STUDIES ON MYCOPLASMA MYCOIDES

Concerning the bactericidal activity of antisera and their property of agglutinating erythrocytes sensitized with antigen(s) of M.mycoides

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STATEMENT AND ACKNOWLEDGEMENTS

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University and, to the best of my knowledge and belief, it contains no material previously published or written by another person except where due reference is made in the text.

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SUMMARY

Sera containing complement-fixing and agglutinating antibody to *Mycoplasma mycoides* were shown to participate in two other reactions described here.

When a fresh bovine serum containing complement-fixing antibody to *M.mycoides* was inoculated with viable *M.mycoides* and incubated at 37°C a reduction in the viable count of up to 4 logarithms was demonstrated. This activity was apparent within 2 minutes. Heating the serum to 56°C for 30 minutes destroyed its activity, which could be replaced by the addition of an unheated serum itself not active in the reaction. The activity depended upon heat-stable and heat-labile factors. The reaction was referred to as a bactericidal reaction but agglutination, or possibly conglutination, appeared to be the cause of the reduction in the viable count. The sera of some cattle and other animals possessed this activity despite the absence of antibody detectable by the complement-fixation and agglutination reactions.

Both the addition of *M.mycoides* polysaccharide to a serum, and the absorption of the serum with this organism reduced the bactericidal activity. From these and other observations it appeared probable that antibody to *M.mycoides* was required in the bactericidal reaction. The relationship of the non specific, heat-labile factor to complement was not determined.

Sera that were active against M.mycoides were also active against some other Mycoplasma species.

The indirect haemagglutination reaction was readily performed with M.mycoides. Sheep, rabbit, fowl, goat, bovine and horse erythrocytes were sensitized with cultures of M.mycoides in a medium without serum, or with polysaccharide isolated from the organism. These sensitised red cells were mixed with sera from which erythrocyte agglutinins had been absorbed with the appropriate red cells. Sera containing complement-fixing antibody to M.mycoides rapidly clumped the sensitized red cells. The indirect haemagglutination reaction carried out on slides with sheep cells optimally sensitized with M.mycoides polysaccharide was of the same order of sensitivity as the standard complement-fixation test for M.mycoides. Absorption of a serum with red cells sensitized with polysaccharide removed detectable agglutinating and haemagglutinating activity and removed or substantially reduced complement-fixing activity. This indicates that the polysaccharide fraction of M.mycoides was involved in all these reactions.

Haemagglutination was inhibited by the addition to sera of polysaccharide recovered from M.mycoides. This was used as the basis of a semi-quantitative test for the presence of this antigen.

A strain of Actinobacillus lignieresi and some Mycoplasma strains were shown to cross react with M.mycoides in the haemagglutination test.

PART I

GENERAL INTRODUCTION

STUDIES ON THE BACTERICIDAL EFFECT OF SERUM

INTRODUCTION

Recovery from contagious bovine pleuropneumonia (CPP) is usually followed by immunity (Turner, 1959). Prophylactic vaccination in the tail tip with living cultures of Mycoplasma mycoides, the causal organism, protects about 97% of vaccinated cattle against challenge with an aerosol of virulent culture. Only 8% of controls failed to develop evidence of pulmonary disease (Turner, 1959).

Following vaccination or during the course of the natural disease, antibody is detectable in the blood. Of the serological tests available, the complement fixation (CF) test (Campbell and Turner, 1936; Campbell and Turner, 1953) is most widely used. It is specific and can detect acute and chronic cases. In fatal cases, complement-fixing antibody may be maintained at a high level until death, otherwise it falls slowly and is still at a detectable level in animals with chronic sequestra containing viable organisms (Turner, 1959).

Agglutinins appear in the blood at about the same time as, or a little before, complement-fixing antibody but may disappear while the animal is still acutely infected (Campbell, 1939). It appears that agglutinin activity may be eclipsed by the antigen present in the blood in such cases (Dafaalla, 1957).

Slide agglutination tests using serum or whole blood have been described (Priestley, 1951; Newing and Field, 1953;

Provost and Queval, 1957; Turner and Etheridge, 1961). While such tests may be of value in detecting antibody in early acute cases, false negative reactions may occur when antigen is present in the blood (Dafaalla,1957) and false positive reactions may occur when animals are infected with Mycoplasma species carrying related antigens (Provost et al., 1959). Heslop (1921) and Walker (1923) described the use of a conglutination test for detection of antibody, but this test has not been generally adopted.

The indirect haemagglutination (HA) reaction (Keogh, North and Warburton, 1947) exploited by Neter (1956) in enteric infections has been widely used in detecting antibody. This reaction differs from the direct agglutination of red cells by some viruses. Erythrocytes may absorb antigens from some bacteria and become specifically agglutinable by homologous bacterial antibody.

The application of this reaction to the detection of antibody to M.mycoides is described.

Although the ultimate test of immunity in experimental work with vaccines is the challenge by a virulent strain of the organism concerned, much useful screening work could be performed with a laboratory test for immunity. Priestley (1952) showed that whole blood or serum from cattle exposed to M.mycoides reduced the viability of an inoculum of this organism. As the presence in the blood of cattle of antibody

detectable in the CF test for CPP does not always indicate immunity to challenge with virulent M.mycoides, the reaction described by Priestley (1952) appeared worthy of further study.

Another reaction of interest was that described by Edward and Fitzgerald (1954). These authors incorporated hyperimmune serum into growth media and demonstrated inhibition of growth of homologous strains of Mycoplasma. Unfortunately it was not clear whether this was true for M.mycoides.

Since the work to be described began, Villemot and Provost (1959a) demonstrated bactericidal activity of antiserum to M.laidlawii (Strain 35), against M.mycoides by Priestley's technique. They did not test the homologous antiserum. They were also able to show inhibition of M.mycoides when the antiserum was incorporated into agar medium as described by Edward and Fitzgerald (1954).

A plate counting technique devised in this laboratory was used to study the bactericidal reaction in more detail.

MATERIALS AND METHODS

The number of viable organisms in samples was counted by a surface-plate technique based on that of Miles and Misra (1938).

Medium

The basal medium was made by adding 2% agar to the buffered digest prepared as for BVF-08 (Turner, Campbell and Dick, 1935). This was melted as required and 30% sterile ox serum was added together with sodium penicillin (100 units/ml). This was tubed in 15 ml amounts and poured into 10 cm Petri dishes. After the medium had set, the plates were dried inverted over their lids at 37°C for 1 hour.

Diluent

Dilutions of material to be counted were made in chilled BVF-08 medium, and the plates were sown within 5 minutes unless otherwise stated. The diluting medium itself supports the growth of M.myoides from minimal inocula.

Dropping Apparatus

Experiments showed that drops of relatively constant weight could be released continuously by a simple apparatus. A 10 ml bulb pipette was reduced in length at both ends to provide a reservoir. To one end, an 18 gauge needle with the tip filed square was attached by means of an adaptor to a four inch length of rubber tubing. Internal surfaces were silicone treated. For sterilization the free end of the reservoir was plugged with cotton wool and the needle was covered with a glass tube. In use, the pipette was clamped vertically, with the needle downwards. Fluid from which drops

were required was drawn into the bulb by means of a suction tube attached to the top end of the pipette. A screw clamp attached to the rubber mid-section controlled the release of fluid. The interval between drops was adjusted to that at which the pipette had been calibrated.

Titration Method

Serial ten-fold or hundred-fold dilutions of the material to be counted were made in BVF-OS medium using separate sterile pipettes for each transfer. The contents of each tube was mixed thoroughly by striking the tube sharply with the finger. Before the transfer was made, the liquid was sucked up and blown out rapidly three times.

Drop Inoculation

On the bottom of the Petri dishes containing the medium, numbered segments were drawn in grease pencil according to the number of drops required on each plate. Drops were then delivered to these plates with the one dropping pipette, commencing at the highest dilution. When the drops were no longer visible on the agar surface, the lids of the Petri dishes were discarded and the bases inverted over sterile glass squares on which the plates were sealed with a mixture composed of equal parts hard paraffin (MP56°-58°C) and soft paraffin. The plates were incubated at 37°C until the colonies grew to a suitable size for counting (usually 4-5 days).

Counting

Colonies were counted with the aid of a plate microscope at 5x magnification.

Assessment of Counts

The viable count was obtained by multiplying the mean colony count from a dilution by the reciprocal of that dilution and by the number of drops/ml (pipette factor) delivered by the pipette.

Test Organism

Unless otherwise specified, the strain of M.mycoides used throughout was the "W5" which was isolated from a field case of CPP in 1936 and has had 20 subcultures in liquid medium since isolation. This strain, of moderate virulence for cattle, is used for the preparation of the broth-culture vaccine issued by the C.S.I.R.O.

Sera

When the term "CF-positive" is used it refers to animals whose sera contain antibody detectable in the CF reaction. The term "normal" refers to animals which have had no known contact with M.mycoides and are apparently in good health.

DEMONSTRATION OF THE BACTERICIDAL PHENOMENON

Preliminary Trials

The serum of an animal, strongly positive to the CF test for CPP following tail tip inoculation with M.mycoides

culture, was available for the first experiments.

Blood was collected from the jugular vein in a vessel containing glass beads and was defibrinated by shaking the bottle. One of two bottles both containing 1.8 ml of the blood was placed at 56°C for 30 minutes then cooled to room temperature. The other was left in the cold, then warmed to room temperature. Then 0.2 ml of a culture of M.mycoides in BVF-OS medium containing about 10^9 viable organisms per ml was added to each bottle. The contents were mixed by gentle shaking and the bottles were placed at 37°C for 1 hour. Mixing was repeated after 30 minutes. Following the incubation period, dilutions of $^1/10$, $^1/1000$ and $^1/100,000$ were made in chilled BVF-OS medium. Thus, 10^{-2}, 10^{-4}, 10^{-6} dilutions of the original culture were available. Commencing at the highest dilution, 2 separate drops of dilutions 10^{-6} and 10^{-4} were placed on each of three plates with a calibrated dropping pipette. The 10^{-2} dilution was delivered to the plates in 0.1 ml amounts with a graduated pipette. As M.mycoides may be present in the blood during the acute phase of CPP blood samples were cultured for free organisms.

Result: Growth of M.mycoides occurred on the plates in the areas sown from 10^{-2}, 10^{-4}, and 10^{-6} dilutions of the heated sample, but there was no growth from even the 10^{-2} dilution of the unheated sample. No M.mycoides were cultivated from the original blood sample.

With another sample of defibrinated blood from the same animal, a similar trial was performed. On this occasion, samples were taken for viable count immediately after the culture was added to the blood, as well as after incubation for 1 hour at 37°C. In addition, blood from a normal cow, the serum of which contained no detectable antibody in any of the usual serological tests, was treated similarly.

TABLE 1

The viable count of an inoculum of M.mycoides before and after incubation with unheated blood and with the same blood heated to 56°C for 30 minutes.

Log. viable counts* /ml.

Time at 37°C (hours)	Normal Blood		CF-Positive Blood	
	Heated	Unheated	Heated	Unheated
0	9.30	9.26	9.06	4.82
1	9.30	9.32	8.48	<3.78

* The viable counts are expressed in this and other tables, unless otherwise specified, as the logarithms to the base 10.

The reduction in count (Table 1) occurring after incubation of M.mycoides in the presence of unheated, fresh, defibrinated, CF-positive blood was thus confirmed. There was a relatively small reduction in the viable count in the presence of heated blood. After incubation with blood from the normal cow however, there was no similar reduction in the number of viable organisms in either heated or unheated samples.

There was a delay of about 10 minutes in making the dilutions and inoculating medium for viable counts from the sample in unheated CF-positive blood and the reduction in count to about $^{1}/_{17000}$th of the original occurred during this time.

It appeared from the second trial that the action of CF-positive blood on M.mycoides could be very rapid and a further test on the same blood was made to confirm this point. Similar techniques were used, and viable counts were were made on samples as soon as possible (1-2 minutes at room temperature) after introduction of organisms to the blood and again after 10 and 20 minutes at 37°C.

TABLE 2

Log.viable count of M.mycoides after 0, 10 and 20 minutes at 37°C in the presence of heated and unheated blood.

Log. viable count /ml.

Time at 37°C (mins.)	CF-Positive blood	
	Heated	Unheated
0.	8,98	<5.78
10.	NOT DONE	<5.78
20.	8.25	<5.78

Again, (Table 2), it was obvious that the reaction occurred very rapidly. The number of viable organisms present 1-2 minutes after being mixed with unheated blood being less than $^{1}/_{5000}$th of the number detected in the

heated blood under the same conditions. There was reduction to 1/5th of the original in the count of the heated blood sample after 20 minutes at 37°C.

As phagocytosis could have been responsible for the drop in viable count, it was important to determine whether plasma or serum, rather than whole blood, showed this effect.

Fresh defibrinated blood from the same animal was centrifuged for 30 mins. at 3,500 r.p.m. at 10°C. The supernatant serum was filtered through cotton wool to withhold white cells (Turner, 1960) and divided into 2 samples, one of which was placed at 56°C for 30 minutes then cooled to room temperature. The other sample was kept in the cold, then warmed to room temperature before inoculation. A culture of M.mycoides was added to both samples and viable counts were made immediately, and after 20 minutes' incubation at 37°C (Table 3).

TABLE 3

The log. viable counts of M.mycoides after incubation for 0 and 20 minutes at 37°C in the presence of CF-positive serum heated to 56°C for 30 minutes and of unheated serum

Time at 37°C (mins.)	Log. viable count/ml	
	Heated serum	Unheated serum
0	9.54	4.56
20	9.32	< 3.78

Stained preparations of the serum failed to reveal the presence of cells.

It thus appeared (Table 3) that a similar, rapid reduction in viable count could be produced by a serum in the absence of demonstrable phagocytic cells.

These preliminary trials demonstrated that whole defibrinated blood, containing CF antibody to M.mycoides possessed the capacity to reduce the viable count of a culture of M.mycoides. The serum obtained by centrifuging the whole blood also had this capacity. The reaction was very rapid, since a very large fall in viable count occurred within 2 minutes of mixing organisms and blood. A blood sample containing no demonstrable CF antibody did not show this effect. Exposure to 56°C for 30 minutes destroyed the bactericidal activity both of whole blood and of serum.

It is proposed, following the example of other workers (Miles and Misra, 1938, Pillemer et al., 1954) to refer to the reduction in viable count shown here as a "bactericidal" reaction, without prejudice to a final assessment of the mechanism of the phenomenon.

DEMONSTRATION OF HEAT-LABILE NON-SPECIFIC FACTOR

It was shown in the previous section that an active serum heated to 56°C for 30 minutes had little or no bactericidal activity. The question asked then was whether this heat lability was a feature of CF-positive sera only.

An attempt was made to restore bactericidal activity to a CF-positive serum rendered inactive by heating it to 56°C for 30 minutes, by adding unheated, normal serum. The serum obtained after centrifugation of the fresh defibrinated blood of a normal animal had previously been shown to be inactive in the bactericidal reaction. Serum from a CF-positive cow (212) was divided into two samples, one being placed at 56°C for 30 minutes, the other kept at room temperature. A sample of serum from the normal animal was treated similarly.

The following mixtures were made from equal volumes of the sera:-

A. Heated CF-positive serum and unheated normal serum,

B. Heated CF-positive serum and heated normal serum,

C. Unheated CF-positive serum and unheated normal serum,

D. Unheated CF-positive serum and heated normal serum.

One volume of M.mycoides culture was added in turn to nine volumes of each of the serum mixtures. Incubation of the mixtures at 37°C followed and viable counts were made after 2 minutes at this temperature (Table 4).

TABLE 4

The restoration of bactericidal activity to a heated CF-positive serum by addition of an unheated normal serum

Serum mixture	Log. viable count/ml
A. Unheated CF-positive + unheated normal	3.00
B. Unheated CF-positive + heated normal	3.30
C. Heated CF-positive + unheated normal	5.28
D. Heated CF-positive + heated normal	9.08

The normal serum, itself unable to reduce the viable count of added culture, was able to restore to heated CF-positive serum part of the activity displayed by the serum before heating. Apparently both CF-positive and normal sera may contain a heat-labile substance contributing to this reaction.

The difference in activity between mixtures A and B was only two-fold, indicating that, in the presence of an active serum, the contribution from unheated normal serum was relatively slight. Yet, when the unheated normal serum was the only source of heat-labile substances (Mixture C), its contribution had a relatively greater effect. The short incubation period of 2 minutes may not have permitted completion of the reaction.

This test was repeated with three further sera from normal animals. Aliquots of an active serum, 349, were heated and mixed in equal volumes with sera 400, 403 and 443. These

sera were also tested alone at this time for evidence of activity. The sera and mixtures of sera were incubated in the presence of M.mycoides culture for 30 minutes at 37°C.

TABLE 5

The restoration of bactericidal activity to a CF-positive serum heated to 56°C for 30 minutes by addition of unheated normal sera

Serum treatments	Log.viable count/ml
Heated CF-positive (349)	9.54
Unheated CF-positive (349)	5.49
Heated CF-positive (349) + unheated normal (400)	6.20
Heated CF-positive (349) + unheated normal (403)	6.41
Heated CF-positive (349) + unheated normal (443)	4.76
Heated normal (400)	9.51
Unheated normal (400)	7.87
Heated normal (403)	9.72
Unheated normal (403)	8.73
Heated normal (443)	9.78
Unheated normal (443)	9.77
Inoculum	9.69

The CF-positive serum (349) was very active in the bactericidal reaction, reducing the viable count of the inoculum by 4 logs. (Table 5). Heating at 56°C for 30 minutes destroyed this activity. Activity was partially restored to the heated CF-positive serum by adding serum 400 or 403. The addition of serum 443 gave the mixture greater activity than was displayed by the unheated CF-positive serum. The normal

sera showed varying degrees of activity when tested alone. Sera 400 and 403 reduced the viable count of the inoculum by less than 2 logs. and 1 log. respectively while serum 443 showed almost no activity. No antibody to M.mycoides was demonstrable in sera 400, 403 and 443 by the CF test or slide agglutination tests.

The property of restoring activity to heated CF-positive serum was found to be present not only in cattle sera but in the serum from a rabbit (Table 6). The test was conducted as for the preceding series.

TABLE 6

Restoration of bactericidal activity to a CF-positive serum heated to 56°C for 30 minutes by the addition of unheated rabbit serum

Serum treatments	Log. viable count/ml.
Heated CF-positive (164)	9.47
Unheated CF-positive	7.53
Heated rabbit	9.61
Unheated rabbit	9.62
Heated CF-positive (164) + heated rabbit	9.33
Heated CF-positive (164) + unheated rabbit	7.01
Inoculum	9.75

This was repeated with another bovine CF-positive serum (349) and the same rabbit serum with the results shown in Table 7.

TABLE 7

Restoration of bactericidal activity to a CF-positive serum heated to 56°C for 30 minutes by the addition of unheated rabbit serum

Serum treatment	Log. viable count/ml
Heated CF-positive (349)	9.54
Unheated CF-positive (349)	5.49
Heated rabbit	9.64
Unheated rabbit	9.53
Heated CF-positive (349) + heated rabbit	Not done
Heated CF-positive (349) + unheated rabbit	5.66
Inoculum	9.69

Thus a rabbit serum itself lacking activity was capable of restoring completely to the heated bovine serum that bactericidal activity possessed by the unheated serum.

OBSERVATIONS ON THE MECHANISM OF THE REACTION

Although unheated bovine CF-positive serum reduced the viable count of a suspension of M.mycoides markedly in a short time, the same serum after being heated for 30 minutes at 56°C failed to reduce the count to the same degree even after overnight incubation with a suspension of M.mycoides.

Slide preparations were made of the serum-organism mixtures from both heated and unheated CF-positive sera, and viewed under a dark ground-microscope. In the mixture

containing heated serum, the M.mycoides organisms appeared similar to those seen in a culture of the organism, i.e. a large number of separate pleomorphic small rods and filaments and very small refractile particles. In the preparation made from unheated serum, however, there was a distinct difference; large bodies apparently composed of myriads of refractile granules were seen. They were more or less oval, with an irregular outline. The background was relatively free from the small particles seen in the heated serum preparation. When unheated CF-positive serum was placed beside a drop of culture of M.mycoides on a slide and viewed by the high dry objective the separate character of the two drops could be observed. When the drops were mixed while under observation, the large bodies were seen to form almost immediately. The conclusion that some form of agglutination was occurring was inescapable. When this was repeated with heated serum no such clumping occurred.

When a test for the bactericidal activity was performed, the tubes of BVF-OS diluent used in the titration were usually incubated at 37°C along with the plates inoculated from them. It was observed that the tubes in which mixtures of M.mycoides and active serum had been diluted were slower to reach maximum turbidity than those in which the mixture containing heated serum had been diluted. Also, when the tubes containing active serum were shaken gently,

large fluffy particles rose from the bottom. This appearance is not usual with growth of M.mycoides in this medium and it did not occur in tubes of medium seeded with the heated serum-organism mixtures. A possible explanation is that in these tubes, the inoculum consists of clumped organisms which fall to the bottom and there multiply, forming microcolonies at first which later become macrocolonies (the fluffy particles) and that eventually an even turbidity develops.

The effect of incorporation serum from CF-positive animals in the growth medium.

Edward and Fitzgerald (1954) showed that the growth of some PPLO strains was inhibited in media containing homologous antisera. The inhibition of M.mycoides is not specifically mentioned in their paper. A trial of Edward and Fitzgerald's method was carried out using BVF agar with 30% ox serum as the growth medium. The normal ox serum was replaced by selected bovine sera. A series of plates was prepared containing:-

(a) Unheated serum 337, known to be bactericidal
(b) Heated serum 337
(c) Unheated serum 349, known to be bactericidal
(d) Heated serum 349
(e) Heated serum 443, known not to be bactericidal
(f) Heated serum W, known not to be bactericidal

The temperature of the serum did not exceed 45°C during preparation of the plates. Also the drying time of

the medium was shortened to 20 minutes at 37°C instead of the usual 1 hour. Six drops of a 10^{-6} dilution of M.mycoides culture were dropped on each plate. When the drops were no longer visible, the plates were sealed and incubated at 37°C.

TABLE 8

The mean colony counts obtained with a suspension of M.mycoides inoculated to agar media containing immune sera

Serum	Activity of serum in bactericidal reaction	Treatment	Mean colony count x 10^6
337	Positive	Unheated	90
337	"	Heated	82
349	"	Unheated	80
349	"	Heated	87
443	Negative	Heated	84
W	"	Heated	86

Thus under these conditions, when sera that were positive in bactericidal tests with M.mycoides were incorporated into growth media, either as heated serum or as unheated serum, no inhibition of the growth of an inoculum of M.mycoides occurred (Table 8).

A further attempt to demonstrate growth inhibition consisted of allowing sera to diffuse from filter paper

discs or "penicillin" cups set in the agar in the manner of an antibiotic assay. No inhibition of growth of M.mycoides was shown by positive or negative sera, whether heated or unheated.

REPRODUCIBILITY OF THE METHOD

It was important to have an appreciation of the variability of the plate counting technique used in this work.

A quantity of a CF-positive serum was heated to 56°C for 30 minutes and was divided into four aliquots each of 1.8 ml. To each bottle was added 0.2 ml of a culture of M.mycoides and the bottles were incubated for 30 minutes at 37°C, the contents being thoroughly mixed every 5 minutes. Viable counts were then made on 1 ml amounts withdrawn from each bottle.

TABLE 9

The mean colony counts of four samples of heated serum, separately inoculated and incubated with M.mycoides

Sample no.	Mean colony count x 10^6
1	27
2	31
3	27
4	25
Inoculum	30

The result (Table 9) indicates the degree of reproducibility of the plate counting method when separate samples of heated serum were tested.

It was desirable that the reproducibility of the method be tested with active sera.

This was done in two ways:

(a) Four separate titrations on the same incubated serum-organism mixture were made.

(b) Four separate samples of serum were inoculated, incubated and then titrated.

(a) An M.mycoides suspension was incubated with two active sera for 30 minutes at 37°C and plate counts were made on four samples of each serum-organism mixture. For comparison, heated control samples of the sera were also tested.

When samples for titration and counting were drawn from the one serum-organism mixture (Table 10), a relatively consistent colony count was obtained in the four replicates on one occasion. On the other occasion this degree of consistency was marred by one aberrant result. It must be pointed out that the reliability of colony counts below ten is low, due to random sampling error (Wilson, 1935).

TABLE 10

Mean colony counts of four aliquots drawn from the one incubated serum-organism mixture

Serum no.	Serum treatment	Mean colony count Dilutions		
		10^{-2}	10^{-4}	10^{-6}
1	Unheated	109	0	0
	"	125	0	0
	"	152	0	0
	"	141	0	0
	Heated	Confluent	Confluent	39
2	Unheated	1	0	0
	"	8	0	0
	"	8	0	0
	"	10	0	0
	Heated	Confluent	Confluent	31

(b) Two active sera were each divided into 5 samples. To each of four samples was added a similar inoculum of M.mycoides. and the mixtures were incubated at 37°C for 30 minutes. The fifth sample of each serum was heated to 56°C for 30 minutes, cooled to room temperature and similarly inoculated and incubated.

When replicate samples of serum were separately inoculated, incubated and the viable organisms present counted, there was greater variation in the counts. (In view of possible agglutination of the organisms by the serum,

the mean counts obtained with serum A (Table 11) (23 to 70) are surprisingly uniform. The results obtained with serum B, however, show one replicate to be widely different from the other three.) It is therefore obvious that conclusions drawn from small differences based on single observations are open to criticism.

TABLE 11

Mean colony counts of aliquots of serum separately inoculated with M.mycoides and incubated at 37°C for 30 minutes

Serum no.	Serum treatment	Mean colony count Dilutions		
		10^{-2}	10^{-4}	10^{-6}
A	Unheated	70	0	0
	"	39	0	0
	"	23	0	0
	"	35	0	0
	Heated	Confluent	Confluent	46
B	Unheated	4	0	0
	"	36	0	0
	"	6	0	0
	"	1	0	0
	Heated	Confluent	Confluent	51

EFFECT OF STORAGE AT -12°C TO -18°C ON THE ACTIVITY OF A SERUM

Freshly drawn and defibrineted blood was centrifuged in the cold. It was Seitz filtered and then distributed

into 5 bottles in 8 ml amounts. Four bottles were placed in the deep freeze at a temperature within the range -12°C to -18°C. The aliquot that was retained was pipetted into four bottles so that each contained 1.8 ml of serum. One bottle was heated to 56°C for 30 minutes, then cooled to room temperature, while the other three were left at room temperature. Then 0.2 ml of M.mycoides culture was added to each of these four bottles and all were incubated in the water bath at 37°C for 30 minutes. Every 5 minutes the samples were shaken to mix the contents thoroughly. At the end of the incubation period, the contents were again mixed and from each bottle 1 ml was removed and diluted 10^{-1}, 10^{-3} and 10^{-5}. Two drops from each dilution were placed on each of three plates which were then sealed and incubated at 37°C. At intervals this was repeated on deep frozen aliquots to observe the effect of storage on the activity of the serum. The mean viable count of the three replicate titrations performed on each occasion are shown in Table 12.

TABLE 12

Effect of storage on the activity of a serum

Time stored at -12°C to -18°C (days)	Log. viable count/ml.		
	Heated serum	Unheated serum	Difference
0	8.77	6.63	2.14
3	7.43	5.43	2.00
10	8.61	6.46	2.15
38	9.08	6.10	2.98

After storage for 38 days at -12°C to -18°C the original activity as estimated by the difference between viable counts on heated and unheated samples was still present (Table 12). The question of the reliability of this expression of activity is discussed later.

OBSERVATIONS ON SERA FROM CATTLE EXPOSED TO M.MYCOIDES

1. Vaccinated Cattle

Vaccination of cattle in Australia against CPP consists of injecting a dose of 0.2 ml of living M.mycoides into the tail tip. After a preliminary lag, antibody demonstrable by CF, agglutination or haemagglutination reactions appears in the serum (Turner, 1959).

Seven normal cattle were bled and vaccinated with M.mycoides. The pre-vaccination serum samples and the sera from subsequent daily bleedings were tested for bactericidal activity and activity in slide agglutination tests.

Method:

The blood was collected from the jugular vein into bottles containing glass beads. The bottles were shaken immediately after collection of the sample until deposits of fibrin were seen. The blood was then centrifuged in the cold for 30 mins. at 3500 r.p.m. The serum was then filtered through either Seitz E.K. pads or bacteriological membrane filters for the dual purpose of ensuring sterility and removing cells. Sera were either tested on the day of

collection or stored at -12°C to -18°C in several aliquots.

The slide agglutination blood test (SABT) and the slide agglutination serum test (SAST) (Turner and Etheridge, 1961) consist essentially of mixing equal volumes of oxalated whole blood (SABT) or whole serum (SAST) and heated suspension of M.mycoides on a slide. In the SABT the suspension is coloured.

Test:

Each serum was divided into two samples and one was heated to 56°C for 30 minutes, then cooled to room temperature, while the other was kept at room temperature. To each sample was added one-ninth of its volume of M.mycoides culture in BVF-OS. The mixture was incubated at 37°C for 30 minutes. The bottles were shaken by hand every 5 minutes. After incubation, 1 ml samples were withdrawn to 9 ml BVF-OS broth held in an ice bath. Two further dilutions, each one hundred-fold, were made by adding 0.2 ml to 19.8 ml of cold diluent, and plates were sown with drops from these dilutions. The difference between the $\log._{10}$ viable count of the heated serum sample and the $\log._{10}$ viable count of the unheated serum sample was used as a semi-quantitative estimate of the activity of the serum.

It can be seen (Table 13) that the sera of two of the seven animals (337,432) were active in the bactericidal

TABLE 13

Bactericidal activity of serum samples from cattle vaccinated with *M.mycoides*

Animal No.	Days after vaccination with *M.mycoides*														
	0	1	2	3	4	5	6	7	8	9	11	12	13	20	28
164	0.1	0.3	0.1	0.2	0.2	0.2	2.9	3.3	3.8	4.1	-	2.4	> 6.1	5.3	3.5
337	3.9	3.6	2.8	4.3	0.5	2.0	3.9	3.4	3.8	3.4	-	0.8	1.5	5.1	4.6
349	0.7	0.8	1.5	0.3	0.7	3.1	4.4	4.7	4.4	5.6	-	2.5	> 6.1	5.0	5.0
407	0.3	0.3	0.2	0	0	0	2.2	3.5	3.7	3.6	-	3.6	3.9	3.6	1.9
432	2.4	3.5	3.5	3.4	2.5	6.2	3.3	2.6	4.2	3.8	-	2.2	3.7	5.2	1.6
439	0.1	1.2	0.2	0.1	0.1	0.2	0.1	0.9	1.3	3.9	2.3	1.6	0.1	4.2	3.9
441	0.2	0.8	0.6	0.2	0	0.2	0.1	0.2	0.9	3.7	2.6	3.1	4.4	5.3	4.8

The measure of activity is the difference between the $\log_{10}$ of the viable counts obtained from heated and unheated serum samples inoculated with *M.mycoides* and incubated for 30 minutes at 37°C.

TABLE 14

Results of slide agglutination blood tests (SABT) and slide agglutination serum tests (SAST) on blood samples collected daily from seven cattle after vaccination with M.mycoides

Animal No.	Test	Days after vaccination with M.mycoides													
		0	1	2	3	4	5	6	7	8	9	11	12	13	14
164	SABT	-	-	-	-	-	-	Tr	4	4	3		4	3	3
	SAST	-	-	-	-	-	-	1	4	4	4		3	4	3
337	SABT	-	-	-	-	-	-	Tr	1	3	4		4	3	4
	SAST	-	-	-	2	1	3	1	3	4	4		3	4	4
349	SABT	-	-	-	-	-	-	2	4	4	4		4	4	4
	SAST	-	-	-	-	-	3	3	4	4	4		4	4	4
407	SABT	-	-	-	-	-	-	-	1	4	4		4	4	4
	SAST	-	-	-	-	-	-	Tr	3	4	4		4	4	4
432	SABT	-	Tr	Tr	-	1	1	1	4	4	3		4	4	4
	SAST	Tr	1	-	2	2	3	2	4	3	3		4	4	4
439	SABT	-	-	-	-	-	-	-	Tr	Tr	1	3	4	4	4
	SAST	-	-	-	-	-	-	-	-	-	2	4	4	4	4
441	SABT	-	-	-	-	-	-	-	-	-	Tr	Tr	Tr	1	Tr
	SAST	-	-	-	-	-	-	-	-	-	Tr	4	4	3	2

test prior to vaccination. The post-vaccination activity was somewhat irregular and any rise that might have been consequent on the exposure of the animal to the organism was masked. Slide agglutination tests performed with the sera of these two animals (Table 14) showed trace reaction present in 432 prior to vaccination and in 337 on the third day after vaccination. These reactions increased in degree with time following vaccination.

The sera of three of the other five animals showed no detectable rise in bactericidal activity until after the 5th or 6th day. The sera of the remaining two animals showed a longer lag, with a rise in activity noticeable on the 9th day following vaccination. It was interesting to note that the serological pattern mirrored the bactericidal activity. The first sign of antibody was found on the 6th and 7th days in the three early serological reactors and 7th and 9th days in the late reactors.

These comparisons of serological and bactericidal activity are based on only seven animals but are consistent with the possibility that antibody is one factor required for a positive bactericidal reaction. During the lag period of the first 5 days the day to day bactericidal activity measured for each animal was consistent, with only two exceptions, serum 349, day 2; serum 439, day 1.

OBSERVATIONS ON SERA FROM CATTLE NOT EXPOSED TO M.MYCOIDES

Victoria is free of contagious bovine pleuropneumonia. Introductions have occurred from time to time and have been effectively controlled by slaughter of serologically reacting cattle, combined with quarantine and vaccination. Thus some residue to immune cattle remain after an outbreak, but experience has shown that the circulating antibody level of a vaccinated animal usually drops to below detectable levels before the elapse of six months. At the time of the work described in this thesis, no outbreak of CPP had been discovered in Victoria for 4 years. We could feel reasonably certain, therefore, that circulating antibody to CPP would be absent. However, from time to time, animals showing slight ($^{1}/10$ - $^{1}/20$) titres to the CF test were found and some six year old cattle have been found to react to the slide agglutination serum test. It is believed that such reactions are not due to antibodies specifically provoked by M.mycoides. They may be due to cross reacting antigens - possibly of other Mycoplasma (Provost et al., 1959). One case of cross serological reaction has been shown to be the result of infection of the animal with a strain of Actinobacillus lignieresi (Turner, 1956).

The sera from eight adult cattle showing no antibody detectable by CF and slide agglutination tests were tested for bactericidal activity. The method followed that

described previously, sera being incubated for 30 minutes at 37°C in the presence of M.mycoides.

TABLE 15

Bactericidal activity of sera from normal cattle

Serum	Log. viable count/ml	
	Heated	Unheated
132	-	7.20
366	-	7.25
381	-	9.79
443	9.78	9.77
400	9.61	7.43
416	9.38	5.94
422	9.74	7.65
403	9.72	8.73
Inoculum	9.68	

Only two of the eight sera (381,413) were devoid of activity (Table 15); that of the others varied from 1 log. to between 3 and 4 logs. In one of these two sera (443) at least, the lack of activity was not due to lack of the heat-labile substance, for the serum was shown to be highly effective in restoring activity to heated CF-positive serum (Table 5).

A further 12 animals were bled and their sera were tested for bactericidal and complement fixing activity.

TABLE 16

Bactericidal activity of twelve normal sera

Serum	Log. viable count/ml	
	Heated	Unheated
1	9.72	8.43
2	9.25	7.67
3	9.36	8.36
4	9.32	8.79
5	9.32	7.11
6	9.72	9.72
7	9.23	6.92
8	9.36	6.95
9	9.28	7.28
10	9.30	8.08
11	9.73	7.62
12	9.46	7.84

It can be seen (Table 16) that only serum number 6 could be classed as having no activity but serum numbers 1, 3, 4 and 8 had very little. None of these 12 sera fixed complement in the standard CF test. Ten of these animals were challenged by contact with animals infected with CPP, but there appeared to be no correlation between bactericidal activity in the serum and resistance to infection.

Thus it appears that the sera of a high proportion of normal animals possess some activity demonstrable in the bactericidal reaction. This raises the question of

specificity of the test, which will be discussed later. However, the degree of bactericidal activity shown by the normal sera is rarely as high as that shown by CF-positive sera.

OBSERVATIONS ON BOVINE SERA CONTAINING ANTIBODY REACTING WITH M. MYCOIDES

In the sera of some animals with pathological conditions other than CPP, the standard serological tests used in the diagnosis of CPP are occasionally positive. The sera of five animals were tested in the bactericidal reaction and the results together with those of serological tests on the sera are shown in Table 17. As far as could be reasonably determined clinically, these animals were not infected with, nor had they been exposed to M. mycoides. They appeared to offer an opportunity to obtain further evidence on the participation of antibody in the bactericidal reaction.

TABLE 17

Bactericidal activity of sera showing cross reactions in serological tests for antibody to M.mycoides

Serum	Log. viable counts/ml		Serological tests		
	Heated	Unheated	OF†	SAST*	SABT∅
743	9.00	5.00	$^{i}/_{10}$	4	4
C 43	9.67	6.08	$^{i}/_{10}$	3	4
A 9	9.25	5.65	$^{iv}/_{80}$	4	4
C 72	9.08	5.72	$^{o}/_{10}$	2	3
C 73	9.00	6.28	$^{ii}/_{20}$	4	4

† The notation used is that of Campbell and Turner (1936) where the numerators indicate the degree of reaction from o to iv and the denominators are the reciprocals of the dilution at which the reaction occurred.

* SAST = Slide agglutination serum test.

∅ SABT = Slide agglutination blood test.

In these tests the degree of agglutination is scored from o to iv. All five sera showed marked bactericidal activity (Table 17).

The conditions from which these animals suffered were:-

Animal	Sign	Organism isolated
743	Vaginitis	Mycoplasma Species
C 43	Not clinical	Nil
A 9	Pneumonia	Corynebacterium pyogenes
C 72	Not clinical	Nil
C 73	Mastitis	Staphylococcus

OBSERVATIONS ON THE SERA OF ANIMALS OTHER THAN CATTLE

Sera from apparently normal animals of other species were tested for bactericidal activity against M.mycoides. Pooled serum samples from 3 rabbits, 2 pigs and 2 rats, and single samples from 1 sheep, 1 fowl, 1 guinea pig and 4 horses were tested by the method outlined above. The activity of the unheated serum was compared with that of the heated serum. Although the tests were conducted on different days, the viable counts of the inocula were about 5.0×10^9 orgs/ml.

TABLE 18

Bactericidal activity of sera from other animals

Serum	Log. viable counts/ml	
	Heated	Unheated
Rabbit	9.61	9.62
Pig	3.30	5.72
Rat	9.74	4.38
Sheep	9.35	5.04
Fowl	9.68	7.20
Guinea pig	9.34	9.49
Horse 1	9.00	7.28
2	9.52	7.40
3	9.67	6.79
4	9.80	7.15

The sera from pigs, rats, sheep, fowl and horses showed some activity in this test (Table 18). It is not intended that these data be considered representative of

the activity of the serum of a species; they serve only to demonstrate that activity can be present.

ATTEMPT TO DEMONSTRATE PARALLEL RISE IN BACTERICIDAL AND SEROLOGICAL ACTIVITY IN A RABBIT

The serum of a rabbit was found to lack activity in the bactericidal reaction, but was capable of restoring activity to a heated bovine CF-positive serum (Tables 6, 7). In order to obtain evidence of the participation of antibody in this reaction, antibody production was stimulated in the rebbit by the injection of M.mycoides culture. Blood samples were collected prior to, and 6 and 13 days following injection and the sera were tested for bactericidal activity (Table 19).

TABLE 19

Bactericidal activity of a rabbit serum before and after an injection of M.mycoides

Days after inoculation	Log. viable count/ml	
	Heated serum	Unheated serum
Pre-inoculation	9.62	9.61
6	9.46	5.78
13	9.41	5.61

The serum samples obtained 6 and 13 days after inoculation were active in the bactericidal reaction (Table 19). No antibodies were demonstrable by CF or agglutination tests in any of the three serum samples.

An analogous rise in bactericidal activity following E.coli infection of mice was reported by Rowley (1954).

A more conclusive test would have been the addition to the rabbit serum of a small proportion of serum with a high CF titre. The opportunity to do this has not arisen since.

SPECIFIC NEUTRALIZATION OF BACTERICIDAL ACTIVITY OF SERUM BY M.MYCOIDES POLYSACCHARIDE

Polysaccharide was isolated from M.mycoides by extraction with warm aqueous phenol. Details of the method and of the subsequent purification of the polysaccharide were described by Buttery and Plackett (1960).

As will be reported under "Inhibition of Haemagglutination", the polysaccharide isolated from M.mycoides was capable of inhibiting the haemagglutination of sensitized red cells by serum containing antibody to the organism. If the bactericidal reaction requires antibody it might be expected that the addition of polysaccharide to an active serum would lower the bactericidal activity.

An equal volume of polysaccharide (480 µg/ml) in normal saline was added to a serum (164) known to have high bactericidal activity. An equal volume of normal saline was added to another sample of the same serum as a control. The undiluted serum was tested unheated and after heating to 56°C for 30 minutes.

TABLE 20

The effect of the addition of *M.mycoides* polysaccharide to bactericidal serum

Serum treatment	log. viable count/ml
Unheated	7.28
Heated	9.32
Unheated + saline	7.00
Unheated + polysaccharide	8.71

There was an apparent neutralizing effect by the polysaccharide (Table 20) sufficient to warrant further experiments.

The above experiment was repeated, using three different sera.

On this occasion the polysaccharide-serum mixtures were allowed to act for 1 hour at 37°C during which time the polysaccharide-saline mixtures were held at the same temperature. One volume of a culture of *M.mycoides* was added to 9 volumes of each sample and the mixtures were incubated at 37°C for 30 minutes with intermittent shaking.

In sera C33 and C10, the bactericidal activity was completely annulled by incubation with polysaccharide (Table 21); in the sera 164 and C42 (Tables 20, 21), the activity was considerably reduced. In sera C42, C33 (Table 21) there was a loss of activity after dilution with

saline. There may have been threshhold amounts of heat labile factor present in these sera and this dilution sufficed to cause a drop in activity.

TABLE 21

The effect of the addition of M.mycoides polysaccharide to active sera

Serum treatment	Serum number (Log. viable count/ml)		
	C42	C33	C10
Heated	9.40	9.18	9.40
Unheated	3.78	6.45	5.53
Unheated + saline	5.21	8.80	5.48
Unheated + polysaccharide	8.25	9.59	9.52

Although the loss of bactericidal activity which occurred in the sera incubated with polysaccharide was probably due to a specific neutralization of antibody, it is possible that the effect was due to a non-specific neutralization of the heat-labile factor. Had there been a suitable serum available as a source of heat-labile factor, the bactericidal activity of a mixture of heated CF-positive serum + polysaccharide could have been tested.

REMOVAL OF BACTERICIDAL ACTIVITY BY ABSORPTION WITH M.MYCOIDES

Another way of demonstrating the specificity of the reaction was by absorption of a serum with whole M.mycoides.

This experiment required a serum that was itself inactive in the bactericidal reaction and that was capable of supplying the heat-labile factor. One such serum was found at the time of writing. A preliminary test was made using this serum as a diluent. A CF-positive serum (C51 - titre 1/160) was heated to 56°C for 30 minutes. This serum was then diluted 1/90 in the unheated inactive serum to make a serum mixture containing a low level of detectable antibody in the presence of heat-labile factor. This test system was examined for bactericidal activity as before.

TABLE 22

Bactericidal activity of diluted CF-positive serum

Serum treatment	Log. viable count/ml
Inactive serum unheated	9.82
Inactive serum heated	9.84
Inactive serum unheated + dilution of CF-positive serum unheated	8.18
Inoculum	9.87

Bactericidal activity was still demonstrable after 90-fold dilution of the CF-positive serum (Table 22). The quantity of heat-labile factor in the inactive serum was unknown and may have been less than optimal.

The serum for the absorption experiment was made by diluting a CF-positive serum (C49 - titre 1/1280) 1/64 in

the unheated inactive serum, then heating this dilution for 30 minutes at 56°C. The organisms for absorbing the serum were recovered from 500 ml of BVF-OS culture of M.mycoides. The pellet was resuspended in a small volume of the supernatant and placed at 56°C for 30 minutes to kill the organisms, which were then packed in an International centrifuge with high-speed attachment at 18,000 g. The pellet of killed M.mycoides was resuspended in one portion of the heated serum and incubated at 37°C for 1 hour, when the organisms were removed by centrifugation. As a control another portion of the heated serum was incubated at 37°C for 1 hour. The absorbed and unabsorbed sera were tested for bactericidal activity in the presence of unheated, inactive serum to supply the heat-labile component.

TABLE 23

The result of absorbing a CF-positive serum with M.mycoides

Serum treatment	Log. viable count/ml
Inactive (heated)	9.71
Inactive (unheated)	9.75
CF-positive (heated)	9.73
CF-positive (heated and absorbed)	9.78
CF-positive (heated) + inactive (unheated)	8.11
CF-positive (heated and absorbed) + inactive (unheated)	9.74
Inoculum	9.71

It was clear that absorption of the CF-positive serum completely removed the bactericidal activity (Table 23).

CROSS REACTIVITY

Bactericidal activity against M.mycoides has been demonstrated with sera in which antibody could not be detected by the usual serological tests. Also, the sera of animals with no history of CPP have shown activity. One hypothesis is that this activity may be due to antibody formed against organisms sharing an antigen with M.mycoides. Of the known pathogenic microorganisms occurring in cattle in areas from which experimental cattle were drawn, only one strain of one species, Actinobacillus lignieresi (Smith 40) has been shown to be serologically related to M.mycoides (Turner, 1956). Mycoplasmata belonging to species other than M.mycoides occur in cattle and it is conceivable that these may possess antigens serologically related to those of M.mycoides, which could stimulate the formation of antibodies active in the bactericidal reaction.

A culture of Mycoplasma strain L2917, isolated from a calf, was used as an inoculum in a bactericidal test of two sera shown to be active against M.mycoides. Serum A was obtained from an animal not known to have contacted M.mycoides and serum B was a frank positive in CF and agglutination tests with M.mycoides. The tests were carried out as before and the results are shown in Table 24.

TABLE 24

Showing the bactericidal activity of sera against M.mycoides and a Mycoplasma strain L2917

Serum	Inoculum	Log. viable count/ml	
		Heated serum	Unheated serum
A	M.mycoides	9.44	6.62
	L 2917	9.40	6.02
B	M.mycoides	9.57	6.14
	L 2917	9.52	4.89

It can be seen (Table 24) that both sera were active against M.mycoides and Mycoplasma strain L 2917. The polysaccharide isolated from Mycoplasma strain L 2917 has been shown to be made up of glucose units rather than galactose as is the polysaccharide isolated from M.mycoides (Plackett and Buttery, 1960). Slight cross reactions were detected by the haemagglutination test on low dilutions of serum.

The following Mycoplasma species were used as inocula in a bactericidal test with a known bactericidal serum.

M.bovigenitalium	From Lister Institute.
M.1316-2	Avian strain from Queensland.
CA 33	Avian strain from South Australia.

TABLE 25

Showing the bactericidal effect of a serum on suspensions of three Mycoplasma species

Strain	Log. viable counts/ml		
	Inoculum	Heated serum	Unheated serum
M.mycoides	9.92	9.43	5.18
CA 33	7.95	8.38	4.67
M.1316-2	8.48	7.46	4.90
M.bovigenitalium	5.34	5.20	< 3.78

It appeared (Table 25) that the M.mycoides antiserum was capable of markedly reducing the viable count of other Mycoplasma species as well as of M.mycoides. This activity could be due to non-specific factors or to the presence in the serum of specific antibody to each of these organisms.

The latter possibility could be tested by absorbing out or neutralizing the activity to M.mycoides and re-testing the serum against the other species. It would then be important to have due regard for the possibility that the heat-labile factor concerned in the phenomenon might be absorbed out or inactivated by these procedures.

If this were the case, it would then be necessary to restore the factor by the addition of a serum carrying no activity against any of the strains yet possessing a high content of the heat-labile factor.

THE EFFECT OF DILUTION OF ANTIBODY ON THE BACTERICIDAL REACTION

A CF-positive serum B49 (CF titre $^{1}/1280$) was heated to 56°C for 30 minutes and then diluted in serial two-fold steps to $^{1}/1024$ in a heated serum (VRI) shown to be inactive in the bactericidal reaction. One part of each of six of the dilutions was added to 9 parts of unheated serum as the source of heat-labile factor. These sera were then tested for bactericidal activity.

TABLE 25A

The effect of dilution of antibody in the presence of constant heat-labile factor on bactericidal activity

Reciprocal of final serum dilution	Serum treatment	CF titre	Log. viable count/ml
10	Unheated		6.23
40	"		6.89
160	"		9.10
640	"		9.84
2560	"		9.78
10240	"		9.90
10	Heated	80	9.33
160	"	< 10	9.33
2560	"	< 10	9.86
10240	"	< 10	9.66
Inoculum			9.91

Dilution of heated serum in the presence of constant heat-labile factor to the point where antibody was no longer detectable in the CF test (dilution of $^{1}/160$) almost completely eliminated the bactericidal activity of the serum. It appeared that the bactericidal reaction was of a similar order of sensitivity as the CF reaction.

In this experiment there were insufficient samples containing antibody to allow evaluation of the plate counting method as a measure of the bactericidal potency of a serum.

DISCUSSION

The reduction in viable count of a suspension of M.mycoides after incubation with an unheated CF-positive serum is not necessarily due to death of the organisms. The failure to obtain inhibition of growth of M.mycoides when sera that were active in the bactericidal reaction were incorporated in solid media supports this. Clumping of the organisms in the presence of the unheated CF-positive serum could be responsible for the observed reduction in the colony count. This possibility was strengthened by the tendency of the organisms to grow as a granular deposit in BVF-OS containing unheated CF-positive sera. When a culture of M.mycoides was mixed with unheated CF-positive serum and observed with a dark-ground microscope, aggregates of organisms were seen to form, making it appear even more probable that agglutination was playing some part in the bactericidal reaction. Attempts to increase the viable count of sera containing clumped organisms by shaking were unsuccessful, and it was shown that the aggregates were difficult to break up by micromanipulation. Since sera heated to 56°C for 30 minutes showed little or no activity, it was postulated that the reaction was probably conglutination rather than simple agglutination. This phenomenon, discovered by Ehrlich and Sachs (1902) and studied by Hole and Coombs (1947) is in effect a rapid and sensitive agglutination, dependent upon the presence of antibody,

complement and a heat-stable factor, conglutinin, which is normally present in bovine serum. The results obtained in this work are consistent with this hypothesis, although the participation of conglutinin is not proven. There is no doubt that a heat-labile factor is necessary for the reaction described here, but at present the relationship of this factor to complement is not known. Conglutination requires the presence of C'1, C'2 and C'4 components of complement (Coombs, Blomfield and Fulton Roberts, 1950). Preliminary attempts to demonstrate the requirement in the bactericidal reaction of heat stable C'4 were inconclusive. As conglutination requires the presence of antibody, the bactericidal activity of sera lacking amounts detectable by the usual serological tests could be explained if the bactericidal reaction is a sensitive test for antibody. This being so, it should be possible to dilute a serologically positive serum to the point where antibody can no longer be detected by other serological techniques, and still demonstrate bactericidal activity. The result of a preliminary experiment (Table 25A) indicated that the sensitivity of the bactericidal reaction was of the same order as that of the CF test on this occasion. However, the sensitivity of the bactericidal reaction could vary with the concentrations of heat-labile factor and of conglutinin present.

While it is realised that the evidence for the requirement of antibody in the bactericidal reaction is not

conclusive, the following observations are consistent with this hypothesis. The appearance of antibody in the serum of cattle vaccinated with M.mycoides was paralleled by the appearance of bactericidal activity. Although many bovine sera without detectable antibody were active in this reaction, the activity was usually of a lower order than in CF-positive sera, and some sera were found to be quite inactive. On the other hand all CF-positive bovine sera tested were active in the bactericidal reaction. This was true of the sera from five cattle which were positive reactors to serological tests for M.mycoides probably as a result of infection with organisms bearing related antigens. The addition of specific polysaccharide to sera that were active in the bactericidal reaction reduced their activity, and no detectable activity remained when M.mycoides organisms were used to absorb a similar serum. It is interesting to note that Osawa and Muschel (1960) found that the bactericidal effect against Shigella dysenteriae of sera that were negative to bacterial agglutination was due to the presence of complement, and of low levels of antibody, although this effect had been attributed by Pillemer et al. (1954) to the action of properdin in the absence of antibody. In the technique used by Osawa and Muschel (1960), immune sera had levels of activity a thousand-fold greater than those of sera referred to as normal.

If the reduction in viable count is due to clumping of the organisms then neither the technique used by Priestley

(1952) nor the use of the plate counting technique employed in this study is likely to give a quantitative estimate of the potency of a serum, unless the number of particles in a clump is directly related to the content of some serum factor. Progress in the understanding of the reaction should follow more quickly when it is possible to test the effect of dilution of one factor in the presence of adequate concentrations of the other factor or factors. This has been hampered by the lack of suitable sera as sources of heat-labile factor.

On occasions there was a reduction in the viable count of the organisms incubated with heated sera whether these contained detectable antibody or not. The order of reduction was usually small compared with that obtained with unheated sera. Whether this is due to slight toxicity of heated sera, to agglutination, to incomplete inactivation of the heat-labile factor or to some other cause is not known.

It is not known whether the phenomenon described here occurs in the animal body. Experiments not mentioned showed that BVF-OS is not an essential component of this reaction. The inocula used usually contained 10^9 viable organisms/ml. As it is unlikely that these numbers would be found in the blood, dilute inocula were tested. Activity was still demonstrable when test sera contained 10^4 ogs/ml. It should also be recalled that the reaction took place in whole defibrinated blood. If the reaction did occur <u>in vivo</u>

one might speculate that the removal of the organisms by phagocytosis might be facilitated, as the number of particles would be substantially reduced by aggregation. There was, however, no correlation between resistance to infection with virulent M.mycoides and the degree of bactericidal activity measured by the plate-counting method in the serum of ten normal animals tested.

Even if the reaction proves merely to be a sensitive test for the presence of antibody, it should be of value in further studies of the Mycoplasma genus and perhaps of other genera.

PART II

STUDIES ON THE INDIRECT HAEMAGGLUTINATION REACTION WITH *MYCOPLASMA MYCOIDES*

INTRODUCTION

The agglutination of erythrocytes by antibodies, chemical substances and extracts of plants has been known for many years. The haemagglutinating property of certain viruses and some bacteria or their products is also well known. These reactions are known as direct haemagglutination.

A distinct phenomenon was demonstrated by Keogh, North and Warburton (1947). These authors showed that when antigens from Haemophilus influenzae were mixed with human erythrocytes the antigens were absorbed to the red cell surfaces and remained attached after the cells were washed to remove free antigen. When homologous bacterial antiserum was added to the modified cells, they were agglutinated. Antigens from many bacteria have been found to be reactive in such a system (Neter, 1956) and this reaction is referred to as "Indirect" or "Passive" haemagglutination (HA) (Neter, 1956). Antigens which attach readily to red cells by simple mixing and incubation are usually of polysaccharide nature. Proteins can be attached by pre-treating the red cells with tannic acid or by providing chemical linkages, e.g. bis-diazotised benzidine (Pressman, Campbell and Pauling, 1942). However, enterobacterial lipo-polysaccharides failed to attach to red cells. Heating to 100°C for 1 hour or treatment with NaOH enhanced the sensitization of red cells (Neter, 1956). It was

suggested that these treatments removed an interfering lipid.

The HA reaction is usually very sensitive, facilitating the demonstration of amounts of antibody and antigen otherwise undetectable. When it was found that the reaction was applicable to M.mycoides, the relationship of HA to existing serological tests and its uses in the examination of antigenic relationships of M.mycoides to other organisms were investigated.

THE HAEMAGGLUTINATION REACTION DEMONSTRATED WITH MYCOPLASMA MYCOIDES

Materials and Methods

The diluent used was saline (0.85% NaCl in distilled water) unless otherwise stated. The HA buffer contained 0.01 M phosphate in saline (pH 7.0).

Sheep red cells stored in Alsever's solution (Cohen, 1951) were washed three times in saline, and packed by centrifugation. One ml of packed cells was added to 24 ml saline and the mixture was added to an equal volume of a 4-day culture of M.mycoides in a semi-defined medium (Rodwell, 1959). After the mixture had stood for 1 hour at 37°C, the red cells were washed twice in saline and re-suspended in 24 ml of saline to make a 4% suspension of treated red cells. Two bovine sera, the one showing complement fixing antibodies to M.mycoides (serum A), and

the other without CF antibody (serum B), were selected. It had been observed that many undiluted bovine sera agglutinated suspensions of sheep red cells. Accordingly these normal agglutinins were absorbed out of the sera. After dilution to 1/5 with saline, the sera were heated to 56°C for 30 minutes to prevent lysis by complement during absorption which was carried out by adding two drops of washed packed sheep red cells per ml of diluted serum and incubating the mixture for 1 hour at 37°C. The serum was then separated from the red cells by centrifugation, and was further diluted in saline by twofold steps. One drop of each serum dilution was placed on one square of a ruled glass plate with a Pasteur pipette. To each drop was added 1 drop of the 4% treated red cell suspension. As controls, one drop of sensitized cells was added to one drop of saline, and one drop of 1/5 serum dilution was added to one drop of untreated 4% red cells. The plate was tilted back and forth to mix the drops.

Results

Immediate clumping of red cells was observed in the higher concentrations of serum A. Two or three large aggregates contained most of the red cells, giving the illusion of flakes of red enamel paint. Clumping was slower with successive serum dilutions and the reaction was substantially complete in 5 minutes after mixing took place. Haemagglutination did not occur in the mixture containing

dilutions of serum B. The controls showed no haemagglutination.

The reactions were graded from negative to ++++(4) according to the number of red cells agglutinated. Although the readings were subjective, the one operator after practice could classify the grades of haemagglutination in a reproducible manner. The end point was taken as that dilution in which about half of the red cells remained unagglutinated. The temperature and humidity vary throughout the year but comparisons were made chiefly on the one day or under conditions that were similar in these respects. Standard sera were included where necessary.

Suspensions of sensitized red cells in saline or HA buffer in all the tests done showed no auto-agglutination.

Substances capable of sensitizing erythrocytes

At the time this work was commenced the chemical microbiology section of the C.S.I.R.O. Animal Health Research Laboratory, Parkville, was studying fractions isolated from M.mycoides. Preparations ranging from cultures of M.mycoides in a semi-defined medium (Rodwell, 1959) to the purified polysaccharide isolated from the organism (Buttery and Plackett, 1960) were obtained from the section. Sheep red cells were sensitized with crude carbohydrate fractions and by the purified polysaccharide. Whole cultures in BVF-OS medium failed to sensitize cells.

This medium contains 10% bovine serum. M.mycoides centrifuged from this medium, washed twice in 0.4 M sucrose phosphate and subsequently frozen and thawed was capable of sensitizing the red cells.

Amount of sensitizing substance

(a) Whole culture

Serial twofold dilutions from 1/2 to 1/128 in saline were made of M.mycoides cultures in the semi-defined medium (Rodwell, 1959). One volume of each dilution was used to sensitize one volume of 6% washed sheep red cells by the method described. After washing, the cells were suspended to 4% and used to test a standard serum containing CF antibody to M.mycoides (Table 26).

The results (Table 26) indicated that the titre of the serum was greatest with cells treated with the highest concentration of sensitizing substance. Thus, to obtain the maximum titre with this material a dilution of 1/2 or even undiluted substance would have to be used. As cultures may vary in content of sensitizing substance, results could only be comparable within a single batch of culture.

TABLE 26

The effect of the concentration of culture used to sensitize sheep erythrocytes on the HA titre

Dilutions of culture	Serum dilutions†						
	16	32	64	128	256	512	1024
2	4	4	4	4	2	1	-
4	4	4	4	3	1	-	-
8	4	4	4	3	-	-	-
16	4	4	4	3	-	-	-
32	4	4	2	-	-	-	-
64	4	3	2	-	-	-	-
128	3	2	1	-	-	-	-
C*	-						

† In this and subsequent tables the dilutions are given as reciprocals.

* Control of normal red cells and highest serum concentration.

(b) Polysaccharide isolated from M.mycoides

Serial doubling dilutions of polysaccharide were made in saline and to 1 volume of each dilution, 1 volume of 4% sheep red cells was added. After incubation of the mixtures at 37°C for 2 hours, the cells were separated by centrifugation, washed twice in saline and then resuspended in saline to 4%. These suspensions of sensitized cells were used in a slide HA test with dilutions of a known positive serum.

TABLE 27

The effect of the concentration of polysaccharide used to sensitize sheep erythrocytes on the HA titre of serum B20

Polysaccharide (µg/ml)	Serum dilutions							
	10	20	40	80	160	320	640	1280
0.16	-	-	-	-	-	-	-	-
0.32	1	-	-	-	-	-	-	-
0.65	4	2	-	-	-	-	-	-
1.3	4	4	4	1	-	-	-	-
2.6	4	4	4	4	3	-	-	-
5.2	4	4	4	4	4	3	1	-
10.4	4	4	4	4	4	4	2	-
20.8	4	4	4	4	4	4	2	-
Control	-							

It appeared that increasing the concentration of polysaccharide above 10.4 µ g/ml failed to increase the HA titre obtained (Table 27). This concentration was referred to as the optimal sensitizing dose.

Conditions for sensitization

Initially, red cells were placed in contact with sensitizing substance for 2 hours at 37°C. Subsequently, it was found that incubation for longer than 90 minutes at 37°C failed to increase the HA titre.

Standardisation of erythrocyte suspensions

In preliminary trials, 4% and 6% cell suspensions were made by pipetting the appropriate amounts of packed washed red cells to polysaccharide dilution. Later the method of standardising red cell suspensions in use in this laboratory for the complement fixation test for bovine contagious pleuropneumonia (CPP) (Campbell and Turner, 1936) was used. A 6% suspension was made up, and 1 ml lysed by the addition of 49 ml of 0.37% ammonia solution. The haemoglobin content was determined by light absorption using an E.E.L. absorptiometer and filter No.625. The red cell suspension was diluted to contain 1.7 g Hb./100 ml. The standardised suspension was referred to as a 6% cell suspension. Sensitization of cells was then effected by mixing equal volumes of 6% cell suspension with appropriate polysaccharide dilutions.

Erythrocytes of other species

Rabbit, fowl, goat and bovine erythrocytes were sensitized with a range of concentrations of M.mycoides polysaccharide. The HA titres of standard positive sera tested with these sensitized cells are shown in Tables 28, 29, 30 and 31. As these tables and Table 26 show, amounts of polysaccharide from 10 to 30 µg/ml sufficed to sensitize sheep, rabbit, fowl, goat and bovine red cells.

Horse and sheep cells were sensitized with 20 µ g/ml

polysaccharide and tested against the same standard serum (B20). The titre with sheep cells was 1/320 and with horse cells was 1/1280. When amounts of polysaccharide greatly in excess of 30 μg/ml were used to sensitize fowl and bovine red cells, the titre apparently decreased. This phenomenon was not investigated, but a possible explanation is tentatively advanced. The washing procedure, adequate for removing unattached polysaccharide when low concentrations were present, was inadequate when greater concentrations were used. Free polysaccharide could thereby inhibit the reaction and reduce the apparent titre.

TABLE 28

The effect of concentrations of polysaccharide used to sensitize rabbit erythrocytes on the HA titre with serum B20

Polysaccharide (μg/ml)	Serum dilution							
	10	20	40	80	160	320	640	1280
2.7	4	4	4	3	1	-	-	-
3.6	4	4	4	3	1	-	-	-
4.7	4	4	4	3	2	-	-	-
6.3	4	4	4	4	2	1	-	-
8.4	4	4	4	4	3	2	-	-
11.2	4	4	4	4	3	2	-	-
15.0	4	4	4	4	4	3	2	-
20.0	4	4	4	4	4	3	2	-
Control	-							

TABLE 29

The effect of concentrations of polysaccharide used to sensitize fowl erythrocytes on the HA titre with serum M79

Polysaccharide (μ g/ml)	Serum dilution								
	10	20	40	80	160	320	640	1280	2560
3	4	4	4	4	3	2	-	-	-
6	4	4	4	4	4	3	1	-	-
12	4	4	4	4	4	4	4	1	-
24	4	4	4	4	4	4	3	1	-
47	4	4	4	4	4	4	3	1	-
95	4	4	4	4	4	3	1	-	-
190	4	4	4	4	3	2	1	-	-
Control	-								

TABLE 30

The effect of concentrations of polysaccharide used to sensitize goat erythrocytes on the HA titre with serum M79

Polysaccharide (μ g/ml)	Serum dilution					
	10	20	40	80	160	320
0.7	-	-	-	-	-	-
1.5	2	1	-	-	-	-
3	3	2	1	-	-	-
6	4	4	3	1	-	-
12	4	4	3	1	-	-
24	4	4	3	2	-	-
47	4	4	3	2	1	-
95	4	4	3	2	-	-
190	4	4	3	2	-	-
Control	-					

TABLE 31

The effect of concentrations of polysaccharide used to sensitize bovine erythrocytes on the HA titre of sera B20 and 24

Serum no.	Polysaccharide (µg/ml)	Serum dilution										
		10	20	40	80	160	320	640	1280	2560	5120	10240
B20	3	4	4	4	4	3	-	-	-	-	-	-
	7.5	4	4	4	4	4	4	3	1	-	-	-
	15	4	4	4	4	4	4	4	3	-	-	-
	30	4	4	4	4	4	4	4	4	1	-	-
	60	4	4	4	4	4	4	4	4	1	-	-
	120	4	4	4	4	4	4	4	3	-	-	-
	Control	-										
24	3	4	4	4	4	4	4	4	3	1	-	-
	7.5	4	4	4	4	4	4	4	4	4	2	-
	15	4	4	4	4	4	4	4	4	4	3	1
	30	4	4	4	4	4	4	4	4	4	4	1
	60	4	4	4	4	4	4	4	4	4	4	1
	120	4	4	4	4	4	4	4	4	4	2	-
	Control	-										

THE USE OF PLASTIC TRAYS FOR HAEMAGGLUTINATION REACTIONS

The incubation period of tests carried out on a glass plate was restricted by evaporation of the fluid. An alternative method was to use larger volumes contained in tubes or in cavities pressed out in plastic haemagglutination trays (supplied by Acrylic Industries, Melbourne).

General method

The reagents were prepared in the manner described for the slide test. Dilutions of serum were made in the wells. To 0.5 ml volumes of serum dilution, 0.5 ml of sensitized red cell suspension was added. The tray was shaken to allow the contents of the wells to mix and allowed to stand covered with polythene sheeting, at room temperature for about 16 hours (overnight).

Selection of sensitized red cell concentration

Dilutions of a reference serum were tested against concentrations of sensitized red cells varying from 0.125% to 2%.

Although the sensitivity increased with decreasing concentrations of sensitized red cells, the reactions were difficult to read when the concentration of red cells was less than 0.5%.

TABLE 32

The effect of the concentration of sensitized red cells on the HA titre

Sensitized red cell concentration (per cent)	Serum dilution								
	32	64	128	256	512	1024	2048	4096	SRC + diluent
2	+	+	+	+	+	-	-	-	-
1	+	+	+	+	+	+	-	-	-
0.5	+	+	+	+	+	+	-	-	-
0.25	+	+	+	+	+	+	+	-	-
0.125	+	+	+	+	+	+	+	-	-
Serum control	-								

Reading of tests in plastic trays

Red cells settled to the bottom, giving rise to different patterns according to the degree of agglutination. Untreated red cells characteristically settle into a compact round button with sharply defined edges, leaving a clear supernatant. The sheep cell suspensions used required about 4 hours to settle adequately. This was not convenient, so a period of overnight settling was allowed.

In a negative reaction, the red cells settled to the bottom of the well as a compact disc with clearly defined edges and the supernatant fluid was clear.

The positive reactions were broadly divided into two types:-

(i) Agglutinated cells settled to the bottom of the well into a disc with feathery edges and left the supernatant clear. This occurred in the presence of strong concentrations of serum.

(ii) The cells settled as a carpet or sheet over the hemispherical bottom of the well. The edges of the sheet often had a crinkled appearance. Gentle tapping increased the degree of crinkling. The carpet appearance could occur in the strongest serum concentrations, but more frequently followed type (i) reaction, separating that type from a negative reaction.

Difficulty was experienced in precisely determining the end point of haemagglutination. It was found, however, that when the plastic tray was tilted to about 60° from the horizontal the difference in behaviour of the cell clumps of positive and negative reactions was apparent. The cells comprising the button of a negative reaction would flow along the tilted side of the well to form a "parachute" appearance within 5 minutes. On the other hand the red cells in a positive reaction did not flow in this way but remained as a central disc or became folded into a half disc. The end point was that dilution in which the cells had flowed half way to the rim of the well, in the time that the cells in the control well had reached the rim. Usually with doubling serum dilutions, only one well showed the intermediate pattern between the positive and the negative pattern.

RELATIONSHIP OF HA TO OTHER SEROLOGICAL TESTS

The results of testing sera from cattle after exposure to M.mycoides by agglutination, CF and HA tests, indicated that whenever the CF test was positive, so was the HA test. Agglutinins were not always detectable despite the presence of high CF and HA titres. Thus to determine the relationship between HA and agglutination and between HA and CF, it was necessary to absorb or to neutralize the activity to one of the tests.

Absorption of sera

(a) Using red cells sensitized with culture of M.mycoides. Sheep red cells sensitized with 4-day-old culture of M.mycoides in a semi-defined medium (Rodwell, 1959) were packed by centrifugation and added to 1/10 dilution of a serum which contained antibody detectable in all three tests.

The mixture was shaken several times during 15 minutes' incubation at room temperature, then the cells were removed by centrifugation. This was repeated until no slide haemagglutination was obtained with the serum. After three absorptions, no HA was demonstrable in the serum which was then tested by agglutination and complement fixation. A similar quantity of 1/10 serum dilution of M79 was treated with normal red cells.

TABLE 33

Effect of absorption of bovine serum M79 with red cells sensitized with culture on serological titres

Test	Serum absorbed with	
	Normal red cells	Sensitized red cells
HA	100	< 10
AGGL	40	< 40
CF	1280	1280

It appeared then that both agglutinins and haemagglutinins were either reduced or removed by treatment with sensitized cells, but the CF activity was unchanged. This suggested that there was a close relationship between the haemagglutinins and the agglutinins, but not between these and complement fixing antibody.

There was insufficient serum after absorption to test the tube agglutination at stronger concentrations than 1/40, but the reaction at that dilution was negative (Table 33).

(b) Using red cells sensitized with polysaccharide from M.mycoides. When a supply of M.mycoides polysaccharide became available, cells sensitized with it were used to absorb further sera. Some of these tests failed to provide conclusive information usually because of insufficient absorption. The indications (Table 34) of two of these

were that CF antibody might be reduced by absorption with sensitized red cells.

TABLE 34

Effect of absorption of sera with red cells sensitized with polysaccharide on HA and CF titres

Serum	Test	Serum absorbed with	
		Normal red cells	Sensitized red cells
369	HA	128	< 8
	CF	256	16
53	HA	128	< 8
	CF	22	< 8

There was a decrease in the CF titres of sera 369 and 53 after absorption with sensitized red cells, and the HA activity was reduced below the detectable level in both. A closer dilution series was required to assess the titres more accurately. Dilutions, based on an arithmetic series, were made of serum 410 before and after absorption with sensitized red cells.

TABLE 35

HA and CF titres on serum 410 before and after absorption with red cells sensitized with polysaccharide

Test	Serum absorbed with	
	Normal red cells	Sensitized red cells
HA	208	< 16
CF	880	208

The use of formalized red cells

Formalin treated red cells were prepared by the method of Te Punga (1959) and optimally sensitized in the usual way with polysaccharide. The serum titre obtained was the same with these cells as with untreated sensitized cells. Packed sensitized formalized cells were then used to absorb serum. Six absorptions using a total of 5 ml packed sensitized cells were made on a reference serum.

TABLE 36

The effect of absorption of B20 serum with formalized cells sensitized with polysaccharide on serological titres

Test	Serum absorbed with	
	Formalized cells	Sensitized formalized cells
HA	320	< 10
CF	640	40
AGG	40	< 10

Again CF activity was reduced while HA and agglutinating activity were removed.

The reduction in HA titre effected by absorption was not necessarily proportional to the reduction in CF titre, but apparently the activity to both tests could be removed by absorption with red cells sensitized with polysaccharide from M.mycoides.

Neutralization

The addition of polysaccharide from M.mycoides to serum had been shown to inhibit HA. It was thought that if sufficient were added to neutralise the HA activity, the CF activity might also be reduced or removed.

Two aliquots of a serum (+ve to HA and CF tests) diluted 1/10 with saline were inactivated and absorbed with normal sheep red cells. Polysaccharide was added in successive small amounts to one aliquot and equivalent volumes of saline were added to the other. After each addition, the mixtures were incubated at 37^{o} for 1 hour. A total of 1.6 ml of polysaccharide (1900 μg/ml) was added to the 2 ml test aliquot, and 1.6 ml of saline to the control. The precipitate formed by antigen and antibody reacting in this system was not removed. After the final incubation period, serial doubling dilutions of both mixtures were made and tested by HA, CF and tube agglutination.

TABLE 37

Titres to HA, CF and agglutination tests of serum J14 after neutralization with polysaccharide

	Test	Control
HA	< 19	19456
AGG	< 19	< 19
CF	1216	4864

There was a reduction in HA activity amounting to almost complete removal, but the CF activity decreased only to one-fourth the original activity (Table 37).

Several similar tests on sera with titres of the order of 20 to the CF test and 160 to HA test have shown that both CF and HA activity can be removed to undetectable levels. The results of these and other tests indicate that the degree of reduction in both systems is not linearly related. Ratios of degree of reduction in CF activity to degree of reduction in HA activity vary from 1 to 2.5 to 1 to 24.

Dafaalla (1957) has shown that the serum from animals acutely infected with CPP may contain antigen detectable in the precipitin ring test. Coincident with the appearance of antigen, the sera lose the capacity to agglutinate a heated suspension of M.mycoides.

The tube agglutination test is rarely positive in high dilutions of serum from infected animals. During the course of infection, the test becomes negative at 1/10 dilution while the CF test may still be positive at high dilutions. It has been considered that the type of antibody reacting in these two tests may be different. That the presence of antigen in the blood lowers the agglutination titre but not the CF titre was noted *in vitro* when small amounts of polysaccharide from *M.mycoides* were added to serum from infected animals. Further additions lowered the CF titre as well as tube agglutination and HA titres. Yet there are sera which, although containing no detectable antibody, will react in high dilutions to CF and HA tests, e.g.

Test	Titre
HA	5120
CF	5120
AGGLN	-

The difference between HA and agglutination could be merely quantative - the agglutination test being a much less sensitive detector of antibody than the CF or HA tests. Circulating antigens would reduce the antibody content of a serum to a level undetectable in the agglutination test but detectable in the CF and HA tests.

Neter (1956) in discussing the indirect bacterial haemagglutination test mentions its sensitivity compared with the agglutination test and says, "Differences may be due in part to differences in the amount of antigen present on the surface of bacteria and of modified red cells. At times the superiority of the HA method is of such magnitude as to appear qualitative rather than quantitative". This author quotes Gaines and Landy (1955) who detected HA antibodies at dilution of 1/960 when no bacterial agglutinin could be demonstrated. Neter found, with Escherichia coli, HA titres 5 to 20 times those of corresponding titres of bacterial agglutinin.

"An explanation of these differences must await the results of future research." (Neter, 1956).

OBSERVATIONS ON THE SERA OF CATTLE EXPOSED TO LIVING CULTURE OF M.MYCOIDES

Living culture of M.mycoides was injected intradermally into the tail tip of 20 cattle in 0.2 ml amounts. The antibody response which followed was measured by HA, standard CF and tube agglutination tests, on blood samples taken periodically. The serological titres obtained provide a way of comparing the response between animals, between days and between tests.

(a) Between animals and between days

It is difficult to obtain a simple meaningful expression to score an individual response, made up as it

is of the variables of time and degree of reaction. The graphs of titres from individual animals may be shown as in Figure 1. The choice of any animal as typical of the group remains subjective. The group mean titres to each test for each sampling can be plotted as in Figure 2, but this gives a pattern to which that of no single individual may conform.

(b) Between tests

Our purpose was primarily to compare the ability of each test to demonstrate antibody present in serum. The between tests comparison seems to be best made by taking in turn the end point titres found in one test and plotting the range of titres given by the other tests on each occasion.

Methods

In each test, serial doubling dilutions from $^{1}/_{10}$ were used. To represent the end point, a scoring method combining the dilution and degree of reaction was evolved by Dr. F. E. Binet. In this system 50 per cent end points (2+) occurring at dilutions $^{1}/_{10}$, $^{1}/_{20}$, $^{1}/_{40}$ for example, were represented by the digits 0, 1, 2 respectively, etc. When the 50% end point fell between dilutions the score was obtained by interpolation. According to the degree of reaction the following increments were added:

Fig. 1

RESPONSE OF ONE ANIMAL TO C F, H A AND AGGLUTINATION.

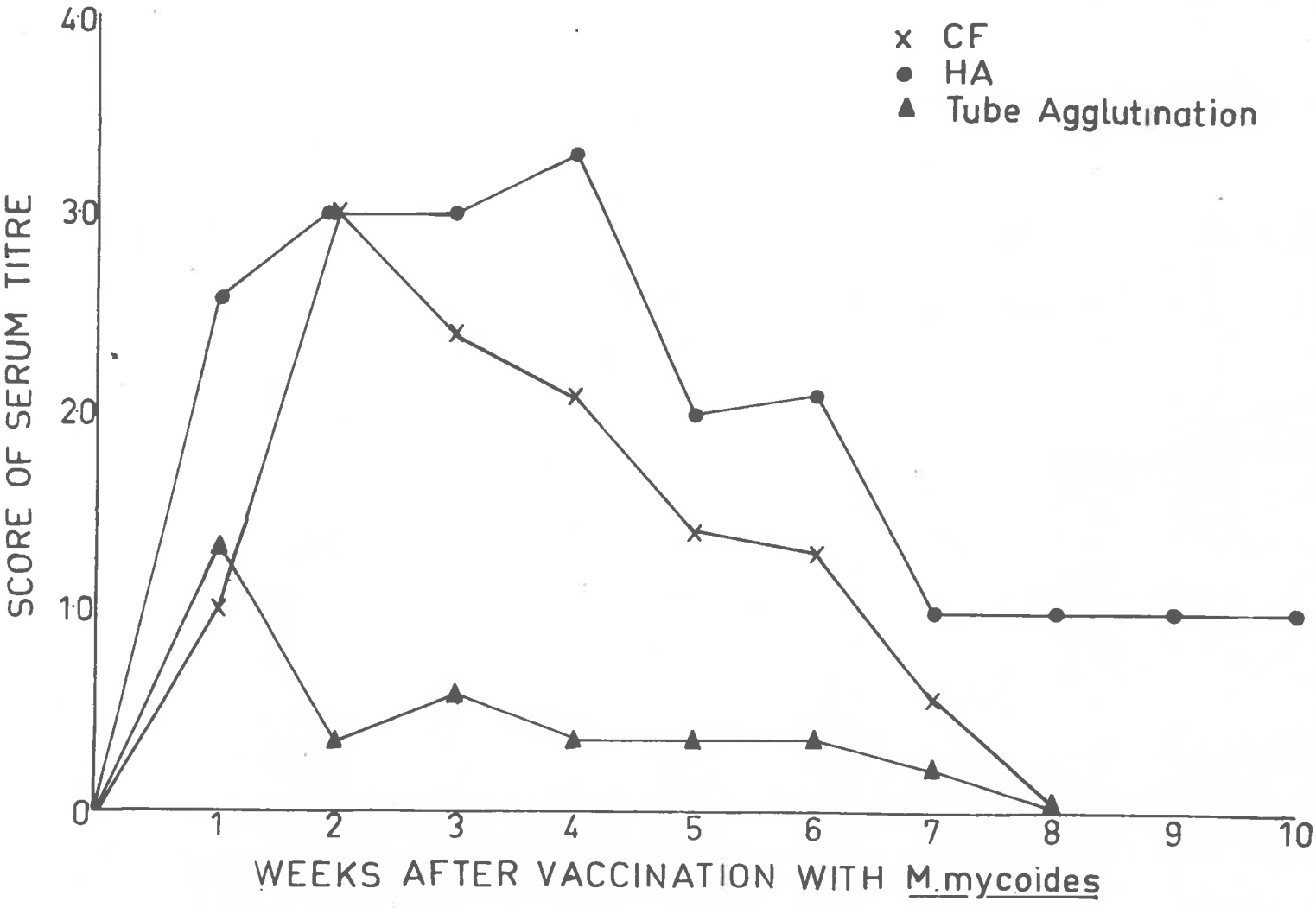
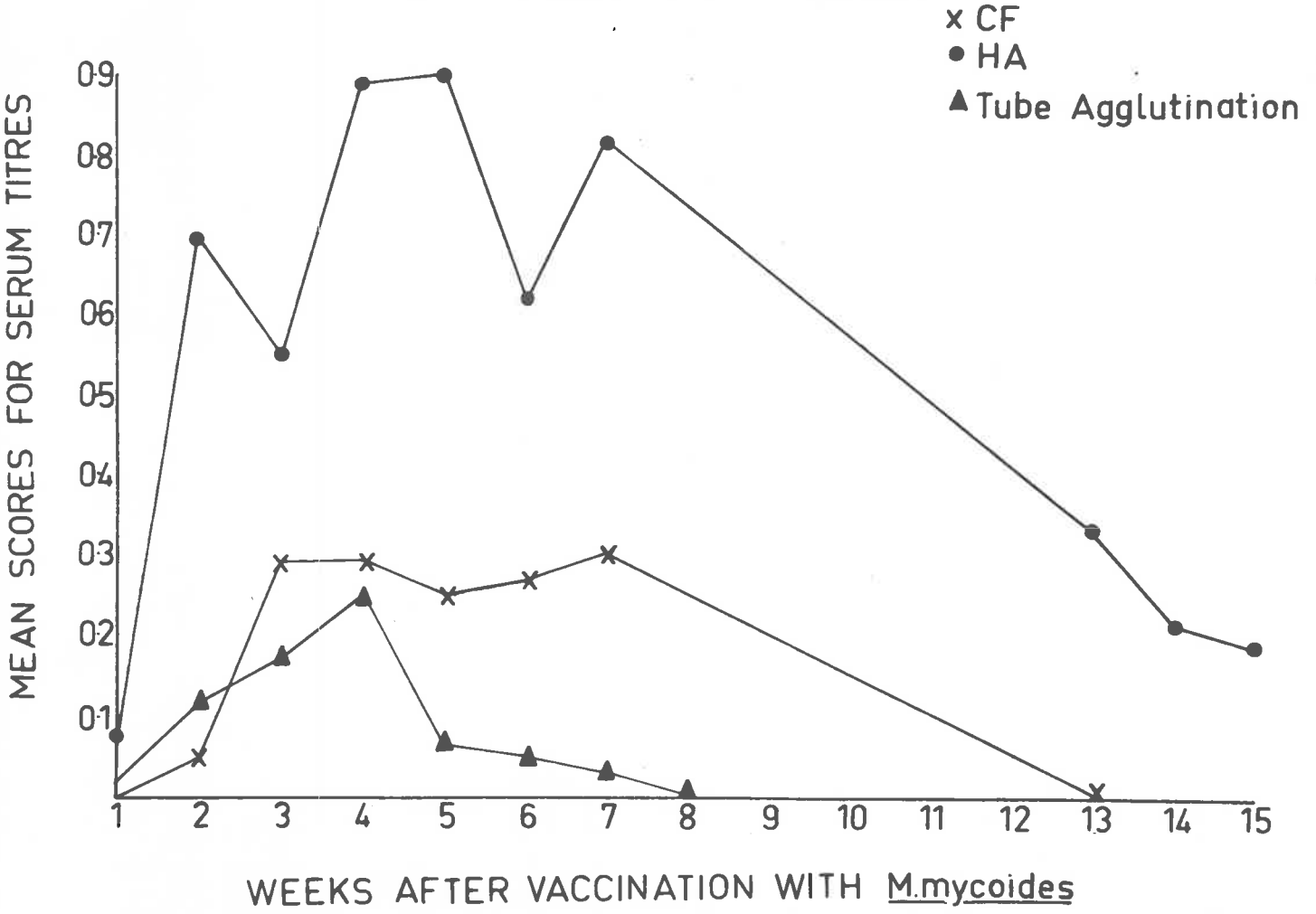


Fig.2

COMPARISON OF THE RESPONSE TO C.F, HA AND AGGLUTINATION
(MEAN SCORES OF 20 ANIMALS)



Degree of reaction in last two dilutions		Increment
3	-	.21
3	Trace	.29
4	-	.32
3	1	.42
4	Trace	.42
4	1	.56

For example:

10	20	40	80	Total score
4	4	2	-	2.0
4	4	3	1	2.42

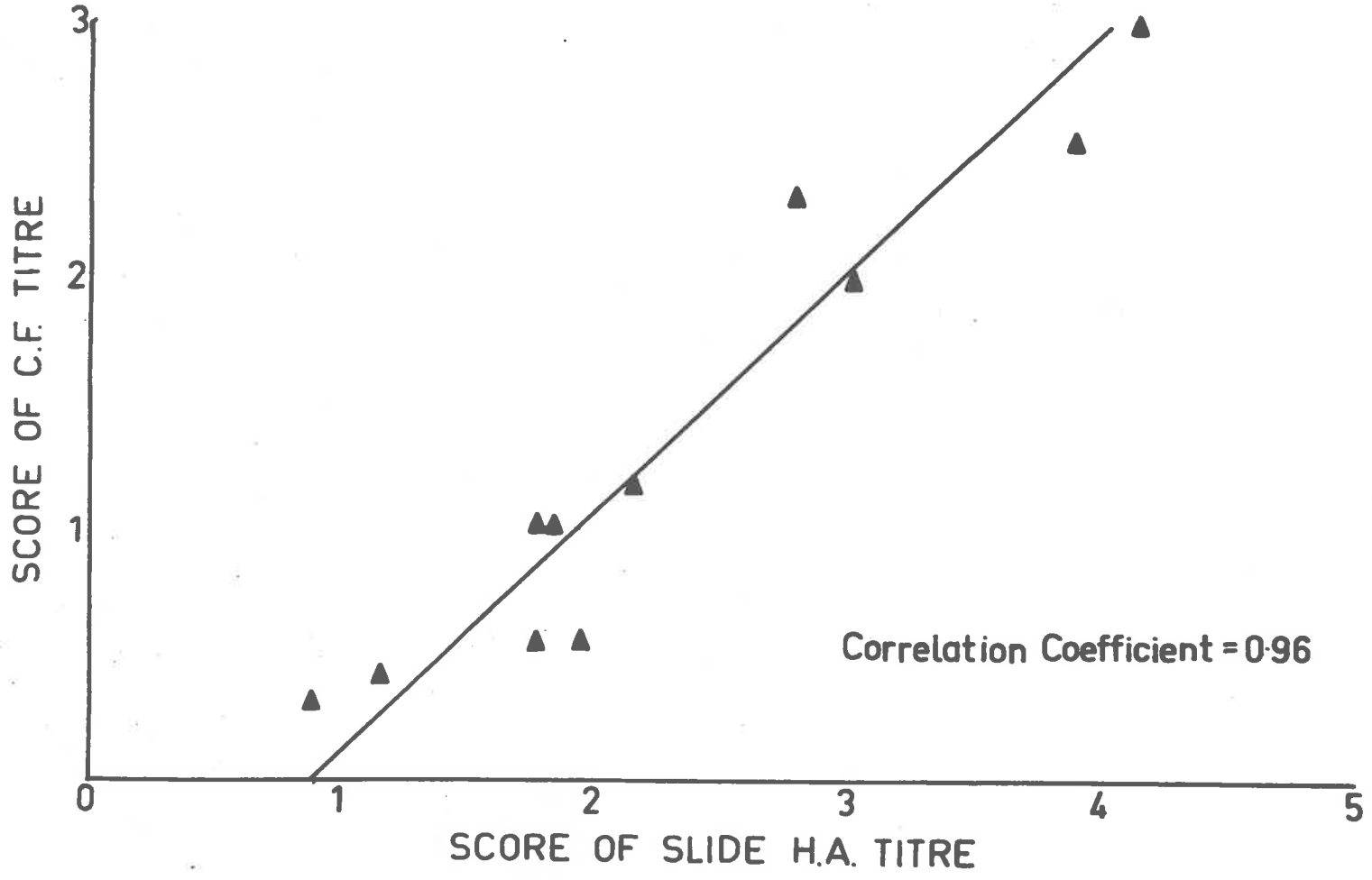
Mean titres were then calculated as arithmetic means of the scores.

(i) A comparison was made between CF titres and the corresponding slide HA titres on the same sera. Each slide HA score is the mean of three or more observations. The correlation coefficient $r = 0.96$ which for 9 degrees of freedom is significant at the 0.01 level. The least squares line (Figure 3) illustrates that the slide HA titres were higher than the CF titres to which they correspond.

(ii) The CF titres were compared with the tube agglutination titres on the same sera. As might be expected from previous observations on the tube agglutination test (Page 70) there was no overall correlation between the titres to these two tests.

Fig.3

COMPARISON OF C F AND SLIDE HAEMAGGLUTINATION TESTS



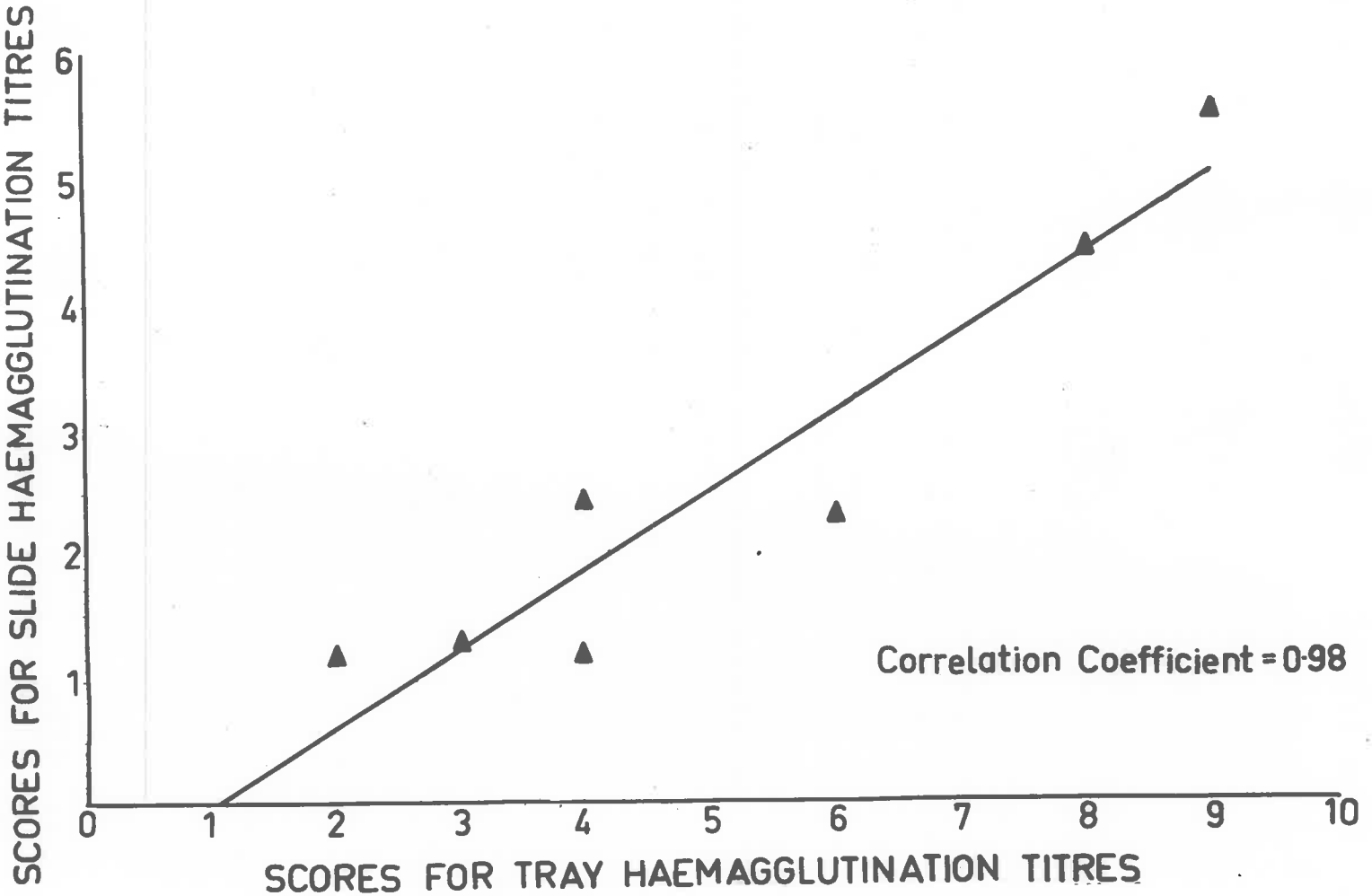
(iii) Comparison of haemagglutination titres obtained by the slide and by the tray methods.

Seven sera were tested by both methods on the one day. When the titres to each test were compared, the correlation coefficient was found to be r = 0.98 which for five degrees of freedom was significant at P = 0.01 (Figure 4). The results of two further sets of bleedings examined in the same way showed that they were significantly correlated. When the mean scores to each test on the seven sera were compared between days, they were found to be perfectly correlated (r = 1). The ratio of slide HA score to tray HA score was 1 : 2. Such consistency between days was not always shown, however, even though there was consistent correlation between the two tests on the one day. Small variations in titre from day to day are not uncommon in serology, especially when twofold serial dilutions are made. Despite this, the finding of some inconsistencies emphasizes the need for careful standardisation of the test if reproducible results are to be obtained.

From the foregoing information, it may be seen that there is conformity between the CF and slide HA titres and between HA and tray HA titres. The gradation of sensitivity in descending order is tray HA > slide HA > CF. However, despite the usefulness of a sensitive test in diagnostic or experimental works, specificity is a major requirement. HA tests in trays have rarely if ever been completely negative

Fig. 4

COMPARISON OF SLIDE AND TRAY HAEMAGGLUTINATION TITRES



at the 1/10 dilution. Normal animals show tray HA titres of the order of 1/40 as might be expected from consideration of Figure 4. The slide test has slightly greater sensitivity than the CF test as performed in this laboratory (Campbell and Turner, 1953), and could be expected to give positive reactions in low dilutions when the CF test is weakly positive or just negative. The determination of a "specific" titre can never be absolute but only negative. If sufficient normal animals were tested, such a "specific" titre could be assessed, above which all reactions could be classed as positive. Neither the HA nor the CF test can distinguish cross reactions from specific reactions.

INHIBITION OF HAEMAGGLUTINATION

Substances used for sensitizing red cells when mixed in suitable concentration with antisera were found to inhibit the haemagglutinating activity of the sera. This demonstrated the specific nature of haemagglutination and offered a method of detecting antigen.

Two methods of testing substances for the presence of antigen were available:-

1. A single concentration of antigen containing material was mixed with successive dilutions of serum, allowed to react, and the haemagglutinating activity of the serum dilution compared with a similar set of dilutions to which diluent had been added.

2. A certain dilution of serum, usually 4 times the concentration at the HA end point, was mixed with dilutions of the unknown, allowed to react and then the haemagglutinating activity of the serum dilution tested by addition of sensitized red cells.

In both cases the neutralizing activity of the unknown antigen preparations was compared with the activity of standard polysaccharide concentrations. Cultures of M.mycoides in serum broth did not sensitize cells, possibly because of the serum content of the medium, but inhibited HA activity of positive serum.

During the preliminary stages of fractionation of M.mycoides by Buttery and Plackett, use of the haemagglutination inhibition reaction permitted the estimation of antigenic activity in the fractions examined. Subsequent to the isolation of polysaccharide (galactan) in relatively pure state, the effect of various treatments designed to yield information on the linkage of the hexose units was measured by haemagglutination inhibition.

Since this work was carried out in collaboration with Dr. Plackett and Mr. Buttery it will not be reported here.

Amount of polysaccharide detected by haemagglutination inhibition

Slide haemagglutination inhibition tests in which

dilutions of polysaccharide were mixed with equal volumes of a dilution of serum corresponding to 4 times the HA end point, showed that amounts of the order of 5 - 10 µg/ml of homologous polysaccharide were detected.

CROSS REACTIONS

Although the CF test described by Campbell and Turner (1936) for the diagnosis of CPP is remarkably specific, very rare false positive reactions do occur. Turner (1956) reported that a strain of Actinobacillus lignieresi was capable of provoking in cattle and rabbits, antibodies that fixed complement with M.mycoides. This strain is referred to as Smith 40 and is the only strain used in this work. Provost (1958) described a serological relationship between the virus of cowpox and M.mycoides. This author also describes the sharing of antigens among several Mycoplasma species and suggests an antigenic classification that could be applied to these strains (Villemot and Provost, 1959b).

Complement fixing antibody to M.mycoides has been demonstrated in this laboratory in sera from several cattle with no clinical sign of CPP. There is no reason to believe that these cattle had been vaccinated with M.mycoides or had ever contacted CPP which has not been detected in this State for 4 years. From one animal whose serum showed a positive CF test with M.mycoides a Mycoplasma strain was isolated

from the vagina. Various Mycoplasma strains were collected from within Australia and overseas and some of these together with some local isolates were examined. It was considered that the HA reaction could assist in the study of cross reactions, particularly in those cases where only unstable suspensions unsuitable for agglutination tests were obtained.

A.lignieresi (Smith 40)

When a preparation of polysaccharide obtained from this strain of A.lignieresi was used to sensitize red cells, the cells were agglutinated by homologous antiserum but not with M.mycoides antiserum. On the other hand, red cells sensitized with polysaccharide from M.mycoides were agglutinated by homologous antiserum and by the A.lignieresi antiserum.

Tube agglutination tests (Campbell, 1938) and CF tests using boiled microbial antigens also displayed this cross reactivity. The titres obtained are expressed as the reciprocal of the highest dilution showing approximately 50 per cent activity in Table 38.

The A.lignieresi CF antigen prepared from this strain lacked activity against homologous antiserum. The A.lignieresi HA antigen, however, although active against homologous antiserum, failed to react against M.mycoides, the heterologous antiserum.

TABLE 38

Cross serological reactions with M.mycoides and A.lignieresi (strain Smith 40)

Antigen	Antiserum	Test		
		CF	HA	AGG
M.mycoides	M.mycoides	1280	2560	640
	A.lignieresi	40	320	320
A.lignieresi	M.mycoides	< 10	< 10	40
	A.lignieresi	< 10	160	160

HA inhibition tests, carried out by first mixing inhibitor with serum, then adding the appropriate sensitized red cells, gave the results shown in Table 39.

TABLE 39

The effect of cross inhibitions on HA reactions with M.mycoides and A.lignieresi (Smith 40)

Antiserum	Inhibitor	Red cells sensitized with	Presence of haemagglutination
A.lignieresi	A.lignieresi	A.lignieresi	Nil
"	A.lignieresi	M.mycoides	Nil
"	M.mycoides	M.mycoides	Nil
"	M.mycoides	A.lignieresi	Present
M.mycoides	M.mycoides	M.mycoides	Nil
"	A.lignieresi	M.mycoides	Present

Further, absorption of the A.lignieresi antiserum with red cells coated with M.mycoides polysaccharide removed all HA reactivity to M.mycoides sensitized cells, but the absorbed serum still agglutinated red cells sensitized with A.lignieresi. Also, absorption of A.lignieresi antiserum with red cells coated with A.lignieresi polysaccharide removed all HA reactivity to these cells but the absorbed serum still agglutinated red cells sensitised with M.mycoides. This is not in accordance with the result of inhibition shown in Table 39.

These apparently anomalous results could be explained if one first postulates that A.lignieresi (strain Smith 40) polysaccharide contains one antigen not related to any present in M.mycoides and one that is closely related to an M.mycoides antigen. If one further postulates that when red cells are sensitized with A.lignieresi polysaccharide this common antigen is masked or rendered unreactive, then, although there was sufficient of the common antigen in the polysaccharide used as an inhibitor with A.lignieresi serum (Table 39) to prevent any subsequent reaction to M.mycoides, with this serum little or no antibody to the common antigen was removed by cells sensitized with this polysaccharide. This postulate would also explain the lack of HA reaction with M.mycoides antiserum (Table 38).

A second preparation of A.lignieresi polysaccharide made by Dr. P. Plackett and Mr. S. H. Buttery from the

Smith 40 strain was tested. Dilutions of polysaccharide were prepared in HA buffer and used to sensitize sheep red cells as described above. No haemagglutination occurred when these cells were mixed with A.lignieresi antiserum. The polysaccharide had failed to sensitize the red cells. The polysaccharide was heated for 60 minutes at 56°C in the presence of 0.25 M. Na OH, cooled and neutralized. Doubling dilutions in HA buffer of treated polysaccharide containing 490 µg/ml to 1 µg/ml were used to sensitize sheep red cells. The sensitized cells were washed in HA buffer and used to test dilutions of A.lignieresi antiserum and M.mycoides antiserum. On this occasion (Table 40) the cells sensitized with A.lignieresi polysaccharide were agglutinated by M.mycoides antiserum.

TABLE 40

HA reactions with A.lignieresi (strain Smith 40) and M.mycoides antisera and red cells sensitized with A.lignieresi polysaccharide

Heated A.lignieresi polysaccharide (µg/ml)	Dilutions of A.lignieresi antiserum							Dilutions of M.mycoides antiserum			
	10	20	40	80	160	320	640	10	20	40	80
500	4	4	4	4	2	-	-	4	4	3	-
250	4	4	4	4	3	2	-	4	4	3	-
125	4	4	4	4	3	2	-	4	4	3	-
62.5	4	4	4	4	3	2	-	4	4	2	-
31.2	4	4	4	4	3	1	-	4	4	1	-
15.6	4	4	4	4	3	1	-	4	4	-	-
7.8	4	4	4	4	3	1	-	4	4	-	-
3.9	4	3	3	2	1	-	-	2	-	-	-
1.9	3	2	1	-	-	-	-	1	-	-	-

Controls of (a) normal red cells and serum were negative

(b) sensitized red cells and negative serum were negative.

This second A.lignieresi polysaccharide preparation differed then by

1. Requiring heat + alkali prior to sensitization
2. Reacting with M.mycoides antiserum.

Heating to 56°C for 60 minutes in the presence of 0.25 N Na OH destroyed the capacity of M.mycoides to sensitize sheep red cells. No other A.lignieresi strains have been examined in this way.

Mycoplasma species isolated from goats

O Goat strain was isolated from a goat in New Guinea, and the Y Goat strain from Queensland (Laws, 1956). Both strains grew well in the semi-defined medium (Rodwell, 1959) and cultures were capable of sensitizing red cells (Table 41). The reactions obtained with these sensitized red cells in HA tests with antisera to M.mycoides and to the Y Goat strain are shown in Table 41 with the corresponding agglutination and CF test results.

It can be seen (Table 41) that both goat strains showed considerable cross reactivity with M.mycoides antiserum.

TABLE 41

The titres of antisera to M.mycoides and the Y Goat strain in HA, CF and agglutination tests with M.mycoides, Y Goat and O Goat antigens

Antigen	Test					
	HA		Aggl.		CF	
	Antiserum		Antiserum		Antiserum	
	Y Goat	M. mycoides	Y Goat	M. mycoides	Y Goat	M. mycoides
Y Goat	10	2560	80	160	†	†
O Goat	< 10	640	10	40	10	640
M.mycoides	10	2560	10	640	10	1280

† The CF antigen made from Y Goat strain was unsuitable

Strain L 2917

This was a Mycoplasma species isolated in Queensland from the joint of a calf. A polysaccharide fraction was isolated from this strain by extraction with warm aqueous phenol, and used as sensitizing substance for sheep red cells.

To find the optimal sensitizing concentration, dilutions of polysaccharide were used to sensitize sheep red cells. These sensitized red cells were tested against dilutions of homologous antisera (Table 42).

It appeared (Table 42) that 30 µg/ml of L 2917 polysaccharide was sufficient to sensitize optimally sheep red cells. Cells sensitized with this concentration were tested with rabbit antisera prepared against M.mycoides.

The homologous titre was demonstrated using red cells sensitized with M.mycoides polysaccharide.

TABLE 42

The effect of concentrations of L 2917 polysaccharide used to sensitize sheep red cells on the HA titre of two homologous rabbit antisera

L 2917 polysaccharide (µg/ml)	HA Titre of rabbit antisera	
	Serum 1	Serum 2
10	128	128
20	128	128
30	256	256
40	256	256
50	256	256
60	256	256
Control: Serum dilution ½ + Normal red cells - negative		

TABLE 43

Cross HA reactions with M.mycoides and Mycoplasma strain L 2917

Red cells sensitized with	Antiserum			
	L 2917		M.mycoides	
	(1)	(2)	(1)	(2)
L 2917	128	128	2	< 2
M.mycoides	10	10	160	80

The M.mycoides antiserum showed very slight haemagglutination with red cells sensitized with Mycoplasma strain L 2917 (Table 43). The reverse cross reaction, using red cells sensitized with M.mycoides polysaccharide, gave an HA end point of 1/10 with both L 2917 antisera. It may be recalled that sera containing bactericidal activity against M.mycoides were also active against Mycoplasma strain L 2917.

DISCUSSION

Although erythrocytes of sheep and other species were readily sensitized with antigens from cultures of *M.mycoides* in the semi-defined medium (Rodwell, 1959), cultures in a serum-containing medium (BVF-OS) failed to sensitize the cells. Serum may have interfered with the attachment of antigen. The purified polysaccharide isolated from these cultures, unlike some enterobacterial lipopolysaccharides, sensitized red cells without prior treatment.

There appears to be a relationship between antibodies responsible for the agglutination, HA and CF reactions, as the activity in these tests may be undetectable after absorption with polysaccharide. Some sera in which the agglutinins were eclipsed by combining with free antigen still showed HA activity, but this could have been a reflection of the quantitative differences between titres obtainable in these tests. The reduction in CF activity appeared not to be proportional to the reduction in HA activity. Even when the HA titre of a serum was substantially higher than the CF titre, some CF activity remained after neutralization of the HA activity by the addition of polysaccharide. This could be explained if complexes of polysaccharide and antibody were able to fix complement although they were unable to agglutinate sensitized red cells. Dafaalla (1957) isolated two separate fractions from

M.mycoides, one being responsible for the major part of precipitating and agglutinating activity and the other for complement-fixing and some precipitating activity. Although the relationship of these fractions to the polysaccharide used here was not studied, the polysaccharide possessed some features of both fractions.

The sensitivity of the HA reaction is dependent on the concentration of the sensitizing substance and on the time of sensitization. The species of red cells and their concentration in the test suspension also had an effect on sensitivity as did the choice of the slide or the tray HA tests. Awareness of these variables and possibly of others could assist in standardising the reaction.

The specificity and sensitivity of the slide test, using sheep red cells optimally sensitized with polysaccharide isolated from M.mycoides, were of the same order as those of the CF test for M.mycoides as performed in this laboratory. Thus, the slide HA test could play a part in the diagnosis of CPP as well as being of value in research. The test is simple to perform, although preparation of the sera and of the sensitized red cells is time consuming. If a carrier of polysaccharide could be found that was stable for long periods and was unreactive itself with bovine sera, the test would be simplified, and might be suitable for field diagnosis of CPP.

The versatility of the reaction is illustrated by the use of sensitized red cells for absorption of antibody in addition to the detection of antibody and of antigen. However, relatively large amounts of sensitized cells are required for absorption, probably because the amount of antigen present on the red cells is small. This may account for the sensitivity of the HA test.

Inhibition of the HA reaction was a useful method of detecting amounts of antigen down to the order of 10 µg/ml. Experiments with this technique were not described as they were performed jointly with other workers.

Cross-HA reactions demonstrated the relationship of M.mycoides to three Mycoplasma strains and to A.lignieresi (strain Smith 40). The ability of the reaction to detect both antibody and antigen prompted its application to this work.

The possible occurrence of antigenic variants of M.mycoides is good reason to examine strains isolated from field cases of CPP for their relationship to the single strain used in the production of the C.S.I.R.O. liquid culture vaccine. The specific inhibition of HA by homologous antigen could be used as a screening test for these strains.

The bactericidal and the indirect haemagglutination reactions described should facilitate further investigations on *M.mycoides* and of the interrelationship of species in the genus Mycoplasma.

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