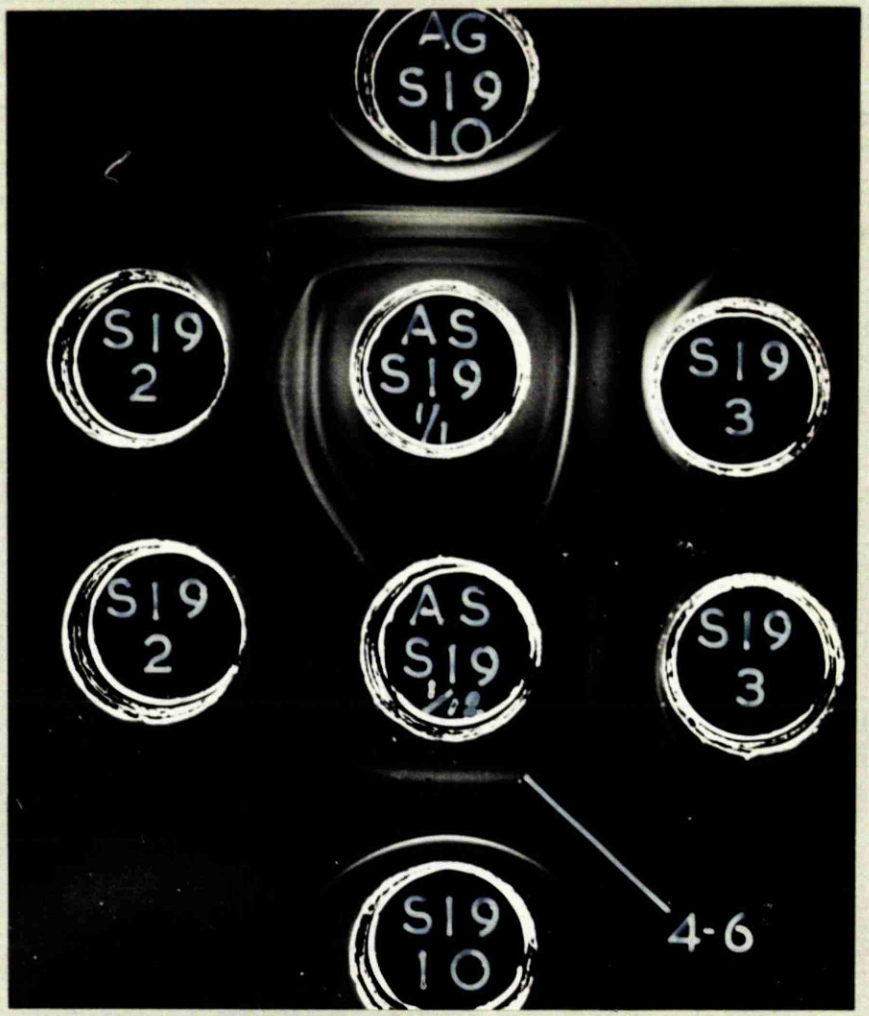


FIGURE 34.

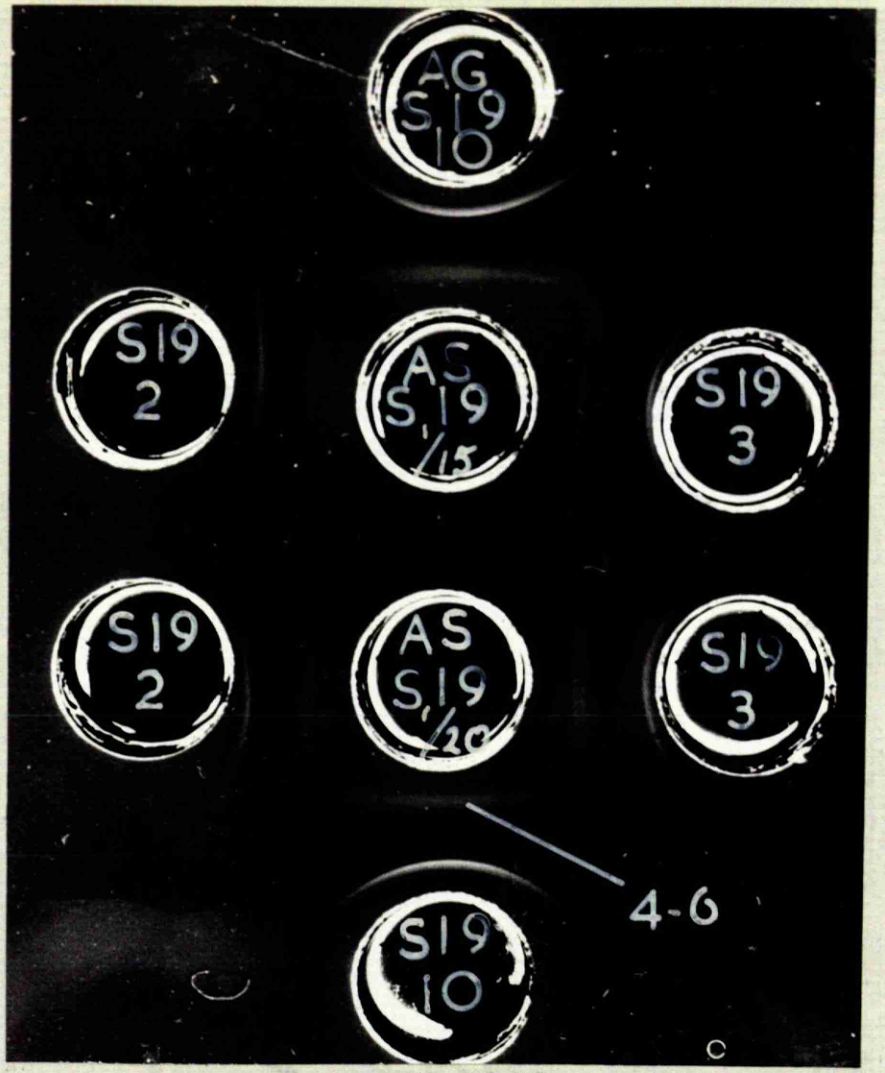


FIGURE 35.

FIGURES 29 - 35.

Titration of the precipitin content of bovine antiserum prepared against S19 Brucella abortus.

The upper half of each Figure (except 35) was used as control to facilitate identification of precipitin lines. Central reservoir contained 0.1 ml. of undiluted S19 antiserum, and the peripheric reservoirs were charged with graded quantities of S19 antigen as indicated.

The lower half of each Figure (except 35) served for the actual titration of S19 antiserum. The central reservoir contained dilutions of S19 antiserum in 0.1 ml. quantity. The peripheric wells were charged with graded quantities of S19 antigen as indicated. The titration of precipitins was carried out against the content of the lowermost peripheric wells and the two reservoirs next to it served as bridges to tie up precipitin lines to that of the control systems.

Arrows and figures denote particular lines of precipitate.

RESULTS

The results of the quantitative titration of precipitating antibody content of Brucella abortus S19 and type 1 antisera are illustrated by figures, whereas those of the other three types of brucella (types 2, 3 and 4) are tabulated in Table 36.

In general, the titre of a precipitating antibody was measured against 10 mg. of S19 antigen unless the demonstration of a precipitin line required the application of higher quantity of the antigen preparation. This applies only to the titration of No.2 and No.3 precipitins in S19 antiserum where the demonstration of the corresponding precipitin lines was only possible against 15 mg. of the antigen.

Figures 29 - 35 show the results of titration of Brucella abortus S19 antiserum. For the easier identification of the various precipitin lines the upper half of the plates (except for Figures 31 and 35) was used as control. The upper central well contained 0.1 ml. undiluted S19 antiserum and the 3 peripheric reservoirs received varying quantities of S19 antigen expected to suit best for the demonstrtion of particular precipitin lines.

The lower half of the plates served for the actual antibody titration. In the central well dilutions of S19 antiserum were delivered as shown in the Figures and the lowermost peripheric well contained the quantity of antigen against which the titration of antibody was carried out. The two peripheric wells on its immediate right and left served as bridges for the tying up of precipitin lines to those of the control system.

Figures 29 and 30 serve only to show the result of diluting the immune serum on the formation of No.2 and No.3 precipitin lines in the presence of 15 mg. of antigen. Precipitin line No.3 is the first of the two lines to fade out, it can be last observed as a hazy line at 1:2 serum dilution (Figure 29) whereas line No.3 is last seen at 1:6 dilution of the serum (Figure 30).

All the other precipitating antibodies were titrated against 10 mg. of antigen. Thus the upper half of Figure 31 shows all the precipitin lines but No.2 and 3, although lines No.10B and C are shown as a hazy band of precipitate in the presence of 10 mg. of the antigen. At 1:2 dilution of the serum (lower half of Figure 31) precipitin lines from No.7 to No.9 become a single line and were last seen as such at 1:8 serum dilution (Not shown in Figure).

Precipitin lines from No.4 to No.6 can be seen as individual bands at from 1:1 to 1:4 serum dilution (Figures 31 - 32). They fuse to form a single broad line at 1:6 and can be last observed as such at 1:30 dilution of the serum. The hazy band of precipitate representing No.10B and C and probably line No.11 can be last discerned at 1:2 serum dilution whereas line No.10A disappears at 1:6 dilution of the serum (Figure 33). The sickle shaped No.1 precipitin line was the most persistent of the precipitate bands in the S19 homologous system. It was last seen at 1:300 serum dilution.

In order to re-examine whether S19 antiserum reacted with antigen M of the type 4 Brucella abortus, 10 mg. of this antigen was applied against dilutions of S19 antiserum. To discourage the wastage of antibody, the serum containing reservoirs were spaced close together linearly, and flanked on both sides with antigen containing wells running parallel to them. The first of the serum containing wells received undiluted type 4 antiserum whereas the others contained dilutions of S19 antiserum. The results showed that, unlike in earlier experiments, a very hazy precipitin band developed as the continuation of line M of the type 4 homologous system. This hazy precipitin band disappeared at higher than 1:6 serum dilution (Not presented in figure).

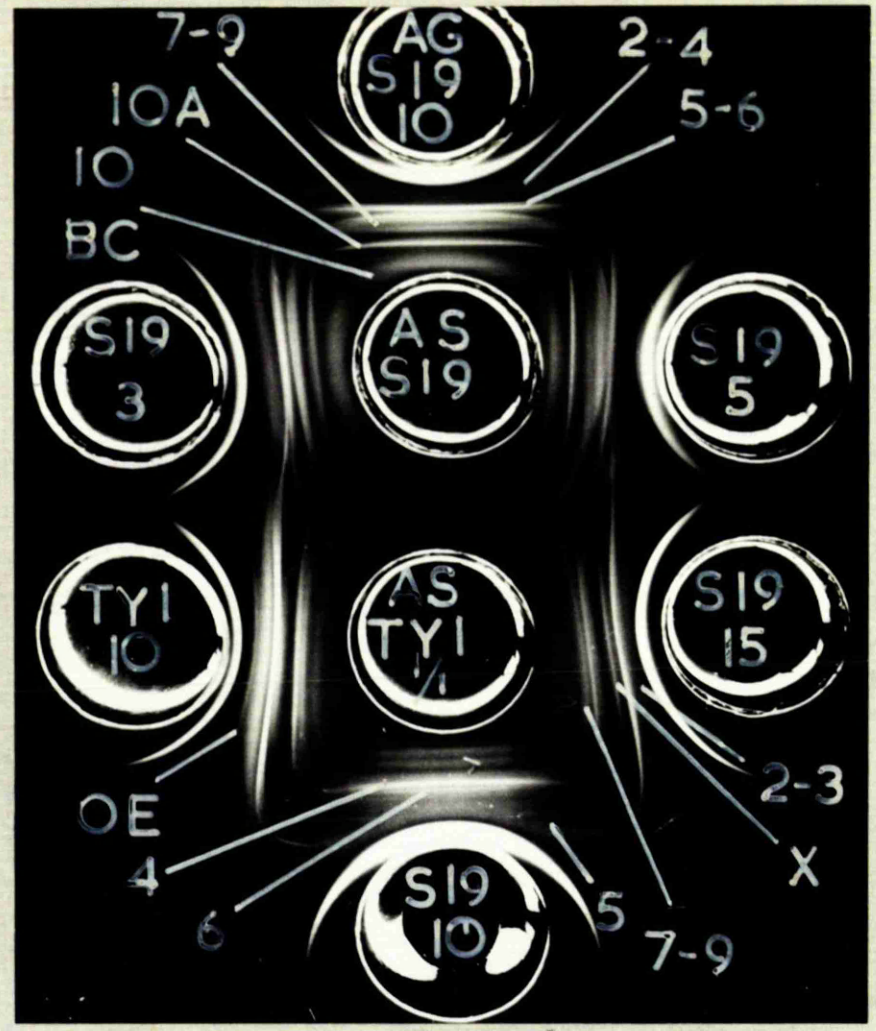


FIGURE 36.

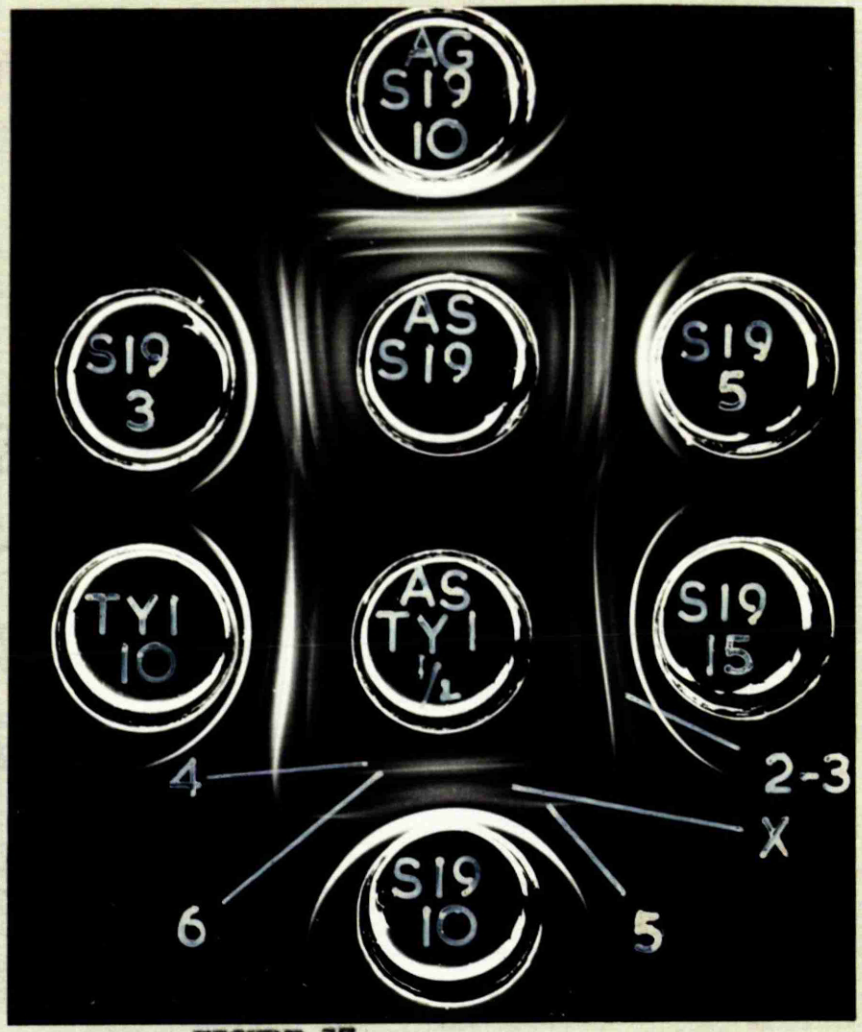


FIGURE 37.

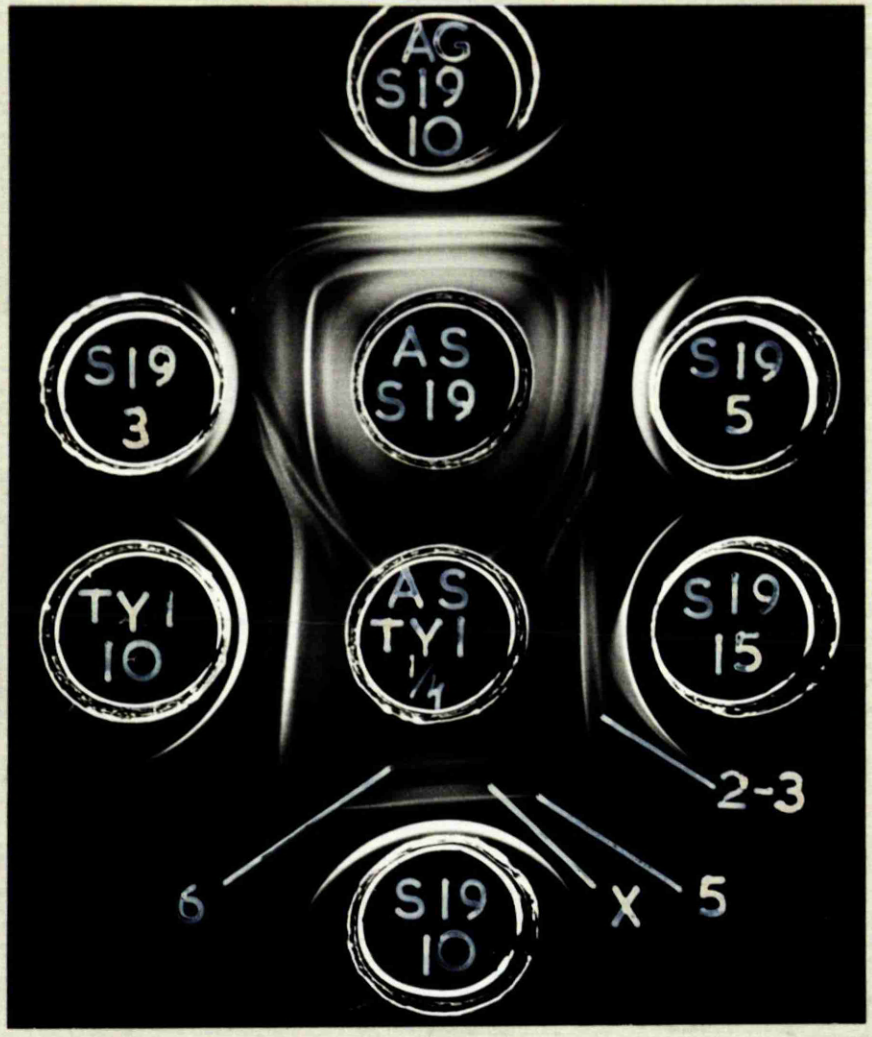


FIGURE 38.

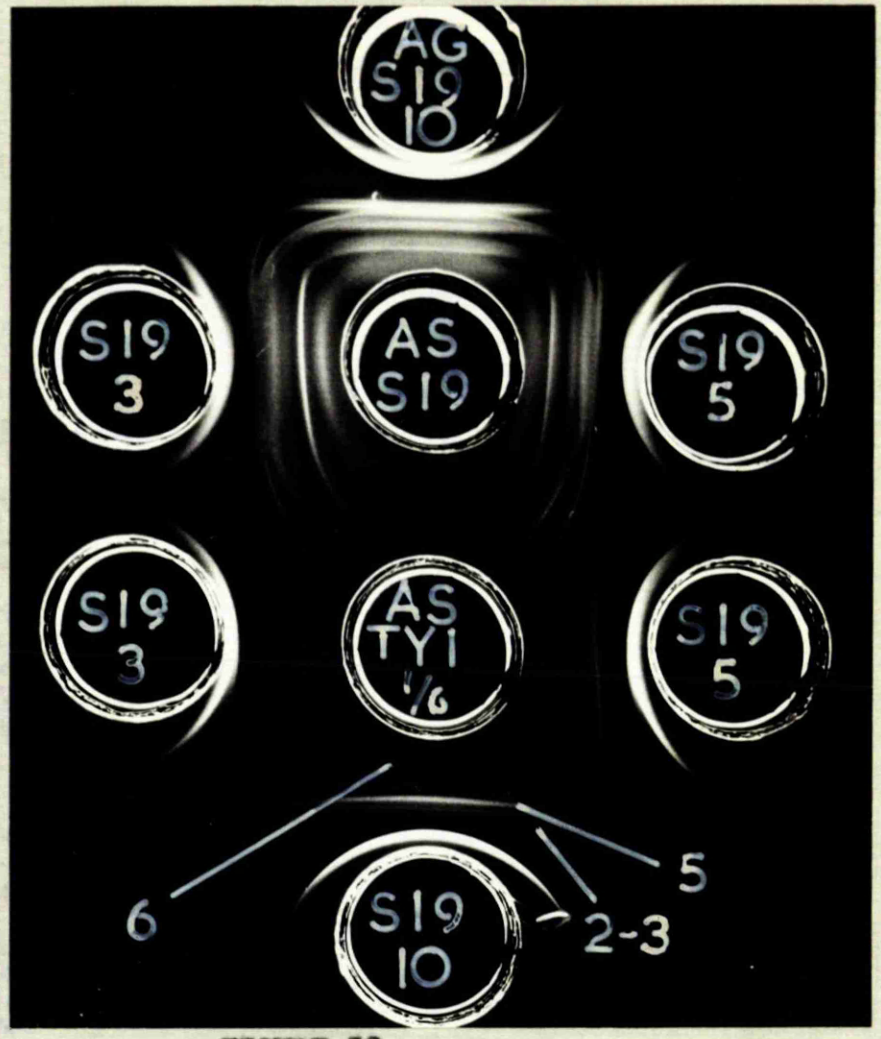


FIGURE 39.

FIGURE 40.

FIGURE 41.

FIGURES 36 - 41

Titration of precipitin content of bovine antiserum prepared against type 1 Brucella abortus.

The upper half of the figures was used as control to facilitate identification of precipitin lines. Central reservoir contained 0.1 ml. of undiluted S19 antiserum and the peripheric reservoirs were charged with graded quantities of S19 antigen as indicated.

The lower half of each figure served for the actual titration of type 1 antiserum, various dilutions of which an 0.1 ml. quantity was placed in the central reservoir as shown. The titration of precipitins was carried out against the content of the lowermost peripheric well (10 mg. of S19 antigen). The reservoir on its immediate right served as a bridge to tie up precipitate lines to that of the control systems. The reservoir on its immediate left was either charged with graded quantities of S19 antigen to serve as a bridge or was charged with 10 mg. of type 1 antigen to facilitate the titration of antibody with no corresponding antigen in S19 preparation.

Arrows, figures and capital letters denote particular lines of precipitate.

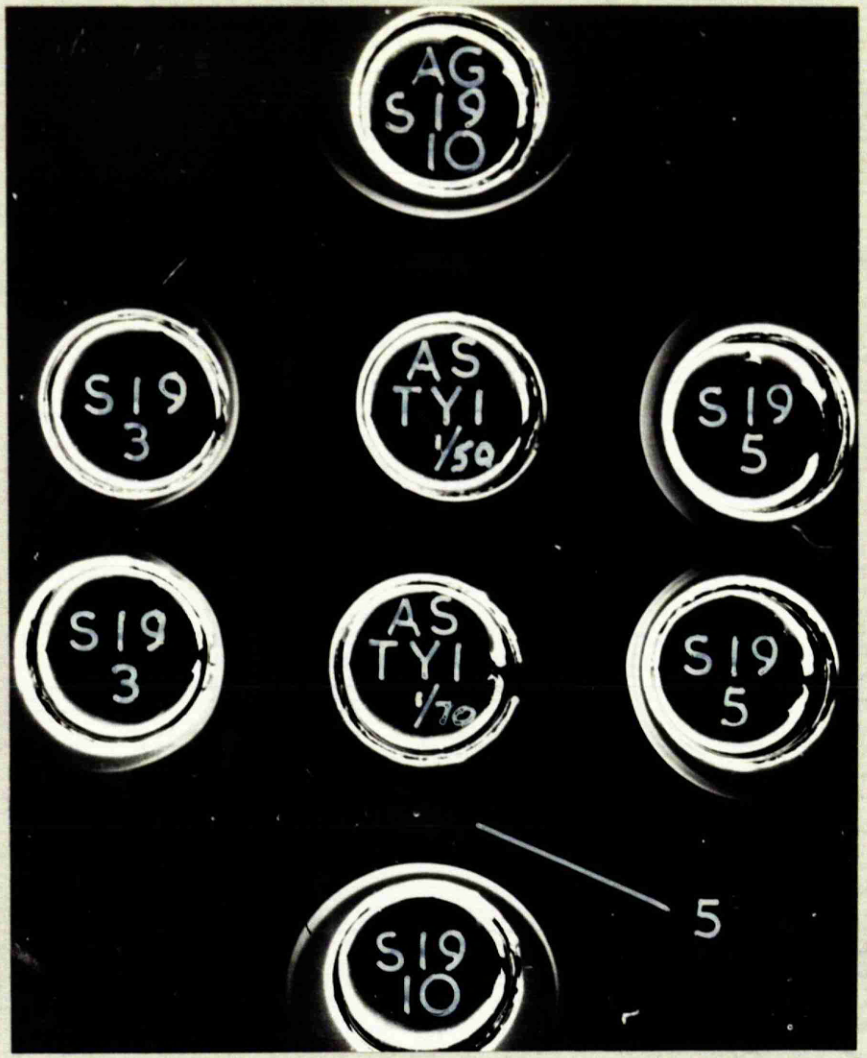


FIGURE 42.

FIGURE 43.

FIGURES 42 - 43.

Titration of precipitin content of bovine antiserum prepared against type 1 Brucella abortus.

Central reservoirs contained 0.1 ml. quantity of the dilution of type 1 antiserum as indicated.

Peripheric reservoirs contained graded quantities of S19 antigen as shown. The titration of precipitinswas carried out against 10 mg. of the antigen and the antigen in the other reservoirs served as bridges to tie up precipitin lines.

To illustrate the method of titrating precipitins in immune sera prepared against the virulent strains of Brucella abortus, the precipitin titration of Brucella abortus type 1 antiserum is presented in detail. Here again the upper half of the plates was used as control to facilitate the identification of the various precipitin lines (exceptions Figures 42 and 43).

The central reservoir in the lower half of the plate received dilutions of type 1 antiserum. The titration of the precipitins was carried out against 10 mg. of S19 antigen which was placed in the lowermost peripheric reservoir. The well on its immediate right was charged with S19 antigen to act as a bridge for tying up precipitin lines with those in the control system. The peripheric well on its immediate left received, in some case, 10 mg. of type 1 antigen to facilitate the titrations of antibody with no corresponding antigen in S19 preparation.

Taking the titration of the various precipitins in numerical order the one contributing to the formation of precipitin line No.1. was again the most persistent. It was diluted out at 1:1000 dilution of type 1 antiserum. This antiserum, as all the others was tested for its ability to react with antigen M of type 4 Br.abortus in identical

way to that used for S19 antiserum. The highest serum dilution at which it was found to form precipitin line with antigen M was 1:50. (Not shown in figures).

Lines No.2 and No.3 were represented by a single band of precipitate when type 1 antiserum reacted with S19 antigen. In Figures 36, 37 and 38 this compound line can be seen only in the presence of 15 mg. of antigen, but at from 1:6 to 1:10 dilution of the serum it also developes in the presence of 10 mg. of antigen. It was last observed at 1:10 serum dilution.

In the control S19 homologous system the precipitin line marked 2 - 4 (Figure 36) is a compound line which splits up into two partial lines in the presence of 5 mg. of antigen. Subsidiary experiments showed that the outer component of the two lines corresponded to line No.4, whereas the inner one corresponded to line No.2. Thus it will be seen that the outer component of line 2 - 4 of the S19 homologous system fuses with a precipitin line in the heterologous system thus identifying line No.4 therein (Figure 36) which is last seen at 1:2 serum dilution (Figure 37).

In the presence of undiluted type 1 immune serum, line No.5 is better seen at 15 mg. of S19 antigen (Figure 36) but

as the dilution of the serum increases line No.5 becomes well defined in the presence of 10 mg. of the antigen (Figure 36). It becomes hazy again at still higher serum dilutions (Figures 41 and 42), this time as a result of antigen excess, before it disappeared at 1:70 dilution of the serum.

Line No.6 in the heterologous system is formed just on the outside of line No.4 (Figures 36 and 37) and persists up to 1:6 serum dilution. It will be noticed, however, that at 1:6 serum dilution the diffuse line No.6 of the heterologous system is no longer the direct continuation of line No.6 of the S19 homologous system but the two lines form a spur. This phenomena was sometimes observed with the application of diluted heterologous serum against S19 antigen just before the disappearance of a particular precipitin line from the heterologous system.

Lines from No.7 to No.9 are represented as a single band of precipitate, and the 3 components (No.10A, B and C) of the compound line No.10 are represented by 2 lines (Figure 36). None of these lines (No. 7 - 10) can be seen at 1:2 serum dilution. The identity of a precipitate line marked X could not be established with certainty. In Figure 36

TABLE 36

RESULTS OF PRECIPITIN TITRATION OF VARIOUS BRUCELLA ABORTUS ANTISERA

Immune Serum	Antigen	Reciprocals of serum dilutions producing precipitate line No.														
		1	2	3	4	5	6	7	8	9	10 A	10 B	10 C	M+	OS++	IE++
Br.abortus S19	Br.abortus S19	300	6	2	(30)			(8)			6	2	2	6	-	-
do Ty.1	do	1000	(10x)		2	70	6	(1)			(1)			50	15	-
do Ty.2	do	250	(50)		8	8	(1)	1	1		-	-	-	8	-	-
do Ty.4	do	200	(200)		1	6	2	20	(10)		2	-	-	50	30	50
do Ty.5	do	200	(4)		1	30	2	-	-	-	1	1	1	50	-	-

\+ Antigen for titrating this precipitin was 10 mg. of ty.4 Brucella abortus.

++ " " " " " " 10 mg. of the homologous organism.

x Figures in brackets indicate compound line of precipitate.

it forms a well defined sharp line which may show the pattern of partial identity with line No.6 of the control system. It persists up to 1:4 serum dilution.

Precipitin line OE was only formed when the homologous antigen was applied against type 1 antiserum. It is best seen at 1:4 and last observed at 1:15 serum dilution.

The results of precipitin titration of antisera prepared against Brucella abortus type 2, 4 and 5 respectively are summarised in Table 36 together with those obtained for S19 and type 1 of Brucella abortus.

Taking Brucella abortus type 2 antiserum first, it exhibited No.2 and No.3 lines as distinct bands of precipitate against 10 mg. of S19 antigen up to 1:4 serum dilution, but at higher dilution than that the two lines fused to form a compound precipitin band. Lines No.6 - 7 and 8 - 9 were represented as single compound lines respectively even in the presence of undiluted type 2 antiserum, whereas those corresponding to line No.10A - C were not present at all. Nor was it possible to demonstrate line OE and IE even if 10 mg. of type 2 antigen was applied against its homologous antiserum.

Brucella abortus type 4 immune serum, reacting with 10 mg. of S19 antigen, formed line No.2 and line No.3 as distinct lines of precipitate up to 1:50 serum dilution when they fused to form a single compound line. Lines No.8 and No.9 fused to form a compound line at 1:2 serum dilution, whereas those corresponding to No.10 B-C were not present at all. Precipitins corresponding to lines N, OE and IE were titrated against 10 mg. of type 4 antigen.

Brucella abortus type 5 antiserum did not contain antibodies to form lines from No.7 to No.9, and those corresponding to precipitin bands No.2 and No.3 were represented by a single line of precipitate.

DISCUSSION

In the foregoing experiment an attempt was made to measure the titre of the various precipitins in immune (bovine) sera prepared against biotypes of Brucella abortus.

To obtain comparable results, the titration was carried out against standard quantity of S19 antigen and the use of any other antigen was only resorted to if a particular antigenic component was known to be either partially different or totally absent from S19 preparation.

By the use of the control system, the identification of precipitin lines was accomplished with comparative ease and certainty. Without the use of the control system this would not have been possible, for the position of precipitin lines changed in relation to each other, depending on the relative concentration of antibody in the various heterologous sera.

To obtain a titre for precipitins contributing to the formation of single lines of precipitate was not always achieved. It was a common occurrence that 2 or 3 distinct precipitin lines of the control system were directly continued

in a single line of precipitate even though the heterologous immune serum being titrated was undiluted. Alternatively, as a result of diluting the immune serum, some of the originally distinct lines of precipitate fused to form a single compound line before they finally disappeared. This alignment of several precipitin lines in the same plane made it impossible to obtain titres for many of the precipitins of the various antisera. Thus, with the exception of antibody contributing to the formation of line No.1, it is not possible to make any reliable deduction of the antigenicity of the various soluble antigens of Brucella abortus on the basis of antibody titre stimulated by them.

There were only 4 different types of precipitin, the titration of which was not the subject of interference. These included precipitins contributing to the formation of lines No.1, H, OE and IE.

The predominant precipitin in all the antisera formed line No.1 with its corresponding antigen. The value of its titre was comparable in all the sera including those prepared against the two melitensis type of Brucella abortus. It will be recalled that the corresponding antigen to precipitin No.1 could not be clearly demonstrated in type 4 or type 5 Br.abortus

when they were applied against S19 antiserum (see experiment No.4 and 5). This contradiction cannot be explained in terms of a simple antigen excess present in type 4 or type 5 preparation, for as little as 0.012 mg. of the acetone-dried cells of these two biotypes was applied against 0.1 ml. of undiluted S19 antiserum (see Experiment No.4) without producing precipitin line No.1.

To resolve the contradiction it is necessary to amend the postulation made about the nature of the antigenic molecule contributing to the formation of line M of a type 4 or type 5 homologous system. It was suggested in Experiment No.5. that this molecule possessed a single determinant group (b) in contrast to the corresponding molecule of S19, which had two determinants (ab). In order to make all the reactions of that antigenic molecule of type 4 or type 5 Brucella abortus explicable, it is necessary to postulate 2 different antigenic determinant groups on that molecule (mb). Furthermore, it need be assumed that only one of the two determinants with (m-) specificity is freely accessible to antibody in an in vitro serological reaction and the second determinant (-b) cannot take part freely in an in vitro reaction due to its situation on or in the antigenic molecule.

It becomes, however, unfolded in the animal body as a very potent antigen stimulating the formation of antibody which can react to a high titre with one of the determinants of No1 antigen molecule of S19 Brucella abortus.

On the basis of these suggestions it would be understandable how type 4 or type 5 antiserum can react with antigen No.1 of S19 to a high titre (1:200) but giving a lower titre (1:50) on reacting with the homologous antigenic molecule, where only determinant '-a' is freely accessible. Equally, it would become comprehensible why S19 antiserum having given an even higher titre (1:300) with the homologous No.1 molecule gave such a low titre (1:6) on forming precipitin line M with type 4 Brucella abortus.

Precipitin forming line OE with the corresponding antigen was detected only in type 1 and type 4 antisera giving a moderately high titre (1:15 and 1:30 respectively) whereas the antibody contributing to the formation of line IE was found only in type 4 antiserum. The absence of this antibody from type 1, 2 and 5 antisera (the corresponding antigen to this precipitin was shown to be present in these organisms, see Figure 28) would suggest that the antigen stimulating its formation is not strongly antigenic in every individual of the bovine species.

In the course of precipitin titration it was noted on occasions that a precipitin line, which gave a reaction of complete fusion (identity) with one of the lines in the control S19 system, formed a pattern of intersection (non-identity) with the same line on diluting the heterologous antiserum.

Crowle (1961) pointed out that this can happen if in one of two adjacent reservoirs (facing a pair of reservoirs containing antigen) antiserum is used at low enough concentration, so that in the area approaching the point of line juncture it cannot precipitate all the antigen diffusing against it, and this reactant penetrates to precipitate antibody diffusing from the more concentrated source of serum producing a spur of precipitate.

EXPERIMENT NO. 7.

ELECTROPHORETIC STUDIES ON THE SOLUBLE EXTRACTS OF VARIOUS BIOTYPES OF BRUCELLA ABORTUS.

Having found an antigenic substance shared by all the virulent strains of Brucella abortus examined but missing from the antigenic structure of the vaccine strain, the next objective of the work was the isolation of this extra antigen (IE) of the virulent strains. It was hoped that a relatively pure preparation of antigen IE may be prepared which might be a basis of a serological technique capable of differentiating vaccinal titres from those caused by superimposed natural infection.

For the fractionation of the soluble components of brucella a method of high resolving power was necessary. Zone electrophoresis in starch gel was shown by Smithies (1955) a very powerful analytical tool for the fractionation and direct visual comparison of human serum components. Furthermore, the removal of protein fractions from the starch gel could be efficiently achieved by freezing and slow speed centrifugation of the gel.

Factors affecting zone electrophoresis, particularly in semisolid media include buffer pH, ionicity, composition of buffer, electric current and voltage, the charge of supporting medium, temperature and the nature of the substance being electrophoresed. It was pointed out by Growie (1961), however, that the knowledge of the mechanism of electrophoresis in semi-solid media is so incomplete that how to electrophorese a given substance must be largely determined by trial and error experimentation. Thus the aim of this experiment is to find a suitable electrolyte system for the efficient electrophoretic fractionation of the soluble components of various Brucella abortus strains.

The electrophoretic system to be adopted should be efficient for the segregation of antigen IE in relatively pure form. Finally it should be feasible to locate and elute the electrophoretic fractions from the gel for additional studies.

MATERIALS AND METHODS

Antigens for electrophoresis

From the acetone-dried cells of five biotypes of Brucella abortus (Types 1-2, 4-5, and S19) soluble antigens were prepared as described in Experiment No.4. After heat-treatment of the cell suspension it was centrifuged at 12000 gs. for 60 minutes at 10°C and the greenish-yellow opalescent supernatant (Brucella extract) collected for use in electrophoresis.

Preparation of starch gel

12 gm. of starch-hydrolysed (Connaught Medical Research Laboratories, Toronto, Canada) was suspended per 100 ml. buffer solution. The suspension was heated over a naked flame until it became a viscous liquid. Continual mixing by swirling was carried out during the whole period of heating. Degassing followed by the application of negative pressure on the liquid, then it was poured into a suitable tray, covered with a sheet of Melinex (Plastic and Organic Chemical Dep., I.C.I. Ltd., Glasgow) to prevent drying and allowed to set at room temperature.

Preparation of Acrylamide gel

7 gm. of Cynogum 41 (B.D.H. Laboratory Chemical Division, Poole, England) was dissolved per 96 ml. of TRIS-Citrate buffer (pH 8.75; Mol. 0.0808) to which 2 ml. of a 10% aqueous solution of ammonium ethyl cyamid and 2 ml. of a 10% aqueous solution of ammonium-peroulphate was added in this order to act as catalysts for gelling, immediately prior to the evacuation of the gel. Degassing followed by the application of negative pressure on the solution which was then poured into a suitable tray and covered with an airtight lid, care being taken not to trap any air bubbles under the lid. The lid itself was fitted with a number of P.V.C. projections one third down on its length (6 cm). Each of the projections measured 10 x 6 x .5 mm. to form slits in the gel to receive the bacterial extract. The thickness of the gel was 7 mm. so that the reservoirs did not penetrate right through the gel.

Electrolyte systems

Various combinations with varying values of pH and of molarity were employed as shown in Table 37.

TABLE 37

ELECTROLYTE SYSTEMS EMPLOYED IN THE ELECTROPHORESIS OF SOLUBLE EXTRACTS OF BRUCELLA ABORTUS

ELECTROLYTE					
in GEL	pH value	molarity	in TANK	pH value	molarity
Phosphate[1]	8.7	.0383	Phosphate	8.70	.0383
do	7.-	.0382	do	7.-	.0382
do	6.2	.0370	do	6.20	.0370
TRIS-Citrate[2]	8.75	.0398	TRIS-Citrate	8.75	.0398
do	7.-	.0387	do	7.-	.0387
do	6.-	.0379	do	6.-	.0379
Borate[3]	9.0	.0383	Borate	9.-	.0383
do	6.15	.0377	do	6.15	.0377
Barbitone acetate[4]	8.60	.0368	Barbitone acetate	8.60	.0368
do	8.60	.0368	Phosphate	9.10	.401
do	8.60	.0368	Borate	9.-	.383
do	8.60	.0368	TRIS-Citrate	8.90	.404
TRIS-Citrate	8.90	.0404	Phosphate	9.10	.401
do	8.90	.0404	Borate	9.-	.383
do	8.90	.0404	Barbitone acetate	8.60	.0736
Borate	9.00	.0383	Phosphate	9.10	.401
do	9.00	.0383	TRIS-Citrate	8.90	.404
do	9.00	.0383	Barbitone acetate	8.60	.0736
Phosphate	9.10	.0401	Borate	9.-	.386
do	9.10	.0401	Barbitone acetate	8.60	.0736
do	9.10	.0401	TRIS-Citrate	8.90	.404

1. ($Na_2 HPO_4$ - KH_2PO_4)
2. (TRIS-citric acid)
3. (Boric acid - NaoH)
4. (OXOID Barbitone-acetate buffer)

Electrophoresis

Electrophoresis of the various bacterial extracts was carried out in a VOKAM 2541 electrophoretic apparatus (Shandon Scientific Co. Ltd. London) at constant voltage, applying a potential difference of 2.5 V drop per cm. of the gel as measured between the wicks. Electrical contact between the ends of the gel and the electrode compartments was made by means of absorbent lint soaked in buffer. Two and six hours of electrophoresis was applied to the extract in the starch and cynogum gels respectively. The electrolyte in the tank was cooled by running tap water in the outer jacket.

The application of bacterial extracts in starch gel was effected by the insertion of Wattman's filter paper, impregnated with the extract, into slits cut in the gel. In the case of the cynogum gel the bacterial extract was applied directly into the reservoir by the aid of a capillary Pasteur pipette. Both the starch and cynogum gels were covered with a sheet of Melinex during electrophoresis to prevent drying. The cynogum gel was cooled during electrophoresis by the aid of a hair dryer blowing cold air onto the Melinex covered gel. After the termination of electrophoresis the gels were sliced horizontally (a very this steel wire was used for the slicing of cynogum gel) and stained.

Staining technique

Staining of starch and cynoglm gels was done with naphthalin black (5% acetic acid saturated with naphthalin black). The stain was applied for approximately 2 minutes to the gels, then decolourised in several changes of the solvent. (5% acetic acid).

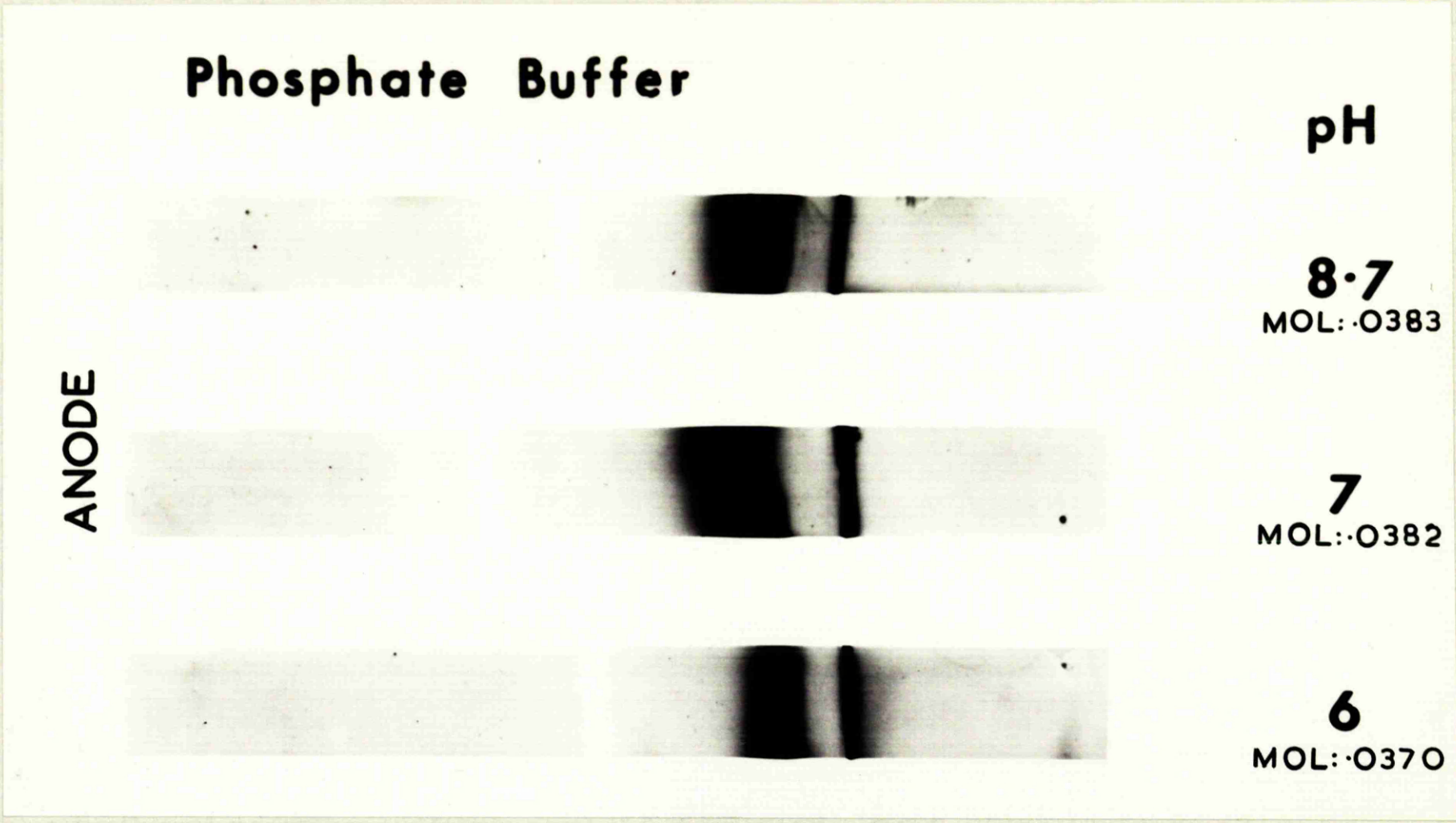


FIGURE 44.

Starch gel electrophoresis of soluble extract of S19 Brucella abortus in a continuous phosphate buffer system of various pH values. Field strength of 2.5 V/cm. gel (constant voltage) was applied for two hours. The gel was stained in naphthalene black.

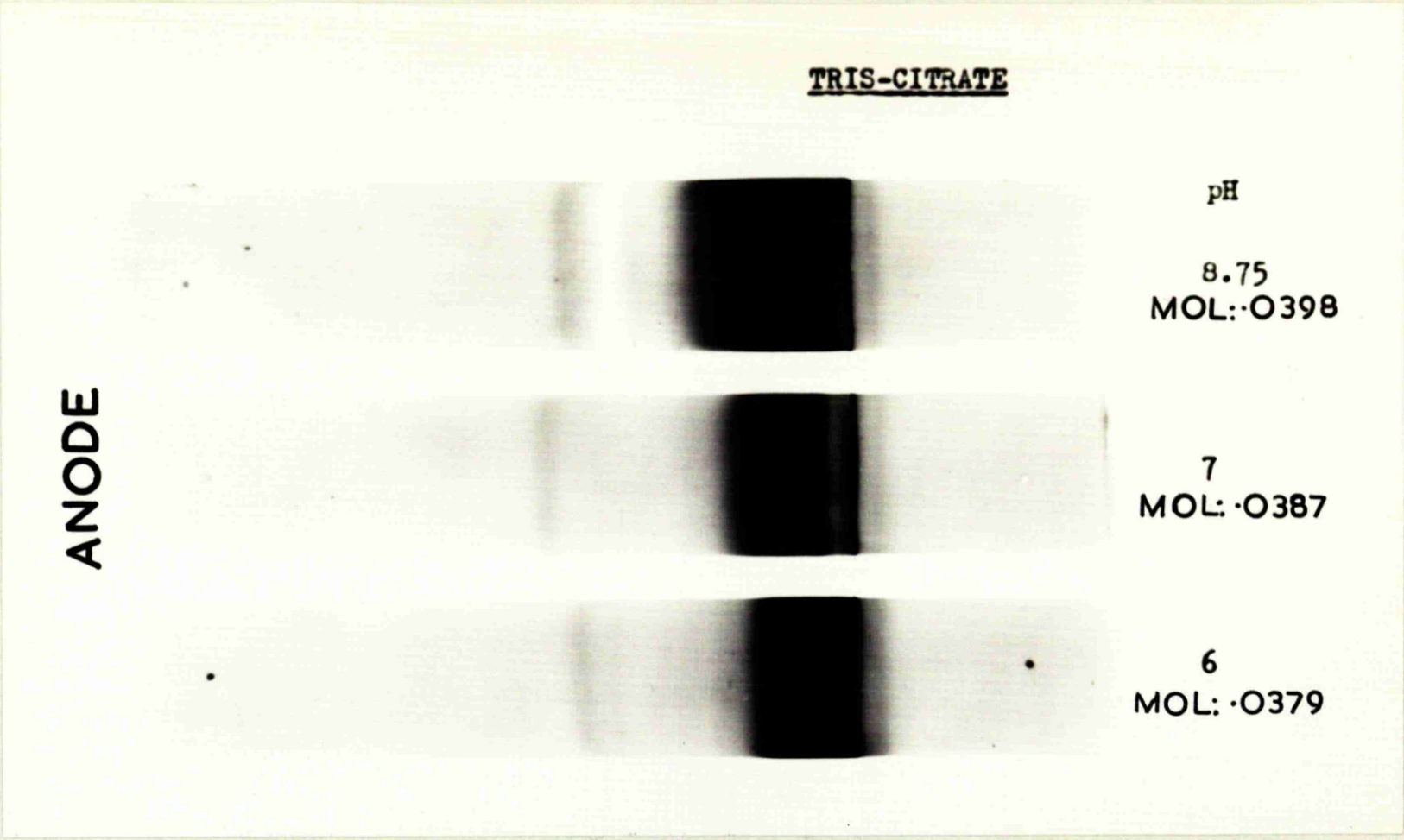


FIGURE 45.

Starch gel electrophoresis of soluble extract of S19 Brucella abortus in a continuous TRIS-citrate buffer system of various pH values. Field strength of 2.5 V/cm. gel (constant voltage) was applied for two hours. The gel was stained in naphthalene black.

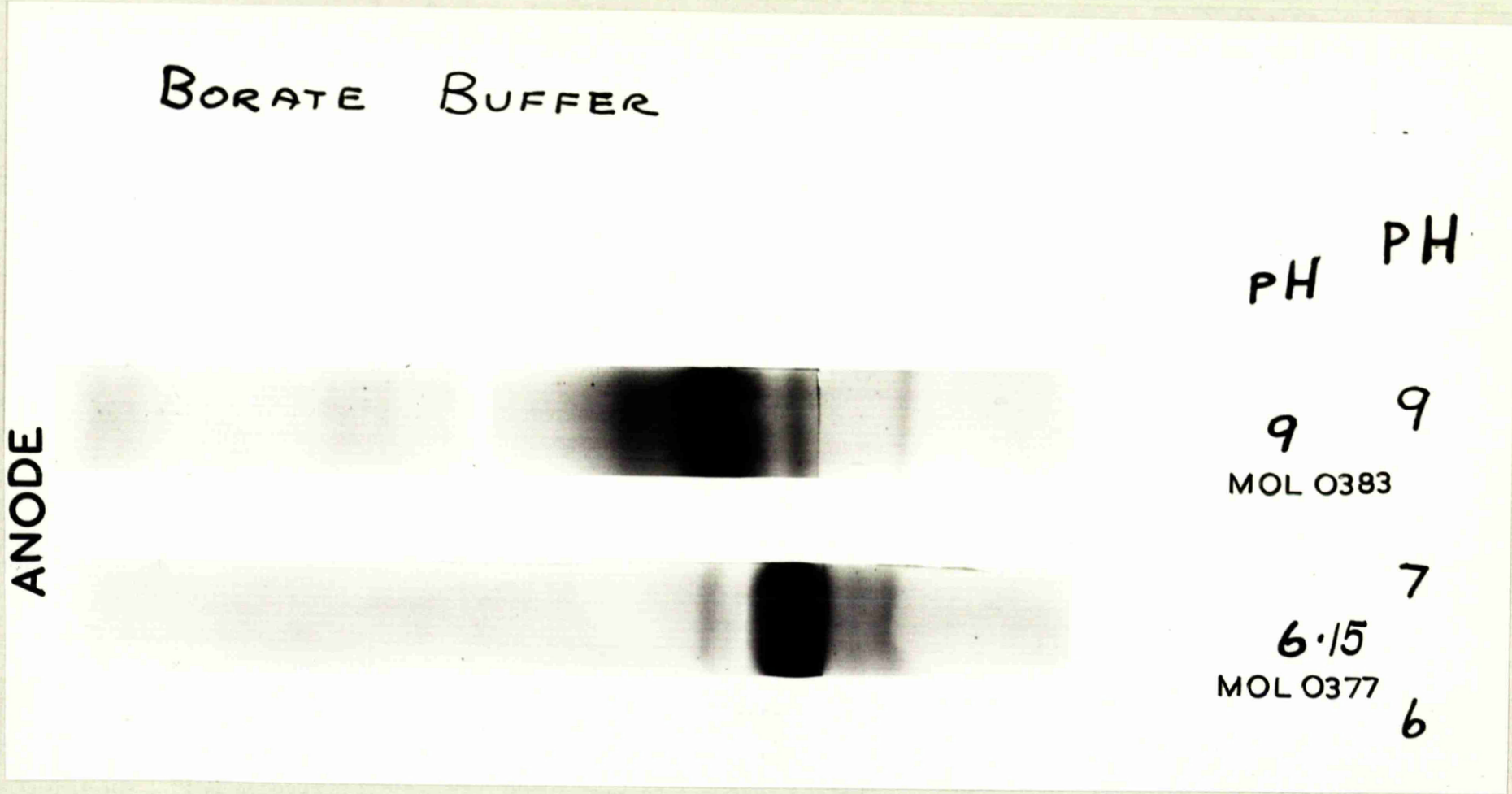


FIGURE 46.

Starch gel electrophoresis of soluble extract of S19 Brucella abortus in a continuous borate buffer system of various pH values. Field strength of 2.5 V/cm. (constant voltage) was applied for two hours. The gel was stained in naphthalene black.

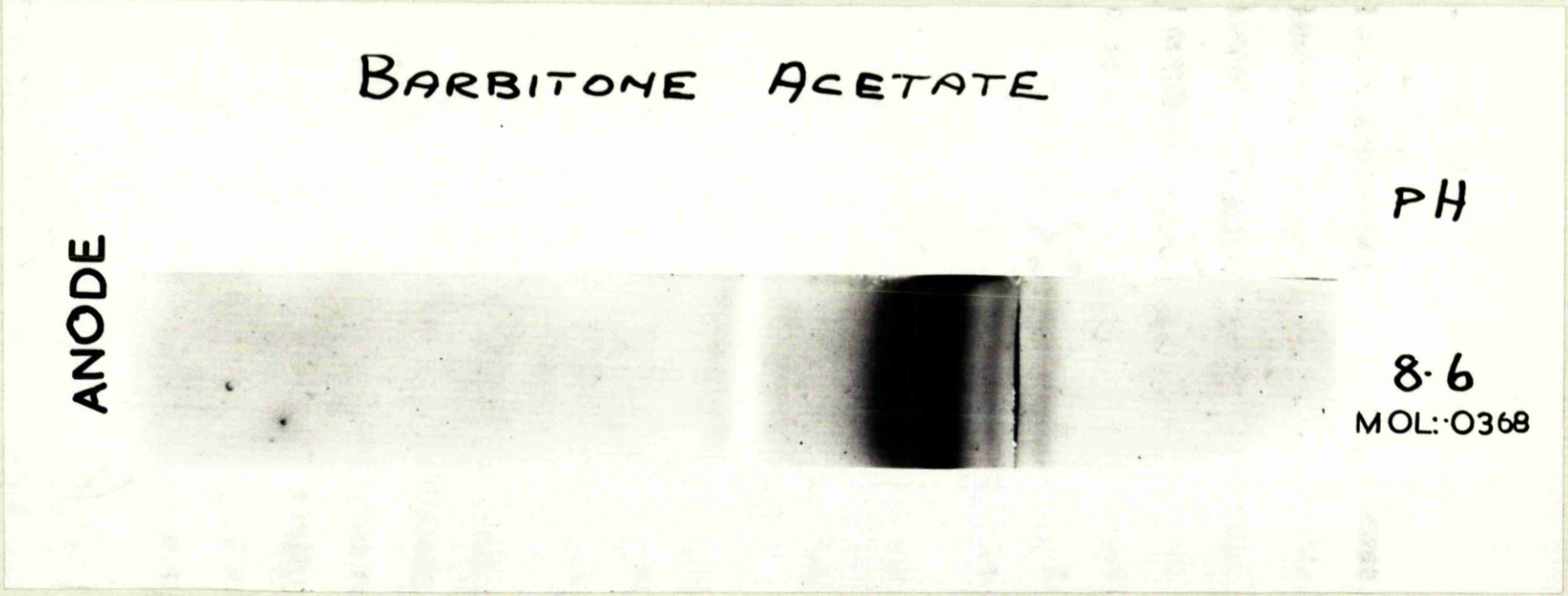


FIGURE 47.

Starch gel electrophoresis of soluble extract of S19 Brucella abortus in a continuous barbitone acetate buffer system. Field strength of 2.5 V/cm. gel (constant voltage) was applied for two hours. The gel was stained in naphthalene black.

RESULTS

In search of a suitable electrolyte system for the electrophoretic separation of the soluble compounds of Brucella abortus the supporting medium of electrophoresis was starch gel and the brucella extract employed was that of S19. These experiments were carried out by using constant voltage at a field strength of 2.5 V/cm. and electrophoreis lasted for 2 hours.

Figures 44 - 47 show the outcome of electrophoreeis using phosphate (44), TRIS-Citrate (45), borate (46) and barbitone acetate (47) buffer respectively in continuous systems, i.e. the electrolyte in the starch gel was identical to that used in the tank in every respect including its pH value as well as its molarity.

In order to obtain information about the effect of the pH of electrolyte on the electrophoretic separation of the various components, the pH of the phosphate (Figure 44) and TRIS-citrate (Figure 45) buffer was adjusted to give readings in the acid, neutral and alkaline regions respectively, at the same time maintaining their molarity comparable.

It will be seen in Figures 44 - 45 that the electrophoretic separation was poorest in an acid medium and that

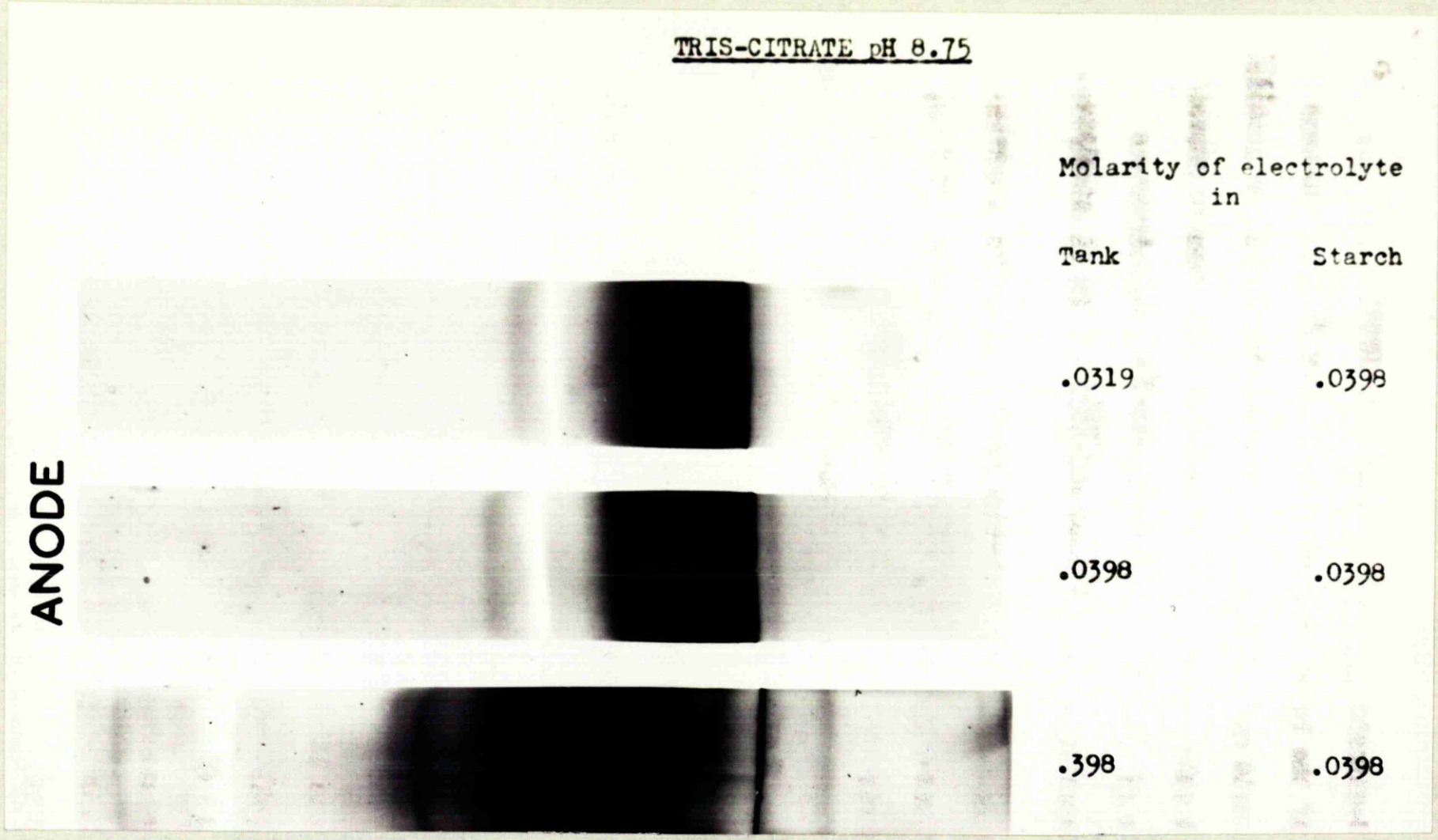


FIGURE 48.

Starch gel electrophoresis of soluble extract of S19 Brucella abortus in a continuous TRIS-Citrate buffer system. The molarity of tank buffer was altered as indicated. Field strength of 2.5 V/cm. gel (constant voltage) was applied for two hours. The gel was stained in naphthalane black.

the TRIS-citrate buffer gave somewhat superior results. The pH of the borate buffer was adjusted to give readings in both acid and alkaline regions, whereas barbitone acetate was used only in the alkaline region. The results showed (Figures 46 and 47) that electrophoretic separation was again superior in an alkaline solution of the electrolyte.

As the electrophoretic resolution of the brucella extract was very poor in all of these electrolyte systems, experiments were carried out to establish whether or not variations in the molarity of electrolytes as used in the gel and in the tank might bring about some improvement.

Figure 48 shows the results of such an experiment. Continuous TRIS-citrate buffer system was used at pH 8.75, field strength 2.5 V/cm., electrophoresis lasted for 2 hours. In the first instance the molarity of tank buffer was slightly lower than that of the buffer in the starch, then it was used in identical concentration and finally the tank buffer was 10 times more concentrated than was the buffer in the gel. All the results, including the results shown in Figure 48, indicated that the application of a buffer more concentrated in the tank than in the starch gel enhanced the rate of migration of the components, without showing a substantial improvement in resolution. Measurements taken

on the field strength during electrophoresis showed that when the molarity of electrolyte in both tank and starch was of comparable value, a drop in field strength occurred amounting to as much as 20% of the original value (from 2.5 V/cm. to 2 V/cm). When the tank buffer was ten times more concentrated than the buffer in the starch , no drop in field strength was observed. This would probably explain the somewhat better rate of migration of components in an electrolyte system where the tank buffer is considerably more concentrated.

The degree of resolution of the electrophoretically active substances of brucella, however, was rather poor in all of these continuous electrolyte systems irrespective of their pH values or molarity.

To improve electrophoretic resolution an attempt was made to combine the various buffers in discontinuous systems by applying a different buffer in the starch to that used in the tank.

The brucella extract used in these experiments was again that of S19. Electrophoresis was continued for 2 hours at a field strength of 2.5 V/cm. The molarity of electrolytes in the starch was approximately 1/10th of that used in the tank. The pH value of all the electrolytes

DISCONTINUOUS SYSTEM.

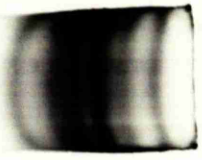
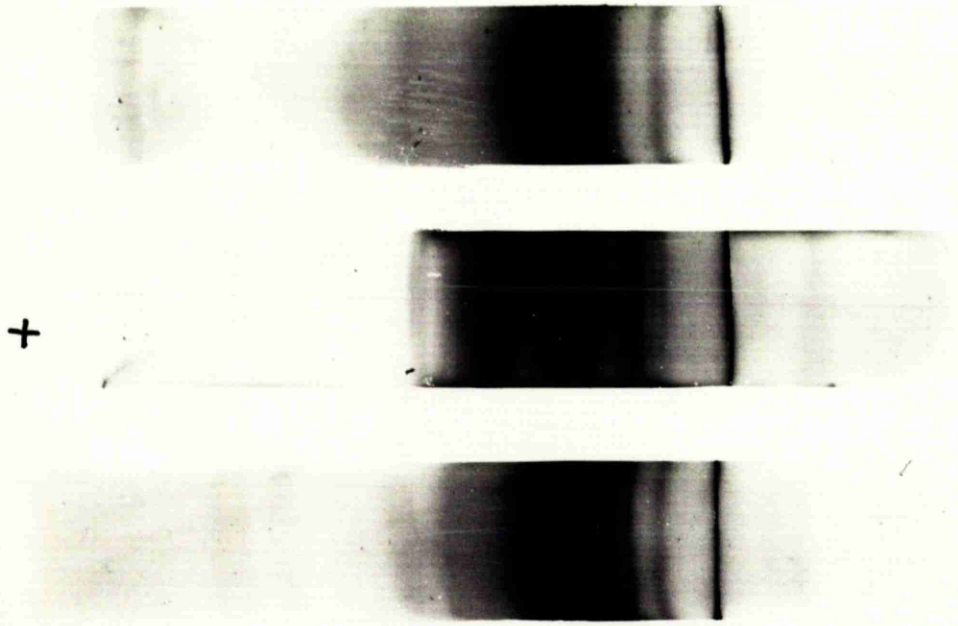
	Electrolyte in STARCH	Electrolyte in TANK
	Barbitone Acetate (pH 8.6, Mol. .0368)	Phosphate (pH 9.1, Mol. .401)
+ 	do.	Borate (pH 8.9, Mol. .383)
	do.	TRIS-Citrate (pH 8.9, Mol. .404)

FIGURE 49.

Starch gel electrophoresis of soluble extracts of S19 Brucella abortus in discontinuous buffer systems. Field strength of 2.5 V/cm. gel (constant voltage) was applied for two hours. The gel was stained in naphthalene black.

DISCONTINUOUS SYSTEM.

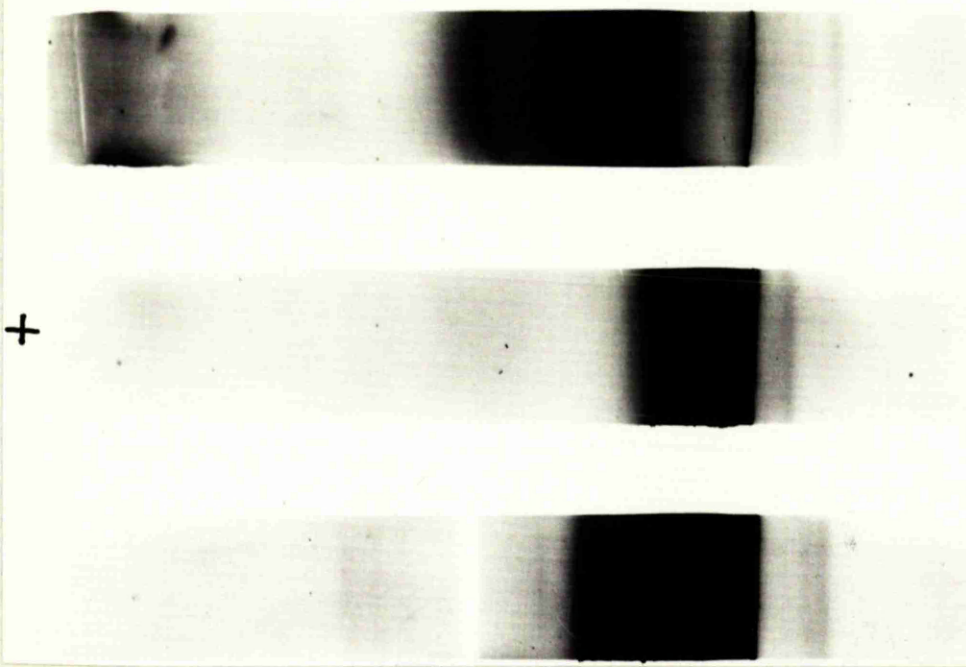


	Electrolyte in STARCH	Electrolyte in TANK
	TRIS-Citrate (pH.8.9,Mol..0404)	Phosphate (pH.9.1,Mol..401)
+	do.	Borate (pH.8.9,Mol..383)
	do.	Barbitone-Acetate (pH.8.6,Mol..0736)

FIGURE 50.

Starch gel electrophoresis of soluble extracts of S19 *Brucella abortus* in discontinuous buffer systems. Field strength of 2.5 V/cm. gel (constant voltage) was applied for two hours. The gel was stained in naphthalene black.

DISCONTINUOUS SYSTEM .

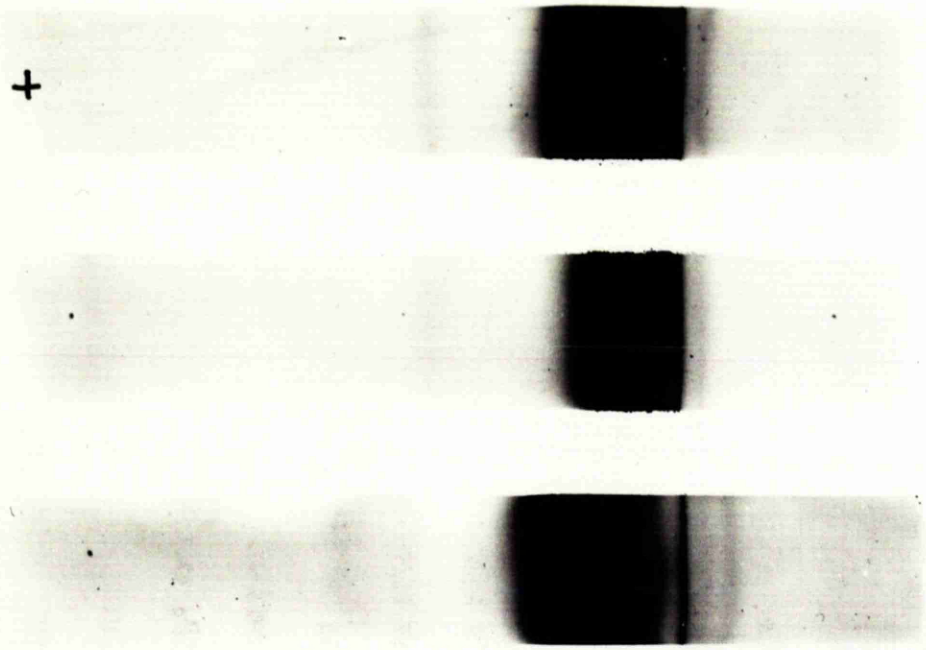


	Electrolyte in STARCH	Electrolyte in TANK
	Borate pH.8.9,Mol..0383)	Phosphate (pH.9.1,Mol..401)
+	do.	TRIS-Citrate (pH.8.9Mol..404)
	do.	Barbitone-Acetate (pH.8.6,Mol..0736)

FIGURE 51.

Starch gel electrophoresis of soluble extracts of S19 Brucella abortus in discontinuous buffer systems. Field strength of 2.5 V/cm. gel (constant voltage) was applied for two hours. The gel was stained in naphthalene black.

DISCONTINUOUS SYSTEM.



	Electrolyte in STARCH	Electrolyte in TANK
+	Phosphate (pH.9.1,Mol..0401)	Borate (pH.8.9,Mol..383)
	do.	TRIS-Citrate (pH.8.9,Mol..404)
	do.	Barbitone-Acetate (pH.8.6,Mol..0736)

FIGURE 52.

Starch gel electrophoresis of soluble extracts of S19 Brucella abortus in discontinuous buffer systems. Field strength of 2.5 V/cm. gel (constant voltage) was applied for two hours. The gel was stained in naphthalene black.

used was between pH 8.6 - 9.1 and the molarity of the various electrolytes was adjusted to approximately the same value. As the molarity of a buffer is not in close correlation with its ionic strength, i.e. conductivity, readings were taken of the passing current at the beginning and end of electrophoresis.

The results of these experiments are presented in four figures (Figures 49 - 52).

It will be seen in Figure 52 that the poorest resolutions, in general, were obtained with the use of phosphate buffer in the starch gel irrespective of the quality of electrolyte in the tank. Indeed, the use of electrolytes other than phosphate in the tank resulted in no improvement of resolution compared to those seen in a continuous phosphate buffer system (Figure 44). The best results, on the other hand, were seen when TRIS-citrate buffer was used in the starch gel in various combinations with other electrolytes in the tank (Figure 50). The best resolution of the various components of brucella was seen in a TRIS-citrate (gel) - borate discontinuous system. (Figure 50, central portion). Not only was the resolution of the various components the best in this sytem but their definition (sharpness of the line) was good and their spacing

TABLE 38

CONDUCTIVITY OF VARIOUS ELECTROLYTE SYSTEMS IN STARCH GEL ELECTROPHORESIS

ELECTROLYTE		Milliamper readings at	
in STARCH	in TANK	0. hour	2 hours
Phosphate*	Borate*	21	22
do	TRIS-citrate	21	22
do	Barbitone-acetate	26	30
Borate	Phosphate	14	30
do	TRIS-citrate	10	9
do	Barbitone-acetate	11	13
TRIS-citrate	Phosphate	14	24
do	Borate	10	5
do	Barbitone-acetate	11	11
Barbitone-acetate	Phosphate	>50	>50
do	Borate	34	40
do	TRIS-citrate	22	22

* For pH values and molarity of buffers see Table 22.

arrangement in relation to each other was the most even. When this same discontinuous electrolyte system was used in a reversed manner, i.e. using borate buffer in the gel and TRIS-citrate in the tank (middle section of Figure 51) the result was much more inferior in every respect.

To show that the improvement of resolution in the TRIS-citrate-borate system was not due to its superior conductivity (ionicity) compared to the conductivity of other systems, the milliamper readings of all the discontinuous electrolyte systems are given in Table 38, as taken at the beginning (0 hour) and at the end (2 hours) of electrophoresis. In all of these experiments, apart from the slight differences in pH values and molarity of the various electrolytes, the variable was the quality of the electrolytes used.

When the current carrying capacity of the various electrolyte systems is compared to the degree of electrophoretic resolution obtained by them, the lack of correlation between the two is quite apparent.

Thus the TRIS-citrate (in starch) borate discontinuous buffer system was adopted for use. The lowering of the pH value of borate buffer to pH 8.0 - 8.2 (mol. 0.349) resulted in slight improvement of resolution of the various

components. The prolongation of electrophoresis beyond three hours resulted in a pattern where the components were separated by slightly larger distances from each other and resolution improved in some extent, but the definition of the lines became increasingly poorer.

When soluble extracts of the 5 biotypes of Brucella abortus were applied side by side in the starch gel and exposed to electrophoresis at a field strength of 2.5 V/cm. for 6 hours using a TRIS-citrate borate buffer system no definite difference in the electrophoretic patterns of the various strains were noted.

In attempts to identify the component in the electrophoretic pattern responsible for the formation of line III in the gel-diffusion plates, experiments were carried out to locate the various electrophoretic fractions in the unstained gel. After the completion of electrophoresis, the gel was horizontally cut into two halves. One half of the gel was stained and decolourised while the second half was kept at $4^{\circ}C$ in the refrigerator. Although by frequent changes of the decolouriser the gel could be freed of non-specifically combined stain within a few hours, the uneven shrinkage of the starch gel in

the stain and decolouriser made calculation unreliable as to the location of a particular component in the unstained half of the gel.

In the course of these experiments it became increasingly apparent that starch gel electrophoresis of the extracts of brucella was not good enough, either for the comparison of electrophoretic patterns of the various strains, or for the location and elution of antigen from the electrophoretically distinct components. A supporting medium for electrophoresis was required which would ensure better resolution of the various fractions of brucella than those seen in starch gel but would not shrink in the process of staining. The choice fell on acrylamide gel which did not shrink at all in 5% acetic acid. During the preliminary trials with the use of this medium, experiments were carried out to determine optimal concentration of cynogum 41 as gelling agent. This included the use of cynogum 41 at from 3, 5, 7% etc. up to 15% concentration.

It was found that at 3% concentration of cynogum in the gel not only was it difficult to handle (sticky, cannot be cut) but the resolution of the components as well as the definition of the individual lines was very poor

(extract of S19 was resolved only into 6 fractions conglomerating just behind the leading fraction). This observation was also true in a lesser degree of the results obtained with the use of a 5% cynogum gel. When the concentration of cynogum 41 was increased over 7% two of the electrophoretic fractions, which were present in 5 and 7% gels, were not detectable. Thus the use of a 7% cynogum gel was adopted for use.

In addition, to the concentration of cynogum 41 in the gel, it was found that the width of the reservoir receiving the brucella extract was critical. The use of reservoirs wider than 0.5 mm resulted in heavy trailing. This could be overcome by mixing the extract with 2% molten agar cooled to approximately 50°C and applying the mixture in the reservoir while still fluid. By this means approximately twice as wide reservoirs (approximately 1 mm) could be used, although the quantity of extract applied could not be materially increased. Thus in later work reservoirs of 0.5 mm. in diameter were used and charged with the brucella extract in solution.

The thickness of the gel was found to be also critical. A maximum thickness of 7 mm. could be used provided the gel was cooled by the constant flow of cold

air over the melinex sheet covered gel. This was achieved by the use of an electric hair dryer.

Trials were carried out to establish the optimal voltage for electrophoresis. In these experiments the molarity of gel buffer was 0.0404 and 0.0908 respectively. Better results were obtained by the use of the more concentrated gel electrolyte (0.0908) even at identical field strength. Although the rate of fraction movement slowed down a little and somewhat more heat generated at the higher ionic strength of gel electrolyte, this was offset by better resolution and definition of the components. The voltage reading on the dial of the power source was very mis-leading. A potential difference of 160 V. on the dial dropped to 32.5 V. as measured by a voltmeter the probes of which were inserted just in front of the absorber lint wicks. As the working distance of the gel (distance measured across the gel between the ends of the two wicks) was 13 cm. the true field strength was $\frac{32.5}{13} = 2.5$ V/cm. Higher voltage than that resulted in over heating which was very difficult to control, lower voltage than 2.5 V/cm. gave increasingly inferior definition of the individual components even if the time of electrophoresis was prolonged to improve resolution.

FIGURE 53

FIGURE 53

Acrylamide gel (7%) electrophoresis of soluble extracts of various biotypes of Brucella abortus.

In the gel TRIS-citrate (pH 8.9, mol.: .0808) and in the tank borate buffer (pH 8.-, mol.: .349) was used at a field strength of 2.5 V/cm. (constant voltage). The gel during electrophoresis was cooled by stream of cold air.

Duration of electrophoresis was 6 hours.

The gel was stained in naphthanene black.

Arrows point out the difference in the electrophoretic pattern of the various biotypes.

Experiments were also carried out to establish the optimal length of time for electrophoresis. It was found that the best results were obtained if electrophoresis lasted for approximately 6 hours at a field strength of 2.5 V/cm. Shorter times than this did not allow complete resolution to take place, and prolonging electrophoresis much beyond 6 hours resulted in loss of definition of the individual components without appreciable improvement in resolution.

During electrophoresis a brown line developed in the gel at its cathode end. In practice it was found that when this line, in the course of its migration towards the anode, overpassed the reservoirs by 80 mm it was time for the termination of electrophoresis. This required approximately 6 hours of electrophoresis.

Figure 55 shows the results of a comparative study on the electrophoretic patterns of the soluble extracts of the 5 biotypes of Brucella abortus.

TRIS-citrate (pH 8.9, mol.: .0803) Borate (pH 8.-, mol.: 0.349) buffer system was used at a field strength of 2.5 V/cm. Electrophoresis was terminated when the brown line was 82 mm. in front of the reservoirs.

It will be seen that up to 21 electrophoretically distinct components may be found in the various brucella extracts. At close inspection of the various patterns it will be noted that the only difference between the electrophoretically resolved components of S19 and that of the virulent biotypes of Br.abortus concerns the fractions which are marked X (S19) and XX (for the virulent strains). This experiment was repeated numerous times, giving always the same result, i.e. a double band of proteins (XX) in the 4 virulent strains which were represented by a single band (X) in the electrophoretic pattern of S19 Brucella abortus.

When 12% starch gel was employed as supporting medium under otherwise identical conditions, the rate of fraction movement was somewhat faster, the generation of heat in the gel was noticeably less and lower voltage had to be applied to the tank buffer to achieve the 2.5 V/cm. drop in the gel. The poorer resolution of brucella extracts and the less even distribution of the various fractions, however, offset these advantages.

The finding of an extra electrophoretic fraction in the soluble extracts of the virulent biotypes of Br.abortus was in support of the earlier observation made with the use of agar gel diffusion tests.

It will be recalled that a precipitate line (IE) common to all the virulent strains of brucella, could not be demonstrated in the S19 homologous system. Attempts aiming to show the corresponding antigen (IE) in S19 organisms or the homologous antibody in its antiserum were also unsuccessful. It was reasonable, therefore, to conjecture that one of the two components (XX) of the virulent strains corresponded to the antigen, contributing to the formation of precipitate line IE.

To ascertain the validity of this hypothesis it was necessary to elute those two fractions (XX) from the gel and examine the eluates for their antigenic identity. This necessitated the accurate location of the fractions in unstained gel. The problem of location was hoped to be accomplished by slicing the gel horizontally into two halves and staining one of them. After decolourisation, the unstained half was to be superimposed on the stained one and the appropriate portion of the gel dissected for elution.

This technique, however, failed because of the very slow rate of decolourisation of the stained half of the gel. Despite frequent changes of the solvent it took at least

48 hours for the gel to loose the non-specifically combined stain, thus allowing the location of the various fractions. During this time diffusion of fractions into each other took place in the unstained half of the gel which itself shrank appreciably even if kept at 4°C in the refrigerator and covered with a sheet of melinex.

Attempts were made to accelerate the removal of non-specifically combined stain from the gel electrophoretically, using 5% acetic acid as electrolyte. The application of electrophoresis for that end, however, was not successful so far, for it either removed all the stain or caused dislocation of the components.

DISCUSSION

The selection of a suitable buffer system for the electrophoresis of a given substance is still largely empirical due to the incomplete knowledge of the mechanism of electrophoresis especially in semi-solid media (Crowle, 1961). As a result of this, various buffers had to be tried in order to find a suitable one for the electrophoretic fractionation of soluble extracts of brucella. All conditions set up for an electrophoretic experiment are aimed at obtaining optimal fractionation.

Among the various factors affecting the electrophoresis of a substance is the pH of the buffer. It is responsible, chiefly, for the direction and rate of fraction movement. In these experiments it was found that, everything else being equal, in an alkaline solution of the buffer the rate of movement of the fractions of brucella towards the anode was somewhat better than in an acid solution, although the direction itself remained unaffected at values ranging between pH 6 to pH 9.

The ionicity (a value equivalent to the current carrying capacity of dissolved electrolytes) of buffer used was not calculated due to the complex nature of

calculation[1]. Instead, the current-carrying capacity of the various buffers was measured under standard conditions. Ionicity affects electrophoresis in various ways. It affects resolution. An increase of ionic strength minimizes reactions between the substance being separated and its supporting medium and between the substance being separated and other substances in solution with it.

Ionic strength is inversely proportional to the rate of fraction movement for two reasons. First, at low ionic strength the current carried will be low, thus high voltage can be applied without the generation of excessive heat. High voltage in turn increased the nett difference in charge between the substance being electrophoresed and the similarly charged electrode resulting in the speeding up of fraction movement. Secondly, the migrating fraction is surrounded by an atmosphere of electrolyte ions of opposite charge so that there is a tendency for fluid around it to move in the opposite direction and aginst the movement of the fraction thus decreasing its mobility. Since this affect is proportional to ionic strength, the lower is the ionicity the less is the slowing

1. Factors affecting ionic strength include: electrolyte disassociation constant, pH, actual salt concentration. temperature, and the influence of one kind of ion on the disassociation of another. (Oxoulo, 1961 pp 29-32).

down effect on fraction movement. Assessing the ionic strength of the various buffers used in these experiments on the basis of their current carrying capacity, the differences in this regard were appreciable (Table 37). However, there was no apparent correlation between the ionicity of the various electrolytes and the degree of resolution obtained by their use in starch gel electrophoresis. When phosphate and barbitone acetate buffers were used in the starch gel in various combination with other electrolytes as tank buffers (Figures 49 and 52) the current carrying capacity of the systems was high yet the resolution was poor in all but the barbitone acetate/phosphate system. (Figure 49 top section). The most likely explanation of this occurrence probably rests with the fact that in these buffer systems the rate of fraction movement was very slow, insufficient for resolution to take place. This explanation, however, is at variance with the result obtained by the barbitone acetate/phosphate system (Figure 49 top section) in which conductivity was one of the highest (Table 38) yet both the rate of fraction movement and the degree of resolution was comparatively good.

Inconsistency between ionicity of buffer on one hand and the rate of fraction movement and resolution on the other hand was emphasised even more by the results

obtained with the use of TRIS-citrate/borate (Figure 50 middle section) and borate/TRIS-citrate systems (Figure 51 middle section). The quality of electrolytes in these two systems was identical and their conductivity of comparable value (Table 38) yet the results obtained by them were very different in terms of both resolution and rate of fraction movement.

This observation directly leads to the effect of buffer composition on electrophoretic resolution. This is one of the most poorly understood factors in electrophoresis. From the results presented here it would appear that perhaps the most important single factor affecting the electrophoretic fractionation of brucella extract was the quality of buffer employed. The application of a continuous buffer system to the brucella extracts did not necessarily give poorer results than those obtained by the use of the combination of these buffers in discontinuous systems. Indeed, some of the results obtained by the use of the combination of these buffers in discontinuous systems were poorer than those obtained by contunuous buffer systems. The use of phosphate buffer in starch gel in various discontinuous systems yielded the poorest results in terms of both resolution and rate of fraction movement. In contrast, the application of

TRIS-citrate in the gel in combination with other electrolytes in the tank gave, by and large, the best results. Even the order of buffers in a discontinuous system is not interchangeable as already pointed out. When all the other factors were practically equal, the TRIS-citrate (gel)/borate system gave better resolution than did the use of borate (gel)TRIS-citrate system.

The field strength, applied in experiments in which efficiency of various buffers were compared, was adjusted to a standard voltage compatible with the use of all the electrolytes without causing overheating. This voltage may not have been optimal for some of the buffer systems (those with low ionicity) but its application was desirable in order to keep down the number of variables in these studies. In those experiments where acrylamide gel was used as supporting medium the aim was to employ ionic strength and voltage which resulted in the most rapid fraction movement together with satisfactory resolution and definition but without generating uncontrollable overheating in the gel.

That the kind of supporting medium in electrophoresis partly controls the type of result which will be obtained is illustrated by the difference of electrophoretic pattern of brucella observed in starch and

acrylamide gels respectively. When electrophoresis was carried out under conditions identical in every respect but the quality of supporting medium, the rate of fraction movement was somewhat faster in 12% starch gel than was in 7% acrylamide gel. The current carried by the electrolyte in the two gels at identical field strength was lower in starch gel resulting in less heat generation. The resolution of electrophoretic fractions of brucella was inferior in starch gel not permitting the demonstration of difference, shown in acrylamide gel, between the fractions of S19 and that of the virulent strains of brucella. The distribution of the fractions in starch gel was quite uneven. Most of the components were distributed in the front half of the path of electrophoresis unlike in acrylamide gel. One of the reasons for the different results obtained with the use of the two gels under otherwise identical conditions may be the phenomena of electro-osmosis.

Electro-osmosis in agar gel is due to the negative electrical charge of the agar itself. Since the agar gel is fixed and unable to move, its tendency to move away from the cathode is counter-balanced by the equivalent actual movement of water towards the cathode which in turn will affect all substances dissolved in water. Electro- osmosis

in both starch and acrylamide gels is stated to be little (Croule, 1961). Its actual value for the two types of gel under the conditions used here is unknown. It would appear that it may have been stronger in the acrylamide gel slowing down fraction movement. But for the superior resolution obtained in acrylamide gel, electro-osmosis in itself cannot be responsible, for it merely shifts the pattern backward or forward without altering the relative position of the components in the pattern (Raymond and Nakamichi, 1962).

Another factor, akin to the medium and affect electrophoretic fractionation, is the molecular sieving action of the gel. According to Raymond et al. (1962) this sieving action of starch and acrylamide gels is comparable provided the concentration of cynogum 41 in the gel falls between 3 - 10%. They suggest that over the range of pore size represented by these concentrations the resolution obtained is unaffected by pore size.

Results obtained in this investigation are at variance with this view. When the concentration of cynogum 41 was cut down to 3% as opposed to 7% in the gel but otherwise conditions were identical, resolution became much poorer and the distribution of the various

fractions was more similar to those seen in 12% starch gel than to those seen in the 7% acrylamide gel. Thus it would appear that the superior resolution of the soluble extracts of brucella in 7% acrylamide gel was, at least in par, due to its molecular sieving effect.

Another medium factor which may affect electrophoresis is viscosity. It decreases with rising temperature allowing the acceleration of fraction movement. The generation of heat was undoubtedly greater in acrylamide gel, but as the viscosity co-efficient of the two types of gel is unknown, no evaluation of the role of this factor is possible.

Despite the demonstration of an extra electrophoretic fraction in the soluble extracts of the virulent biotypes of Brucella abortus, the ultimate objective of electrophoresis was not achieved.

Due to difficulties of locating the various fractions in unstained acrylamide gel, the elution and identification of electrophoretic fraction XX of the virulent strains became impossible. Without the conclusive demonstration of the antigenic identity of one

of the two fractions (XX) to antigen IE of the virulent strains, the presence of this extra factor in the electrophoretic pattern cannot be taken as definite evidence to prove the qualitative difference in the antigenic structure of S19 on one hand and that of the 4 virulent biotypes of Brucella abortus on the other. In spite of that, the combined results of antigenic analysis, by means of the gel diffusion test and the results of the comparative studies by electrophoresis, do seem to indicate an extra soluble substance shared by the virulent strains examined but absent in S19 Brucella abortus. Work in progress, in regards of speedy decolourisation of stained acrylamide gel holds out some promise of success, which in turn may make it possible to obtain this extra component in relatively pure form.

SUMMARY AND CONCLUSIONS OF PART 3

In the foregoing part of this thesis an attempt was described aiming to extract the water soluble antigens of various biotypes of Brucella abortus in order to subject them to comparative analysis. The aim of the analysis was the detection of any antigenic difference which may exist between the various biotypes which may constitute a basis for a diagnostic test capable of differentiating vaccinal titres from those caused by superimposed natural infection of cattle. For the comparative analysis the precipitation reaction in agar gel and the electrophoretic technique were employed. The results of the investigation may be summarised as follows:

1. Various methods of preparing water soluble antigens from Brucella abortus were compared and one of them - using cold acetone treatment of the cells - was slightly modified and adopted for use. By this means up to 13 soluble antigenic components may be extracted from Brucella abortus.

2. Optimal conditions for the precipitation reaction of brucella in agar gel were established. It was shown that 1% agar in the gel, buffered at pH 8.4

and containing .2% NaCl was the most suitable. Experiments in regard to distribution of patterns of reservoirs and the distances separating them showed that the best results may be obtained by placing the immune serum in the centre of no more than 4 peripheric wells which should be 6 mm away from the central reservoir. An adjustable gel cutter was described, suitable for cutting duplicate gels with a high degree of accuracy.

3. Immunisation experiments of rabbits showed that viable Brucella abortus was a much better immunising agent than was its acetone dried derivative. Six intramuscular injections of a viable suspension (each containing 15 x 10^9 cells) administered at one week intervals resulted in highly potent immune serum which was best obtained between the 6th and the 12th day after the last injection.

4. Quantitative examination of the soluble antigens of those biotypes of Brucella abortus which occur in Great Britain showed that the various biotypes possessed up to 13 soluble antigenic components. These antigens differed in their relative concentration in the bacterial extract of different origin. Two of the strains examined (both melitensis type organisms) did not possess an antigenic

component characteristic of typical Brucella abortus.

5. Comparative studies of the precipitate systems of the various biotypes of Brucella abortus revealed two antigenic components in some of the virulent strains partially different from those present in the vaccinal strain of brucella. Furthermore, the four virulent biotypes of Brucella abortus (accounting for over 90% of field infection of cattle in Great Britain) posses at least one antigen which is common to all of them but is absent in the vaccinal strain.

6. The titration of precipitating antibodies in bovine sera by the aid of soluble antigens of brucella was not always possible due to difficulties inherent in the technique used. Nevertheless, it was shown that the two partially different antigens of the virulent strains were strongly immunogenic whereas the extra antigen of the virulent biotypes was not in every individual of the bovine species.

7. Zone electrophoresis in starch and acrylamid gels was employed in an attempt to isolate the extra antigenic component of the virulent strains of Brucella abortus in relatively pure state. Among the various

buffer systems examined, one was capable of resolving the soluble fractions of brucella to a high degree. The comparative electrophoretic patterns of the biotypes revealed an extra component shared by all the virulent strains but absent in S19 Brucella abortus. The elution of the component from the gel, however, was not successful, thus the identity of the extra electrophoretic fraction to the extra antigen of the virulent strains of brucella could not be established.

The results of these studies show that antigenic differences do exist between the vaccinal strain of brucella and those causing natural infection of cattle. It is felt that the isolation and purification of the extra antigenic component of the virulent strains is worthy of every effort, for it may constitute the basis of a serological method by which the brucella-antibody content of bovine sera may be examined qualitatively rather than quantitatively in order to differentiate vaccinal titres from those caused by superimposed natural infection.

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