THE FATE OF THE T_1 STRAIN OF *MYCOPLASMA MYCOIDES* VAR. *MYCOIDES* FOLLOWING VACCINATION OF CATTLE

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D E C L A R A T I O N

A) I Walter Nyamony Masiga hereby declare that this thesis is my original work and has not been presented for a degree in any other University.

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S U M M A R Y

Studies have been conducted on the fate of the T_1 strain of Mycoplasma mycoides var. mycoides (M. mycoides) in an attempt to elucidate the immunity to Contagious bovine pleuropneumonia (CBPP) following vaccination of cattle.

IN VIVO STUDIES WITH T_1 STRAIN OF MYCOPLASMA MYCOIDES PRIMARY VACCINATION

A) SUBCUTANEOUS ROUTE

B) TAIL TIP ROUTE

M. mycoides was recovered from lymph nodes draining the tail region of cattle vaccinated with the T_1 strain of M. mycoides for up to 71 days. M. mycoides antigens were not detected until 3 days after vaccination using the Agar Gel Diffusion Test and became wide spread in the animal tissues by the 10th day and were detected up to 204 days after vaccination. The earliest time at which complement fixing (C.F.) and agglutinating antibodies were detected was 5 days. Both CF and agglutinating antibodies persisted for up to 23 and 24 days following vaccination respectively.

The maximum CF-titre was 1/2560. Bacteraemia was detected 8 hours after vaccination of cattle with the T_1 strain of M. mycoides using the subcutaneous route and was not detected beyond 50 hours following vaccination. No bacteraemia was detected following tail tip route of inoculation. It is suggested that viable organisms stimulate immunity against Contagious bovine pleuropneumonia and associated antigens maintain the immunity.

IN VIVO STUDIES WITH THE T_1 STRAIN OF M. MYCOIDES: SECONDARY VACCINATION

The highest CF-titre was 1/320 following secondary vaccination of cattle with the T_1 strain of M. mycoides. The agglutinating antibodies persisted for up to 8 weeks and the highest Slide Agglutination Serum test (SAST) reaction recorded was lower than that reported following primary vaccination. The majority of cattle did not respond serologically following revaccination. Following revaccination, M. mycoides organisms were recovered from the regional lymph nodes and the tail of some animals. In the majority of cattle this isolation was positive within 7 days but in one animal the organism was recovered 22 days after revaccination. M. mycoides antigens became wide spread by the 4th day following revaccination.

An "in-contact " trial of 9 months duration was carried out to determine the effect of revaccinating cattle six months and 1 year after primary vaccination and to determine the effect of prolonged challenge on vaccinated cattle. It was shown that the T_1 broth culture vaccine protected some cattle against CBPP for at least 2 years.

THE EFFECT OF POST VACCINATION TREATMENT WITH THE ANTIBIOTIC TYLOSIN ON THE IMMUNITY PRODUCED BY THE T_1 STRAIN OF MYCOPLASMA MYCOIDES:

An "in-contact" challenge of 3 groups of cattle vaccinated with the T_1 strain of M. mycoides indicated that immunity to CBPP was not developed in all animals one month after vaccination. Animals vaccinated with T_1 strain of M. mycoides and treated with "Tylosin" one week after vaccination were all resistant to challenge. Three of 11 animals treated similarly one month after vaccination had CBPP lesions. All the animals vaccinated and not treated with the drug were resistant to challenge. With the exception of six animals the remaining 18 controls developed CBPP.

OBSERVATIONS ON THE IMMUNITY TO CONTAGIOUS BOVINE PLEUROPNEUMONIA

Cells producing antibodies to galactan were detected in spleens and lymph nodes of cattle after primary vaccination with T_1 broth culture vaccine. These precipitating antibodies were found in lung and lymph node material, but not in the sera of cattle after vaccination or revaccination. In contrast to this precipitating antibodies were not detected in unvaccinated cattle.

IN VITRO STUDIES WITH THE T_1 STRAIN OF M. MYCOIDES

In vitro experiments have shown that phagocytosis of the T_1 strain of M. mycoides organisms occurs, and it is postulated that release of these organisms by the leucocytes concerned, is the cause of the intermittent bacteraemia demonstrated by sequential blood sampling of cattle vaccinated by the subcutaneous route.

It has also been shown that blood, plasma and serum from normal bovine animals adversely affect the T_1 strain of M. mycoides.

CHAPTER 1

INTRODUCTION

Contagious bovine pleuropneumonia (C.B.P.P.) may be defined as an acute, subacute or chronic disease affecting mainly cattle. The disease may be characterised by a combination of the features of brochopneumonia, interstitial pneumonia and sero fibrinous loba pneumonia. The aetiological agent is Mycoplasma mycoides var. mycoides (M. mycoides).

The disease has an irregular and long incubation period with variable mortality and morbidity rates. In practice the disease is transmitted by inhalation of finely dispersed bronchial secretions expelled during coughing. The disease can also be transmitted by direct contact between susceptible cattle and contaminated fodder.

The disease has been rated as one of the most important diseases in Africa as it causes great economic losses to livestock industry.

Following the control of Rinderpest, Contagious bovine pleuropneumonia is the most important animal disease in Africa and several international projects have been undertaken in Africa to study the disease. The disease is of particular importance in areas of East Africa where the nomadic groups

of people are found. The majority of cattle in Kenya are to be found in these areas.

Cattle with chronic Contagious bovine pleuropneumonia are usually emaciated and have low market value. With the recent introduction of the Kenya veterinary department policy of breeding beef cattle in nomadic areas and fattening them in Contagious bovine pleuropneumonia-free areas, it is of uttermost importance that the disease should be controlled in these nomadic areas. Kenya spends a great deal of money on financing Contagious bovine pleuropneumonia testing programmes to prevent the introduction of diseased cattle into Contagious bovine pleuropneumonia clean areas. Approximately two million cattle in Kenya are vaccinated against the disease annually.

It has been established that cattle which have been vaccinated with the T_1 strain of M. mycoides become resistant to Contagious bovine pleuropneumonia on challenge. An unusual feature in vaccinated animals is that circulating antibodies are present for a maximum period of 3-4 months. After this period circulating antibodies are not usually detectable by conventional serological methods. However, these animals are resistant to a severe challenge one year later. The immune mechanisms, nevertheless, have remained unknown. LLOYD (1967) suggested that possibly cell mediated immunity may be playing a part in the immunity to this disease.

DAVIES and HUDSON (1968) suggested that some local factor was responsible for the immunity to this disease. This obvious deficiency in the information available about the immunity to Contagious bovine pleuropneumonia was as a great stimulus to this investigation as was the possible deliterious effect of treating animals vaccinated with T_1 broth culture vaccine with broad spectrum antibiotics on the field soon after vaccination.

The present study was designed, therefore, to find out the fate of the T_1 strain of M. mycoides following vaccination of cattle and subsequently to show what part the organisms with its antigens play in the initiation, development and maintenance of the immunity to Contagious bovine pleuropneumonia.

The study was conducted in 6 parts:-

1. The investigation of persistence of the organism in blood following subcutaneous and tail tip inoculations.
2. The effect of blood components on the T_1 strain of M. mycoides in vitro.
3. The persistence of the organism in bovine tissues following primary vaccination.
4. The persistence of the organism in bovine tissues following revaccination.

5. The serological response following primary and secondary vaccination.

6. The investigation into a possible local factor that may be responsible for the immunity against this disease.

CHAPTER 2

LITERATURE REVIEW

I. INCIDENCE AND GENERAL IMPORTANCE OF CONTAGIOUS BOVINE PLEUROPNEUMONIA

Contagious bovine pleuropneumonia in Europe was first recorded in the 16th century (CURASSON, 1936) but HUTYRA, MARECK and MANNINGER (1949) have suggested that it probably occurred for the first time in the 17th century. The disease was not definitively described until 1765 (BOURGELAT cited by CURASSON, 1936) and hence the earlier reports might be treated with caution. It is probable that Contagious bovine pleuropneumonia originated from the central highlands of Europe. REYNAL (1873) noted that the disease was contained in mountainous areas of Europe from 1765 - 1792, being enzootic in Switzerland, France and Italy. With the advent of international wars and trade, Contagious bovine pleuropneumonia crossed borders in central Europe and thereafter overseas (DUJARDIN-BEAUMETZ, 1943 and MC'FADYEAN 1925). During the Napoleonic wars in the nineteenth century, cattle brought into Belgium and Holland introduced the disease into these countries (TURNER, 1959). Holland, for instance, lost

600,000 cattle between 1830-1840 (CURASSON, 1936). By 1880 Contagious bovine pleuropneumonia was localized in the North and Seine counties (CURASSON, 1936). MC'FADYEAN (1925) records that the disease was introduced into England in 1840 by cows sent from the Netherlands to the Dutch Consul in London. The disease, however, had been recorded earlier by BARKER (in 1736, cited by HUTYRA, MARECK and MANNINGER, 1949). By the time Contagious bovine pleuropneumonia was eradicated from England in 1898 Britain had suffered great losses: 200,000 cattle in 1860 alone and a million animals over 6 years. During the 49 years in which the disease inflicted Britain, the country suffered a loss of approximately £3,000,000 (COPE, cited by MC'FADYEAN, 1925). Contagious bovine pleuropneumonia was introduced into Spain in 1846, apparently reached Austria and Hungary between 1878 - 1890, and was recorded in Germany at the beginning of the 19th century (SANDER, 1910 cited by CURASSON, 1936).

By means of rigorous stamping out policies, Contagious bovine pleuropneumonia was almost eradicated from Europe by the end of the 19th century, with the exception of Russia, Polland, Portugal and Spain. The disease was eradicated from England in 1898; Germany in 1903; Austria and Hungary in 1892; and France in 1902. During the 1914 - 1918 war, Austria and Germany became infected again via Rumania and Polland and were not free until 1923 (CURASSON, 1936). Contagious bovine pleuropneumonia spread from Europe

to America, South Africa and Australia in the mid-nineteenth century (BEAUDETTE, 1946 and HENNING, 1932), and subsequently, from Australia to Asia; to India in 1910; to China in 1919; and reached Japan from Korea in 1924 (HUTYRA et al., 1949). The disease was eradicated from America in 1892, South Africa in 1916, and Japan in 1932.

Information taken from the records of the Food and Agricultural Organization (F.A.O.) of the United Nations indicates that Contagious bovine pleuropneumonia is still present in Spain, India, China, Australia and Africa.

II. CONTAGIOUS BOVINE PLEUROPNEUMONIA IN AFRICA

Contagious bovine pleuropneumonia was introduced into Sudan from the East in the mid-nineteenth century, and it disappeared in 1899. Nevertheless, it re-appeared in 1911, and is now enzootic in the country. The disease was widespread in Egypt at the close of the nineteenth century but seems to have disappeared very rapidly; in 1933 one case was confirmed in a quarantine station (CURASSON, 1936), and several cases were reported in 1971 (IBAR 1971). The history

of the disease in Ethiopia and Somalia is not clear. However, by the close of the 19th century Contagious bovine pleuropneumonia was present in these two countries. CHASA (1930) cites that the disease existed in Bechuanaland around 1876. Having disappeared for a while, it was re-introduced in 1918, coinciding with a human epidemic of Spanish influenza which led to herders dying and hence the cattle wandering unattended. The disease was eradicated in 1928. Zambia encountered Contagious bovine pleuropneumonia from Angola in 1915. It is clear that the disease was eradicated in 1921 after placing Barotseland under lengthy quarantine. Recently, Contagious bovine pleuropneumonia was reported in the northern Province of Zambia (IBAR 1972). Contagious bovine pleuropneumonia reached Rhodesia around the same time as in South Africa, i.e. 1854, and by 1894 there had been some 28,000 cases. NACHTIGAL cites the disease in Chad in 1870 (CURASSON, 1936). BAINCS (cited by MONOD, 1894) reported the disease for the first time in French West Africa, and described vaccination programmes by the natives. Contagious bovine pleuropneumonia was introduced into Senegal in 1861, and existed in the Francophone countries about the same time. Sudan seems to have been the focus of the disease, disseminating it to other surrounding territories (CURASSON, 1936).

Current FAO reports indicate that Contagious bovine

pleuropneumonia is enzootic in central equatorial Africa in a band between latitudes 19°N and 2°S. Essentially, the disease still persists in regions where slaughter policies cannot be carried out, either for social and economic reasons, administrative difficulties or where cattle populations cannot be closely supervised.

III. CONTAGIOUS BOVINE PLEUROPNEUMONIA IN EAST AFRICA

Contagious bovine pleuropneumonia was apparently endemic in Tanzania up to the end of 19th century (HAYES, 1926). Although the disease had disappeared, it was re-introduced during the 1st World War by cattle from Kenya. It is known to have been endemic in Uganda by the close of the 19th century (CURASSON, 1936) but generally disappeared by 1932, except for the Karamojong district, where it is still endemic.

Apparently, the first documented case of Contagious bovine pleuropneumonia by veterinarians in Kenya was that of a short-horn bull presented by Lord Delamere to Dr. Atkinson in 1901. This bull is reported to have died of Contagious bovine pleuropneumonia (HUXLEY, 1948, 1953).

It has also been reported that native cattle bought from Kavirondo Nyanza in 1901 by Lord Delamere, who had taken land at Equator Ranch near Njoro, introduced the Contagious bovine pleuropneumonia "virus" into this area. Contagious bovine pleuropneumonia afflicted many head of cattle in this area including Lord Delamere's own cattle, and became widespread around Nairobi by 1905. This necessitated the slaughter of native cattle in this area in an attempt to save exotic stock (Ministry of Agriculture Annual Report 1905 - 06).

Contagious bovine pleuropneumonia was reported around Nairobi, Baringo, Naivine, Embu, Ukambani and Laikipia by the close of the first decade of this century. After 1st World War, Contagious bovine pleuropneumonia was reported in Ngong, Kajiado, Njoro, Gilgil, Rumuruti, Nyeri, Nandi, Naivasha, Uasin Gishu, Lumbwa, Nyanza, Kisumbu, Eldoret, Suk, Trans-Nzoia, Subukia, Solai, Samburu, Thika, Meru, Ndeiya Location of Kiambu District, Machakos District, Maragwa, Mombasa and Songor (KARIUKI, 1971). Following the use of the egg vaccine (SHERIFF and PIERCY, 1952) coupled with rigorous slaughter policy, Contagious bovine pleuropneumonia was not controlled in the areas mentioned above with the possible exception of Samburu area. A notable effect of the egg vaccine was the large number of tail reactions following its use in Kenya Masailand.

The disease situation was much the same in the sixties in the areas of Masailand mentioned above and new outbreaks were reported at Kinoo, Kabete, Muguga and Ngecha in Kiambu District (KARIUKI, 1971). In 1966 an outbreak of Contagious bovine pleuropneumonia occurred in cattle owned by the Livestock Marketing Board of the Ministry of Agriculture held at Archers Post (BYGRAVE, MOULTON, SHIFRINE and STONE, 1968).

The disease came under control in Kenya Masailand by the close of the sixties. Currently the disease is confined to the Northern parts of Kenya. Few foci of outbreaks of the disease have been reported from 1970 up to date (Kenya Veterinary Division Reports 1970, 1971 and 1972).

In East Africa Contagious bovine pleuropneumonia has not been officially reported in Tanzania since 1964; or Uganda since 1966. The disease has been reported sporadically to the northern areas of Kenya. Following a rigorous vaccination programme Contagious bovine pleuro-

pneumonia is under control in Tanzania and Kenya Masailand, and similar measures are being taken in the Northern Kenya regions.

IV. TAXONOMIC POSITION OF THE CAUSATIVE ORGANISM OF CONTAGIOUS BOVINE PLEUROPNEUMONIA

The causative agent of Contagious bovine pleuropneumonia was unknown to the end of the 19th century. In 1898 NOCARD, ROUX, BORREL, SALIMBENIENT and DUJARDIN-BEAUMETZ reported that Contagious bovine pleuropneumonia was caused by a living agent "virus". When material from the lungs of a natural case was put in collodion sac which contained meat extract and inserted in the abdominal cavity of a rabbit the contents of the sac were found to be cloudy 15-20 days later. Nothing was seen in the cloudy fluid

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when it was examined under a microscope and yet the fluid was infectious. Subsequently, it was found that infectious material recovered from a natural case could be filtered through earthen-ware candles and then grow in a meat and pig stomach extract bovine serum medium. NOCARD, ROUX, BORREL, SALIMBENIENT, and DUJARDIN-BEAUMTZ (1898) confirmed that Contagious bovine pleuropneumonia was caused by a microbe not previously described. NOCARD, ROUX, BORREL and DUJARDIN-BEAUMETZ (1898) completed their study of the microbe and described methods of vaccination.

DUJARDIN-BEAUMETZ (1900) succeeded in growing the organism on solid media. BORDET (1910) described the colony morphology of the organism, which was subsequently in the same year named Asterococcus mycoides (BORREL, DUJARDIN-BEAUMETZ and JEANTET ET JOUAN, 1910).

Because the infectious agent passed through Berkfield filters it was considered a virus (BARNARD, 1926, SMILES, 1926, ØRSKOV, 1927; NOWAK, 1929; WROBLEWSKI, 1931; LEDINGHAM, 1933

KLIENEBERGER, 1934, SHOETENSACK, 1934, TANG, WEI and EDGAR, 1936; TANG, WEI, McWHIRTER and EDGAR, 1935; TURNER, 1935 and MERLING-EISENBERG, 1935), despite the fact that the organism had been shown to grow on cell free media (NOCARD, ROUX, BORREL and SALIMBENIENT, 1898; DUJARDIN-BEAUMETZ, 1898, BORDET, 1910 and BORREL et al., 1910). Somewhat later, organisms were isolated from goats and dogs (BRIDRE and DONATIEN, 1923, 1925 and SHOETENSACK, 1934) and the name pleuropneumonia like organisms (PPLO) was assigned to the group. NOWAK (1929) suggested the name "Mycoplasma" to replace Pleuropneumonia and EDWARD and FREUNDT (1956) supported the use of this name as a genus name in the order Mycoplasmatale within the family Mycoplasmataceae. The causative agent of Contagious bovine pleuropneumonia is now known as Mycoplasma mycoides var. mycoides (M. mycoides) and throughout this text the organism will be referred to as M. mycoides.

V. CONTAGIOUS BOVINE PLEUROPNEUMONIA AND VACCINATION AGAINST THE DISEASE:

A) LIQUID VACCINES:

In 1852 WILLEMS introduced a preventive method against Contagious bovine pleuropneumonia, which consisted of inocu-

lation into the tail using "virus" collected from the pleural cavity or lung of naturally infected cattle. The process of obtaining the virus was interesting: "The affected beast was selected and the side of the chest affected determined by auscultation; the animal thrown down; the affected side uppermost and then bled to death by severing the carotids and the jugulars - care being taken not to incise the trachea. The animal was then skinned and the abdomen incised along the mid-line. The animal was eviserated carefully to avoid contaminating the pleural fluid with either blood or ingesta. An incision was then made through the diaphragm at its uppermost point relative to the position of the animal. If putrid odour was found the virus was not collected but if the pleural cavity contained sweet smelling clear yellowish fluid some of it was collected with a sterile spoon and put in a sterile bottle using a sterile funnel. The liquid which was collected was then filtered through filter paper to remove the coagulum so as to prevent the blocking of the needle during inoculation operations. The dose used was 1 ml. inoculated at the tail tip". An inflammatory reaction at the site of injection usually followed between 8 - 14 days. If the swelling advanced along the tail then the skin about 6 inches above the swelling was cauterised or the swelling was scarrified.

PASTEUR in 1883 modified Willems method by first inoculating susceptible cattle subcutaneously with the pleural fluid from Contagious bovine pleuropneumonia diseased animals and then removing material (lymph) from inoculated areas for prophylactic vaccination of other animals. Thus Pasteur used a one stage passage of the agent. This process was made use of in Australia and many institutes were created in European countries to produce vaccines using Pasteur's method. The results using either Willems or Pasteur's methods were not encouraging owing to uncertainity of the reactions, and, of course, they had the obvious danger of transmitting any other blood borne diseases (KNOWLES, 1927). However, in the 1920's Pasteur's method was still in use, and HENRY (1924) in Australia, recommended the use of this type of vaccine in countries where the disease was widespread. However, many other workers found that lymph vaccine was too virulent to be used. ROBIN (1925) stated that it was not so much the loss of the tail that farmers resented but the possible loss of the hide as well.

The Kenya Veterinary Laboratory maintained several strains of M. mycoides by cattle passage and for supply of lymph material for vaccination. The results obtained were not wholly satisfactory as the nature of reactions were not uniform in character and in many cases uncontrolable with the result that the organism spread along the route of the tail and sometimes beyond. The percentage of mortality

varied from 0 to 28.8 per cent.

HALL and BEATON (1931) working in Nigeria also observed severe reactions following subcutaneous inoculation of cattle using lymph material. Animals that succumbed to the effect of injection always had a bacteraemia, emphasising the spreading nature of virulent strains of M. mycoides when used as vaccines (WILLEMS, 1900). The spreading nature of the organism had also been observed by NAKAMURA, FATAMURA and WATANUKI (CAMPBELL, 1936) who demonstrated the organism not only in typical lesions of Contagious bovine pleuropneumonia but also in the blood, liver, spleen, kidney and lymph nodes. CAMPBELL (1936) confirmed the wide distribution of a virulent M. mycoides throughout the body of infected animals but failed to demonstrate it in urine. However, BELLER and TAHSSIN-BEY (1926 - 27) did find the organism in urine and demonstrated transplacental transfer following the inoculation of pregnant ewes with the organism. The latter observation was also observed by CAMPBELL (1936) following injection of a pregnant heifer via the peritracheal fascia. CAMPBELL (1936) also succeeded in producing specific Contagious bovine pleuropneumonia lesions in lungs of cattle following intravenous inoculation of ground-up lung lesions of natural cases. DAUBNEY (1935) working at the Kenya Veterinary Laboratory also produced typical Contagious bovine pleuropneumonia when he added agar to the virulent M. mycoides either in lymph or pure culture

and inoculated cattle intravenously. From this it can be seen that, although lymph materials did not often cause typical Contagious bovine pleuropneumonia lesions especially when tail inoculation was employed (HINDMARSH, 1933), it did under certain circumstances (HUDSON, 1965). Thus any type of virulent vaccine was potentially infectious.

The incidence of polyarthritis following tail tip vaccination had been known since 1890. NOCARD (1903) reported polyarthritis in France in calves following tail tip vaccination. In Australia where C.S.I.R.O. Laboratory culture vaccine "(BVF-OS culture of the "V_5" strain)" was used reports varied; thus HINDMARSH, WEBSTER and STEWARD (1943) in New South Wales found that joint swelling was "not unusual" in calves whereas MAHONEY (1954) in Queensland did not observe them in calves inoculated when 3-7 months old. Many workers agreed that tail reactions did not occur in calves; for example tail swellings had not been observed in France, U.S.S.R. or in New South Wales by the cited authors and MAHONEY (1954) in Queensland had observed reactions in calves aged 7 months or more. TURNER and TRETHEWIE (1961) studied tail reactions, serological responses and the incidence of swollen joints following the vaccination of various age groups of cattle. They found that reactions following tail-tip inoculation of calves were rare, and of such a mild nature which could only be detected

by careful palpation of the tails. M. mycoides could be recovered from the shallow zones of necrosis. The tail-tip reactions were observed in 20 out of 30 adult cows but were rare in calves. Detectable swellings occurred in only 5 out of 196 calves injected when aged from 7 - 253 days. Specifically infected and swollen limb joints developed in calves inoculated with V_5 at ages up to 50 days; and appeared from 7 - 239 days after inoculation, 24% occurring between 11 and 20 days and 21% between 30 - 60 days. The incidence in the 7 day old group was 40%, in the 20 - 30 day old group, 37% and in the 40 - 50 day old group 7% after which age the susceptibility appeared to cease. The lesion was classified as a non-puvulent, serofibrinous, poly-arthritis and teno-synovitis, usually terminating in spontaneous healing with residual fibrosis. In the groups of calves inoculated when aged 7 days or 20 - 30 days a positive Complement fixation test (CFT) was almost wholly restricted to calves with swollen joints. In the older groups and in adults, positive CFT occurred in most of those in which tail swellings occurred and in about one fifth of those without detectable tail reactions. Joint involvement appeared to have no relationship with tail reactions. The author stated that after tail tip inoculation of calves or adults a transient bacteraemia may be demonstrated within 5 minutes and that this may persist for about 2 hours. Thus the opportunity for

establishment in joints of susceptible calves may be restricted to this short bacteraemic period. Using other strains others have observed arthritis in older animals. DUJARDIN-BEAUMETZ (1900) observed extensive polyarthritis in a calf inoculated intracerebrally when four months old. MEYER (1908 - 1909) in South Africa observed severe polyarthritis in adult cattle when tail inoculated with pleural exudate from a case of the disease. BENNETT (1929) also recorded the arthritis form in 3 out of 81 adults, inoculated behind the shoulder.

In subsequent studies TRETHEWIE and TURNER (1961) observed vegetative endocarditis (valvulitis) and myocarditis as sequelae to post-inoculation arthritis following vaccination with the V_5 strain. Swollen limb joints occurred 3-4 weeks after vaccination with the V_5 strain in 28% of calves inoculated when 6 - 50 days of age. The joint infections usually underwent spontaneous cure but vegetative endocarditis on the aterioventricular and aortic semi-lunar heart valves occurred; typically as a delayed phenomenon in 28% of calves which had developed joint lesions. Myocarditis usually of a mild interstitial type occurred in most cases of joint involvement. The authors seem to have been the first ones to record a joint-heart syndrome to a mycoplasma species. They did not determine whether heart localization

occurred during the short bacteraemic phase but it seemed more likely that it was secondary to involvement of the joint. Thus the authors support the finding of a bacteraemia following vaccination of cattle with M. mycoides strains. Vaccine trials were also carried out to determine the immunity in relationship to age at inoculation. Some calves died due to joint or heart involvement. The survivors of calves (7 - 50 days old) inoculated in the tail-tip with the living culture of V_5 had a high incidence of immunity that was not significantly different from that of adult vaccinates, when they were exposed to challenge 16 - 24 months later. However, in view of the high incidence of polyarthritis and heart lesions vaccination in the first weeks of life was not recommended. Immunity in calves vaccinated from 5 - 9 months old was less and reactions to vaccination (CF and tail reactions) was also much less.

When the T_1 broth culture and the KH_3J vaccines have been used, however, such spreading has not been observed and instead localization of the agent in regional lymph nodes has been observed (HUDSON 1965, LINDLEY and PEDERSEN, 1968, DAVIES, 1969, MASIGA and MUGERA, 1973). CAMPBELL (1938) noted that a lasting immunity was maintained when animals were vaccinated with a strain of organism possessing relatively high degree of virulence; that immunity was

maintained for more than 12 months when a fairly virulent strain was used; less than 12 months when a comparatively avirulent strain was used. PRIESTLEY (1955) supported CAMPBELL's (1938) work after experiments with a broth culture vaccine resulted in a low-grade immunity. Further, DAFAALA and MASRI (1961) found avianized vaccines to confer a far too short an immunity in Sudanese cattle. Recent experiments with the T_1 strain of M. mycoides which is an avirulent strain, resulted in solid immunity to cattle for up to 1 year although the immunity is waning by 2 years (WINDSOR, MASIGA and READ, 1972). In addition, animals vaccinated with the T_1 strain of M. mycoides in this laboratory have responded to vaccination within 3 - 4 days (DAVIES, 1969, MASIGA and MUGERA, 1973), which supported the early work of CAMPBELL (1936).

Broth attenuated vaccines were an improvement on the old crude methods of vaccination. WALKER (1921) developed a broth culture vaccine using the 31st broth passage. This vaccine induced such mild reactions than the lymph vaccine, but seemed as immunogenic. WALKER continued to produce and test broth vaccines using different strains of M. mycoides obtained from various areas of Kenya.

He concluded that:-

a) The character of the reaction produced by broth culture vaccines varied according to the generation of the

subcultures.

b) The virulence (local reaction) of a culture vaccine remained constant for at least 25 generations whereas subsequent subcultures produced less marked reactions.

c) The passage through cattle of lymph materials did not produce any apparent variation in virulence.

Following vaccination with Walker's vaccine there was always an inflammatory reaction 8 - 14 days later. The swelling either remained localized or spread up the tail. Thus WALKER also observed the spreading nature of virulent M. mycoides following vaccination. As a warning WALKER recommended that because the "virus" of pleuropneumonia tended to spread to sites of injury, the animals had to be handled carefully during vaccination: surgical operations were to be avoided for some time before and after vaccination; branding was not to be carried out as wounds or marks within the reach of the tail could be contaminated; cattle were not to be put through the crush if their tails had been cauterized and that reacting animals were not to be worked. WALKER observed that inoculation of the calves resulted in swelling of the joints thus confirming further the spreading nature of Contagious bovine pleuropneumonia agent. He also stated that necrosis of the tissues often occurred. WALKER noted that the duration of immunity was different in individual animals but observed that generally the immunity lasted about a year. Most of WALKER's observations

are still true today.

KNOWLES (1927) while recommending the use of Walker's vaccine in Sudan, stressed that:-

a) The virulence of the culture could be degraded sufficiently to render its use safe.

b) After the attenuation of the vaccine it was still immunogenic.

c) Broth culture vaccines could be prepared in any quantity.

d) Contamination with extraneous bacteria could also be avoided whereas with lymph vaccines such contamination was not uncommon.

BENNETT (1932) confirmed that cultures of several generations were fairly safe and had a good efficacy. He disagreed with the idea that a local reaction had to be seen so as to confirm immunity. Recently animals which have shown no post-vaccination tail reactions have been solidly immune on challenge (DAVIES, MASIGA, SHIFRINE and READ, 1968, GILBERT, DAVIES, READ and TURNER, 1970 GILBERT and WINDSOR, 1971, WINDSOR, MASIGA and READ 1972, MASIGA and READ, 1972, MASIGA and WINDSOR, 1973 and WINDSOR and MASIGA, 1973).

BENNETT (1932) also noted there was no apparent difference in immunity of a single and double vaccinated cattle when exposed 12 months after vaccination. PURCHASE (1939) however, found that using broth cultures of 11th to 47th generation, immunity was established 36 - 44 days after double vaccination

whereas animals given only 1 injection of the same vaccine failed to show any degree of immunity until about 106 days. Other workers (HAYES, 1926; WALKER, 1922; BENNETT, 1932; SHIRLAW and KRISHNA IYER, 1946) had found that the use of cultures below the 16th generation was dangerous and those older than 73rd generation were of diminishing potency. Between these stages of attenuation there was little variation in immunizing value although BENNETT (1932) and SHIRLAW and KRISHNA IYER (1946) had stated that earlier culture vaccines gave a more satisfactory immunity than the later. MASIGA and WINDSOR (1973) confirmed that double vaccination of cattle using the 46th passage of the T_1 strain protected these animals against challenge 1 year and $1\frac{1}{2}$ years later. However, animals vaccinated once and challenged at 2 years resisted the infection just as well. Thus both dosage and the stage of attenuation seemed to play a part in the immunizing potency of the culture vaccines.

PRIESTLEY (1952) and PRIESTLEY and KARIB (1954) observed that live organisms had to be inoculated to elicit immunity and therefore postulated that the inoculated organisms must multiply in the tissues in order that immunity may set up. They further argued that on one vaccination animals would be immune and that organisms introduced into the animal on revaccination would be killed off.

CAMPBELL (1938) demonstrated a high degree of resistance in cattle as early as 3 days after vaccination with broth culture. He found that immunity in Contagious bovine pleuropneumonia lasted longer than 12 months whether single or double vaccination was employed. After the second vaccination the response was rapid and antibody titre stayed high for long time. CAMPBELL, however, observed that some strains caused smaller reactions than the others, and detected complement fixing (CF) antibodies between the 3rd and 7th day after tail tip vaccination of cattle. He demonstrated peak titres between the 10th and 14th day but the animals became negative to the test between the 7th and 14th week. DAVIES (1969) and MASIGA and MUGERA (1973) detected CF antibody 5 days after tail tip vaccination. The later authors could not detect CF antibody beyond 30 days. However, generally in this laboratory CF titres are negative by the 8th week.

Passaging of M. mycoides to produce a safer vaccine was further investigated by other workers. METTAM and FORD (1939) vaccinated Nigerian cattle using the 120th passage of Contagious bovine pleuropneumonia organism and found it immunogenic. After the 185th passage, however, the vaccine was no longer efficacious. It also appeared that different breeds of cattle varied in their susceptibility. MASIGA and READ (1972) found that Zebu and exotic types of animals were equally susceptible to Contagious bovine pleuropneumonia and that the T_1 broth culture vaccine

was equally efficacious in the 2 types of animals. Similar findings had been observed by LE ROUX (PURCHASE, 1939) who stated that it was not so much the breed of cattle per se, as the local environment which affected the reactions and this point of view was supported by CURASSON (1936) who also failed to discover any correlation between different breeds and their reported reaction to culture vaccines.

Broth culture vaccines became acceptable as good reports were published by many workers. WEBSTER (1945) reported on a broth culture vaccine prepared at C.S.I.R.O. (V_5) in Australia. The vaccine caused very few untoward reactions and the immunity conferred was good. However, GRAYSON (1950) recorded severe reactions which often terminated in loss of tail or even death of the animal when either lymph or the C.S.I.R.O. broth culture vaccine was used in cattle in Victoria, Australia. He nevertheless, stressed that the V_5 strain had satisfactory protective values against Contagious bovine pleuropneumonia.

WEBSTER (1945) confirmed the findings of ALBISTON, GREGORY and JOHNSTONE (1932) who found that inoculation of animals in Victoria, Australia which had Contagious bovine pleuropneumonia in the incubation stage had no influence on the course of the disease. MASIGA and ROBERTS (1973) also showed that vaccination of cattle already infected with Contagious bovine pleuropneumonia had no influence on the course of the original disease. PIERCY (1958) stated that vaccination will not eliminate the chronic

carrier with a sequestrum. However, it may raise the resistance so that they are less likely to break again and become infective.

Broth culture vaccines have been widely used, but with varying results. In 1956 PRIESTLEY looked into the whole problem of vaccinating animals against Contagious bovine pleuropneumonia in the Sudan and concluded that BENNETT's (1932) culture vaccine was not efficacious. KNOWLES (1960) observed that when Nigerian cattle were vaccinated with broth culture vaccines against Contagious bovine pleuropneumonia the vaccination stressed the animals and led to flarring up of latent infections. He observed that the vaccine caused severe local reactions which resulted in a permanent mark. HUDSON (1964) postulated that the occurrence of severe reactions be attributed to enhanced susceptibility of groups of cattle. He suggested that this is not a permanent state and that the reverse could also occur in that occasionally cattle can be so resistant that they do not respond to vaccination. He added that this state was also temporary and these animals could be fully receptive to vaccine and also natural infection a few weeks later. HENDERSON (1945) on the other hand cited by GRIFFIN and LAING (1966) hypothesised that vaccinated animals passed through a hyper-susceptibility period and that after vaccinating susceptible herds they should be separate from possible infected herds so as to avoid being infected during this phase.

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From 1953 the KH_3J strain was used in Nigeria either in broth form or in lyophilized form with agar-gel adjuvant added. The protection was observed to take four weeks to develop and the duration of immunity was said to be 12 months. The avianized T_1 strain was tried and although it gave rapid and good protection the reactions experienced were too severe.

BROWN, GOURLAY and MACLEOD (1965) developed the T_1 broth culture vaccine from the 45th egg passage of the T_1 strain of M. mycoides. MINOR (1967) showed that the T_1 vaccine could be used to control an outbreak of Contagious bovine pleuropneumonia. During 1964 there was a massive epizootic of Contagious bovine pleuropneumonia in Karamoja. Because of the size of the outbreak it was not possible to keep up with CF testing and cattle owners with animals with a positive CFT but no clinical signs could not be persuaded to put their animals in quarantine. Also there was limited grazing and water in the quarantines and they had to be abandoned. Vaccination was the only method of control and 713,956 vaccinations were carried out between September 1964 and December 1965. There was no objection by cattle owners to the use of the vaccine because reactions were very few. A few tails were lost but not necessary due to the T_1 broth vaccine as other cows not vaccinated were seen without tails. It was not possible to accurately assess the degree of cover, but no new outbreaks occurred and vaccination figures exceeded the 1963 census figure.

HUDSON (1968a) showed that first herd vaccination of cattle in Northern Australia with V_5 vaccine led to a level of herd protection similar to that achieved by vaccination of dairy cows in Victoria. Secondly the KH_3J vaccine containing egg fluid proved as an efficient vaccine for the protection of cattle of differing susceptibility in Africa and other countries as it proved in all Victorian cattle in Australia. HUDSON pointed out that using the KH_3J strain an animal could become protected without showing any clinical and serological response to vaccination.

HUDSON (1968b) also described the keeping qualities of the V_5 vaccine used in Australia. He found that the calculated efficiencies of the vaccines based on pathology score (Ep) and total score (Et) suggested that vaccine stored 3 months at 37°C was of lower immunizing value than fresh vaccine but of higher value than vaccine that had been stored for only one month at 37°C. However, statistically the differences between the scores in the different vaccinated groups were not significant. Thus all the vaccines gave a similar degree of protection. SHIFRINE, STONE and DAVIES (1968) showed that the serologic response of cattle to T_1 vaccination was transient as measured by the CFT and the Slide Agglutination Serum test (S.A.S.T.). A larger number of sera were positive in the SAST (12/21) than therapid CFT (9/21). All cattle were negative by 30 days of vaccination in the CFT whereas the reaction persisted in SAST up to 86th day. SHIFRINE et al.

(1968) found no detectable anamnestic response when cattle were revaccinated after one month but found an increase in antibodies in cattle revaccinated after 3 months as measured by a variety of serologic tests. These authors found no reactions at the site of inoculation in cattle inoculated either subcutaneously or by the tail tip route.

DAVIES, MASIGA, SHIFRINE and READ (1968) described two vaccine trials which were the first of their type in East Africa since the advent of the T_1 broth culture vaccine in 1965. The results of the two trials, showed, with one exception that all animals were resistant to challenge one month after vaccination with the T_1 vaccine. Not only were there no lung lesions indicative of Contagious bovine pleuropneumonia in the vaccinated group, but cultures failed to reveal M. mycoides in any part of the respiratory tract. DAVIES et al. (1968) suggested that among the factors responsible for immunity in Contagious bovine pleuropneumonia one might exert a bactericidal effect in the upper and lower respiratory tract. Other Mycoplasma were identified in the respiratory tract suggestive that the effect was not due to the normal cleansing processes of lung but to a factor specific to M. mycoides. The organism was recovered from turbinates, tonsil, larynx trachea, bronchi lung parenchyma of one vaccinated animal . However, it could not be established whether the isolated strain was T_1 or Gladysdale.

DAVIES and HUDSON (1968) compared the relationship between growth inhibition (GIT) and immunity in Contagious bovine pleuropneumonia; they confirmed that complement fixing, agglutination antibodies gave no indication of immunity. In several experiments, the authors failed to detect GIT, CFT or SAST antibodies in sera of vaccinated cattle. On challenge these cattle were found either potentially immune or solidly immune. The authors found one animal out of 4 animals which had a GIT titre after vaccination. The four unvaccinated animals had GIT titres and yet they all succumbed to Contagious bovine pleuropneumonia. Although there was no correlation between growth inhibition titres and immunity, a close temporal relationship between the development of growth inhibition, complement fixation and agglutination titres was found. DAVIES and HUDSON concluded that the resistance to Contagious bovine pleuropneumonia does not involve circulating antibody but is more probably due to some form of cell mediated immunity.

SHIFRINE and BEACH (1968) found that inoculation of an inactivated virulent culture of M. mycoides with adjuvant into cattle did not confer immunity to the cattle, instead it sensitized them so that on subcutaneous challenge these cattle had a Willems reaction after the first or second day instead of 7 days in average cattle. The cattle inoculated with similar numbers of living virulent culture of M. mycoides

adjuvant developed lesions at the inoculation site. These cattle were totally immune to challenge inoculation.

DAVIES (1969) found that M. mycoides persisted in the regional lymph nodes draining the tail of normal cattle for 14 days after vaccination. Except for one animal in whose trachea M. mycoides was recovered, the organism was restricted to the regional lymph nodes and the site of inoculation. Serological reactions were positive by 5 to 7 days after vaccination and continued until 28 to 30 days. In another experiment cattle were stressed with caprinized rinderpest virus and then vaccinated with the T_1 strain. The organism was recovered from regional lymph nodes of one animal 42 days after vaccination. This animal had a small necrotic lesion involving the bony tissue at the site of inoculation. Serological reactions developed at 7 to 8 days after vaccination with the exception of one animal which had a SAST reaction 3 days after vaccination. The serological reactions persisted in one animal up to 42 days in the animal which had a necrotic lesion. This result suggests further that serological reactions become prolonged in animals which have post vaccinal reactions. DAVIES failed to recover M. mycoides from blood and there were no lung lesions seen at post mortem examination. HUDSON and LEAVER (1965) slaughtered 80 cattle at 4 and 6 weeks after vaccination with the V_5 broth vaccine and failed to find lung

lesions or to cultivate M. mycoides from the respiratory tract. HUDSON (1965) was able, however, to recover the organism from a regional lymph nodes 3 months after vaccination with the KH_3J strain. However, when agar (DAUBNEY, 1935) and egg (HUDSON and LEAVER, 1965) were added to M. mycoides cultures when given parenterally Contagious bovine pleuropneumonia was caused.

DAVIES, STONE, and READ (1969) were able to produce disease by passaging 3 strains of M. mycoides. The passage was done by the endobronchial method. After 5 passages in cattle, the T_1 strain produced Contagious bovine pleuropneumonia lung lesions similar to those seen with virulent strains under similar conditions. The V_5 strain produced a Contagious bovine pleuropneumonia lung lesion in the first passage. Repeated passages of the KH_3J strain could only produce a small Contagious bovine pleuropneumonia lesion. These results showed that even avirulent strain could revert to virulence on passaging through cattle especially so when the cattle are under stress.

DAVIES and GILBERT (1969) stated that contact trials designed to evaluate the potency of the T_1 vaccine have shown that immunity lasts for at least 12 months. Safety experiments had shown that the organism localized in regional lymph nodes and did not get to the respiratory tract. Field trials indicated that post vaccinal reactions were minimal.

GILBERT, DAVIES, READ and TURNER (1970) carried out vaccine trials in which they showed that six months after vaccinating 16 cattle with the T_1 vaccine, they were resistant to challenge. Only 2 vaccinated animals had a transient CFT. However, challenge 12 months after vaccination did not indicate a solid immunity, especially since M. mycoides could be isolated from the respiratory tract of 3 vaccinated cattle, although no Contagious bovine pleuropneumonia lesions were present in these cattle. One vaccinated animal developed a transient CFT. It appeared therefore that by 12 months the immunity conferred by the T_1 vaccine was already waning.

WINDSOR, MASIGA and READ (1972) challenged animals 2 years after primary vaccination and 6 of the vaccinated animals showed a CFT titre, the first appearing two weeks after the start of the trial and the last 17 weeks after exposure. However, the vaccinated group gave no other clinical response to challenge. On post mortem examination five of 6 animals had typical, although small, sequestra. While no lesions could be detected in the sixth animal. M. mycoides was isolated from the 5 animals showing lesions. In the 12 months trial GILBERT et al. (1970) had demonstrated that immunity as measured by incontact challenge is beginning to wane 12 months after vaccination. The results obtained by WINDSOR et al. (1972) confirmed this and indicated that the immunity had declined still further.

STONE (1970) used T_1 vaccine to vaccinate a group of calves, each with different levels of circulating antibody. None showed any change in antibody levels following vaccination. However, when the calves were revaccinated 58 - 86 days after birth at a time when all were negative serologically, all had an antibody response within 10 days. The author concluded that passive colostral antibody would require about 60 days to be cleared otherwise the immune response to vaccination might be interferred with. He found in these experiments that the first antibody was IgM and the end was IgG.

GILBERT and WINDSOR (1971) carried out dose response experiments with the T_1 broth vaccine. The serological response was greater in animals which received 10^7 and 10^9 organisms per dose than those vaccinated with 10^5. The authors found after challenging the animals 6 months post vaccination that animals which received 10^7 and 10^9 organisms per dose of the vaccine were protected while those given 10^5 organisms were sensitized.

B) FREEZE DRIED VACCINES

Methods of attenuating M. mycoides to render the organism useful as a vaccine were attempted by many workers. SHERIFF and PIERCY (1952) working at the Kenya Veterinary Laboratory

obtained lymph virus from a natural case of Contagious bovine pleuropneumonia in Tanganyika. The lymph material was inoculated subcutaneously into an ox (1236) and the resultant lymph virus was designated the T_1 strain. When the lymph virus from that animal was inoculated into 2 other animals subcutaneously a local reaction was produced in one of the animals and it was concluded that the T_1 strain was comparatively avirulent. The T_1 strain was then passaged through embryonated eggs. Cattle were immunized successfully using the 9th egg passage material by the subcutaneous route. These inoculations were rarely followed by undesirable reactions. When the strain was passaged further to the 34th generation in eggs no further reduction of virulence was observed. The 32nd generation was found ideal as it caused no untoward reactions and was efficacious. In their experiments, SHERIFF and PIERCY (1952) could not correlate CF titres with immunity, an observation which is still valid since animals negative to the CFT have been found immune on challenge (DAVIES et al. 1968, GILBERT et al. 1970, WINDSOR et al. 1972, MASIGA and READ, 1972 and MASIGA and WINDSOR, 1973). Apart from occasional variations in its virulence, the avianized strain of T_1 showed promise of being a safer, more stable and more effective immunizing agent than vaccines used at that time for the control of Contagious bovine pleuropneumonia. SHERIFF and PIERCY (1952) concluded that the egg vaccine posessed as good if not better

immunizing properties than culture vaccine. They found that severe reactions or death following vaccination were common in cattle which had an early or active Contagious bovine pleuropneumonia. MASIGA and ROBERTS (1973) later found that vaccination of cattle with Contagious bovine pleuropneumonia in its early stages enhances the course of the disease and an extreme type of Contagious bovine pleuropneumonia is experienced. SHERIFF and PIERCY, however, found no Contagious bovine pleuropneumonia lesions in cattle which died following vaccination. Their observation therefore seemed to suggest that it was generally during the incubation period or in very early stages of the disease that cattle reacted violently to vaccination.

HYSLOP (1955) working in Kenya found that animals were completely protected within five weeks following vaccination with an avianized vaccine. The vaccine after 1½ years storage induced immunity in cattle which was indistinguishable from that produced when the batch was initially tested. PIERCY and KNIGHT (1957) discredited culture vaccines and suggested that attempts to produce satisfactory culture vaccines against Contagious bovine pleuropneumonia showed that the quality and duration of immunity induced was largely dependent on the virulence of the organism used. Conversely the safety of such vaccines varied inversely with their virulence. They recommended egg vaccines and

recorded that the T_3 strain at the 11th egg passage produced no untoward reactions in 10 cattle when inoculated via the tail tip. The animals were subsequently immune as tested by a severe subcutaneous challenge with a virulent M. mycoides. They further recommended that a vaccinating dose of 37,000 organisms to induce 95% protection of cattle. Using the 46th egg/broth passage a minimum of 10^7 organisms is required (GILBERT and WINDSOR, 1971). PIERCY and KNIGHT (1958) suggested that too high passages of the avianized T_1 strain of M. mycoides failed to stimulate an immunity sufficient to withstand exposure to virulent outbreaks, although the 46th passage of the same organism conferred immunity that withstood severe outbreaks (MINOR, 1967). PIERCY and KNIGHT also found that when the more virulent T_3 strain was used all animals were solidly immune to a number of subsequent challenges. Since some reactions were noted, the authors suggested a further passage of the organism.

PIERCY (1958) reviewed the use of Walker's (Kenya) and Bennett's (Sudan) broth culture vaccines over 30 years. He supported PRIESTLEY's (1955) view that the broth culture vaccine used since 1932 had not succeeded in eradicating the disease in Sudan. The author repeated PIERCY and KNIGHT's (1957) comment that "In Kenya the overall results of repeated vaccinations with attenuated cultures were so disappointing

that in many instances native stockowners became indifferent or even slightly antagonistic to further campaigns". The main disadvantage of the liquid vaccines was their short shelf life, usually one month or less. PIERCY (1958) observed that the site of inoculation was selected to limit the reaction that followed; Anglophone countries in Africa selected the tail tip and Francophone countries advocated the muzzle. The author cited the work of SHERIFF and PIERCY (1952, 1953) and later that of PIERCY and KNIGHT (1956, 1957) who investigated the possibility of using dried egg vaccines in cattle in East Africa. A safe avianized T_1 strain vaccine was produced, which represented the first Contagious bovine pleuropneumonia vaccine that could be tested in cattle before field use. Successful application of avianized vaccines was achieved in the late 1950's by KNOWLES in Nigeria, MENDES in Angola, PROVOST in Equatorial Africa and BOWLER in Ethiopia. Several workers had reported that about 10% of the animals vaccinated seemed naturally resistant. PIERCY (1958) suggested that this semi-resistance led to the failure of the avianized T_1 vaccine to immunize a proportion of cattle since the attenuated organisms were unable to propagate satisfactorily in these animals. To overcome this, the more virulent T_3 strain was used although it caused severe tail reactions in the field.

PIERCY and KNIGHT (1958) tried immmizing cattle with avianized vaccines by first administering the T_1 strain, with a second inoculation of the T_2 strain a month later. This was to overcome the reactions and also to immunize semi-resistant animals. They obtained encouraging results.

DORMAN (1962) recorded that avianized T_1 and T_2 vaccines appeared to stop the development of further clinical cases in Kenya Masailand during a cycle of outbreaks. Still, there were some severe reactions with the vaccines and anaphylactic shock was noted in 3% of the animals (IBAR 1964). Severe reactions following vaccination with avianized vaccines were aslo recorded in Uganda (IBAR 1964). HAMMOND and BRANAGAN (1965) noted that the anaphylactic reactions that followed revaccination were probably due to the egg component. Anaphylactic reactions were so severe in cattle in some areas that finally use of the vaccine was discontinued.

HUDSON and TURNER (1963) used incontact trials to evaluate the efficacy of Contagious bovine pleuropneumonia vaccines. The animals were vaccinated with both broth and egg vaccines through the tail tip. Swellings of the tails were rarely detected before the 7th day, but almost invariably by the 21st day. The reaction usually regressed from the 21st day unless Gluteal involvement had occurred. Lack of a serological response had led previous workers to recommend

that cattle in Kenya receive a series of 3 injections of vaccine. HUDSON and TURNER (1963) found that the egg vaccine produced lesions but the broth vaccines did not, primarily because the egg component of the vaccine formed an embolus in the lung vessels leading to ischaemia and necrosis. Using the V_5 strain, neither broth nor egg products conferred absolute protection on all animals, but the effect on the clinical disease was striking. Although egg vaccine produced an earlier response than the broth vaccine, after one month the protection was similar.

ORUE (1964) described the prophylaxis of Contagious bovine pleuropneumonia in French speaking West Africa by the use of avianized culture vaccines (Ovo-vaccines). He concluded that while the lyophilised ovo-vaccine had good shelf life and immunogenic properties, the veterinary services preferred the use of broth culture vaccine for the latter had been proved efficacious and was well accepted by the stock owners. The author, however, maintained that the ovo-vaccine injected intradermally had good efficacy and inocuity.

LINDLEY (1967) described an experiment in which he compared the F and KH_3J strains of M. mycoides vaccines against Contagious bovine pleuropneumonia. The animals were vaccinated with either freeze dried egg KH_3J vaccine or the wet F strain vaccine and challenged by subcutaneous inoculation with

a virulent strain. The results showed that the strains were similar in immmogenic potential, but the KH_3J vaccine induced severe local reactions. One animal inoculated with the F strain developed a severe "Willems" reaction and died while none given the KH_3J strain died.

HUDSON (1968) described the development of a satisfactory and safe vaccine in which the avirulent KH_3J strain mixed with 20% brain suspension was found to be an efficient immunizing agent against Contagious bovine pleuropneumonia compared with the V_5. The product was stable at ambient temperatures for a reasonable period, it was easily freeze dried and the freeze dried vaccine kept well. GRAY and TURNER (1955) reported successful results with a dried vaccine without adjuvant. However, PRIESTLEY (1954) showed that dried organisms did not survive in tissues unless an adjuvant was added. He in fact stated that by using agar-gel as an adjuvant the number of organisms necessary to set up immunity is much less than anticipated and quoted 5×10^3 organisms per dose. Despite this finding CAMPBELL (1954) (cited by GRAY and TURNER, 1954) recorded a 97% protection when liquid vaccine wqs used and GRAY and TURNER (1954) recorded an 80% protection rate when the freeze dried vaccine was used. Thus liquid vaccine appeared to have better protective value. It should be noted however, that a dose of Campbell's liquid vaccine contained 10^9 organisms whereas the

freeze dried vaccine contained only 10^6 organisms. GILBERT and WINDSOR (1971) have recommended a minimum vaccinating dose to contain 10^7 organisms.

PRIESTLEY and DAFAALA (1957) working in Sudan found that old vaccines lost their efficacy and developed a freeze dried vaccine with adjuvants added. They found that inoculation of cattle with dried live Contagious bovine pleuropneumonia organisms reconstituted in 0.5% agar resulted in a rapid and efficient immunity to artificial challenge. The dried vaccine could be stored without any loss of potency for at least 14 months, even at a temperature of 37°C and probably for very much longer period at ice-chest temperature. They noted that immunity was established within 3 weeks, that an appreciable degree of immunity still existed at 12 months, and that by using an adjuvant, the number of organisms required to produce immunity was very much smaller than was anticipated. Severe reactions were seen when the vaccine was inoculated subcutaneously into the tail, and they noted that the agglutinating antibody induced was not necessarily the protective antibody. They concluded that other antibodies were involved in the immunity, or another protective factor was involved in the immunity. They failed to elicit an immunological response using dead vaccine. They observed that the serological response depended on the number of organisms given; the greater the number of organisms the faster the appearance of the antibodies. The finding that when agar was added

to the vaccine fewer organisms elicited protection is very suggestive that the agar protected the organisms for a longer time in the animal body. In fact, the authors suggested that where 5,000 organisms in broth or dried vaccine were sufficient to immunize cattle, 500 in agar were protective.

ORUE and DOUTRE (1971) reported 3 incontact trials in Senegal, using cattle vaccinated subcutaneously with 1 ml. of a freeze dried T_1 vaccine. Nearly all vaccinated cattle converted serologically. The first trial was carried out 3 months after vaccination, all animals were immune and had secondary serological reactions. After $7\frac{1}{2}$ months all animals were immune and only 1 of 15 animals had a transient serological reaction. In the trial carried out 14 months after vaccination, all vaccinated animals had a serological reaction and one died of Contagious bovine pleuropneumonia and one had Contagious bovine pleuropneumonia at post mortem examination. The authors concluded that T_1 freeze dried vaccine gave a solid immunity for up to 12 months. Unfortunately no trial was carried out after 12 months of vaccination and animals were not slaughtered 6 weeks after the first appearance of CFT reaction but they either died or were slaughtered at the end of the experiment. No estimate of Hudson and Turner scores were given and no efficiency of the vaccination was calculated.

C) COMBINED CONTAGIOUS BOVINE PLEUROPNEUMONIA AND RINDERPEST VACCINES

Live vaccines have been used in liquid form, freeze dried with or without adjuvant and they have also been used in combination with Rinderpest vaccine. BROWN and TAYLOR (1966) showed that simultaneous vaccination of cattle with rinderpest tissue culture vaccine with the T_1 broth culture vaccine resulted in immunity against both diseases. All 25 cattle vaccinated with TCRV were resistant on challenge. Of 19 cattle given Contagious bovine pleuropneumonia vaccine alone four reacted compared with 3 of 20 given Contagious bovine pleuropneumonia and rinderpest vaccine together and 18 out of 20 challenge controls reacted. None of the cattle given Contagious bovine pleuropneumonia vaccine alone or together with rinderpest vaccine produced complement fixing antibodies during the 3 weeks following vaccination but 3 animals given Contagious bovine pleuropneumonia vaccine alone and one animal given both vaccines possessed complement fixing antibodies against M. mycoides at the time of challenge 45 days after vaccination.

LINDLEY (1967) vaccinated cattle simultaneously with Contagious bovine pleuropneumonia vaccine with goat adapted Rinderpest vaccine. One group of 50 cattle was vaccinated with goat adapted rinderpest virus vaccine, another group of 50 with dried KH_3J vaccine, and a third group with both.

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LINDLEY (1967) vaccinated cattle simultaneously with Contagious bovine pleuropneumonia vaccine with goat adapted Rinderpest vaccine. One group of 50 cattle was vaccinated with goat adapted rinderpest virus vaccine, another group of 50 with dried KH_3J vaccine, and a third group with both.

No difference in the resultant immunity to Rinderpest could be detected between the two groups given Rinderpest vaccine whether alone or in conjunction with Contagious bovine pleuropneumonia vaccine. However, there was a marked difference in the resultant immunity between the two groups given Contagious bovine pleuropneumonia vaccine. Animals in the group which had been vaccinated with both Contagious bovine pleuropneumonia and Rinderpest vaccines developed subcutaneous reactions on Contagious bovine pleuropneumonia challenge. Furthermore, the results indicated that in the simultaneously vaccinated group there appeared to be a correlation between those animals which had a temperature reaction to Rinderpest vaccine and the subsequent absence of immunity to Contagious bovine pleuropneumonia. BROWN and TAYLOR (1966) had noted that animals vaccinated simultaneously with the T_1 broth culture vaccine and rinderpest tissue culture vaccine responded as if the two vaccines had been separately administered. PROVOST (1969) also produced a vaccine in which the Rinderpest and Contagious bovine pleuropneumonia vaccines were mixed. He chose the KH_3J strain because it did not cause reactions in Chad cattle. Equal volumes of KH_3J and Rinderpest vaccine were mixed in 5 ml. amounts and dried in 20 ml. bottles. The incontact trial results were not given but the trial suggested a protection in the region of 67%. In the following year (1970)

PROVOST, BORGEDON, and QUEVAL reported a combined freeze dried rinderpest/Contagious bovine pleuropneumonia vaccine in which KH_3JSR and rinderpest virus obtained from E.A.V.R.O. were used. Vaccinated animals were challenged 7 months after vaccination: 2 vaccinates had circulating antigen and 7 of eight had positive CFT reactions. In the second trial one vaccinated animal out of 26 developed Contagious bovine pleuropneumonia, 2 had circulating antigen and 11 had CFT reactions. HUDSON and TURNER score for the first trial was 67% and for the 2nd 23%. The incidence of Contagious bovine pleuropneumonia in Chad dropped from 200 per annum to 10 and consequently the authors suggested that the drop in incidence was due to a good vaccination cover. From the percentages of the efficacy of the vaccine it is obvious that at 7 months the protection was not so bad for the first trial but very low indeed for the 2nd trial. Experiments in this laboratory have recorded an efficacy of between Ep 67% and Ep 100% (DAVIES et al, 1968, GILBERT et al., 1970, WINDSOR et al., 1972, MASIGA and READ, 1972, MASIGA and WINDSOR, 1973).

D) DEAD VACCINES

Inactivated vaccines have been used and the results in general have been disappointing. Inactivated vaccines have

been prepared using formalin (CURASSON and HANRAS, 1930, WALKER, 1930, SHIRLAW and KRISHNA IYER, 1946; SHIFRINE and BEACH, 1968); Saponin (CURASSON, 1935) aluminium hydroxide and formalin (MENDES, 1957); beta-propiolactone and alhydrogel (BROWN, 1966); Saponin or digitonin (PROVOST, PERREAU and QUEVAL 1963). SHIRLAW and KRISHNA IYER (1946) observed that formalized lymph vaccines caused severe hypersensitiveness when animals were challenged other than immunity. Both control and vaccinated animals died when challenged. At this time SHIRLAW and KRISHNA IYER (1946) made the important observation that when live vaccines were used there was cross immunity between the strains. They made this observation when animals vaccinated with Assam 2 strain were solidly protected against Assam 1 strain on challenge and these strains were immunologically related to strains they received from Australia and Kenya. Vaccination with dried and heat-treated muscle vaccine was also a failure. These workers also tried to assess the immunity using a direct agglutination test without any success. SHIFRINE and BEACH (1968) also met with failure when they attempted to vaccinate cattle with dead vaccines. PROVOST, PERREAU and QUEVAL (1963) had found lysed M. mycoides not to be efficacious. However, some protection has been observed when inactivated vaccines have been given in large doses (GYAZIN and SHCHERBAKOV, 1954; BROWN, 1966).

VI. PHAGOCYTOSIS

In considering protection against any disease phagocytosis must always be considered. HUDSON (1968) was unable to repeat the phagocytosis work of PROVOST and VILLEMOT (1961). He therefore suggested that immunity in Contagious bovine pleuropneumonia was not dependent on conventional phagocytosis mechanisms. He had nevertheless, reported at the Third Session of the Joint FAO/IOE/OAU Expert Panel on Contagious bovine pleuropneumonia held in Khartoum 1967, that Australian workers using roller-tube technique, demonstrated phagocytosis of the triated-tagged V_5 organism. In some trials phagocytosis occurred in blood from both immune and susceptible donors. CRUE, MEINERY and THIERY (1961) reported the lymphotropic behaviour of M. mycoides. Australian workers concluded that some factor other than antibody in immune animals could influence the results. At the same meeting it was stated by workers at Chad that virulence of strains could affect the results of roller-tube technique. With fully virulent strains phagocytosis was hardly ever observed and with less virulent strains phagocytosis was observed with blood from immune animals. MASIGA and BOARER (1973) observed phagocytosis using the T_1 strain with normal bovine blood.

HUDSON (1968) suggested that the mechanism of immunity in Contagious bovine pleuropneumonia was not dependent on the conventional phagocytosis mechanism. Moreover, he suggested that classical concepts of immunity could not be applied to Contagious bovine pleuropneumonia, and that it was unwise to assume that resistance to contact challenge was due to the same mechanism as resistance to subcutaneous challenge. The latter hypothesis was supported by the work of DAVIES and HUDSON (1968) who considered that a local factor in the respiratory tract was responsible for the immunity.

Looking through the literature it can safely be concluded that dead vaccines have been found useless after vaccination. Some Contagious bovine pleuropneumonia vaccines have caused local reactions which have extended to gluteal area while others have caused no reaction at all. The serological responses have been detected within the first week of vaccination and have persisted up to 8 weeks. However, animals with severe reactions have had persistent serological responses. Humoral antibodies have been found to play no part in immunity against Contagious bovine pleuropneumonia. The immunity has lasted on average for about one year following vaccination with various vaccines against Contagious bovine pleuropneumonia although periods of 3

years have been reported. The period in which immunity has developed has not been ascertained although many authors have cited 3 weeks after vaccination. The mechanism of immunity however, has not been worked out.

CHAPTER 3

IN VIVO STUDIES WITH T_1 STRAIN OF MYCOPLASMA MYCOIDES VAR. MYCOIDES

PRIMARY VACCINATION

A) Subcutaneous route

B) Tail tip route

INTRODUCTION

Various Mycoplasma mycoides var. mycoides (M. mycoides) strains have been used as vaccines to control Contagious bovine pleuropneumonia (C.B.P.P.) in many countries. Investigations, on the persistence of some of these strains, following primary vaccination of cattle, have been carried out by several workers.

TURNER and TRETHEWIE (1961) observed a bacteraemia soon after primary vaccination of cattle with the V_5 strain of M. mycoides, as did HUDSON and LEAVER (1965) following the tail tip inoculation of a V_5 egg-vaccine. HUDSON (1965)

stated that the KH_5J strain persists in regional lymph nodes for up to 3 months after vaccination, although LINDLEY and PEDERSEN (1968) could only recover the strain up to 2 months. DAVIES (1969a) and MASIGA and MUGERA (1973), using the T_1 strain of M. mycoides recovered the organism from tissues 14 and 71 days respectively, after primary vaccination.

Cattle vaccinated with the T_1 broth culture vaccine have been found, on challenge, to be solidly immune to CBPP (DAVIES, MASIGA, SHIFRINE and READ, 1968, GILBERT, DAVIES, READ and TURNER, 1970, WINDSOR, MASIGA, and READ, 1972, MASIGA and READ, 1972 and MASIGA and WINDSOR, 1973). However, the immune mechanisms involved remain unknown (GILBERT and WINDSOR, 1971). LLOYD (1967) failed to transfer immunity by injecting susceptible cattle with bovine immune sera prior to subcutaneous challenge; and consequently suggested that the immunity involved in CBPP was probably of a cell mediated type. ROBERTS, WINDSOR, MASIGA and KARIAVU (1973) demonstrated cell-mediated immune reactions in cattle infected with CBPP and failed to do so in cattle vaccinated against the disease.

This chapter records the intermittent bacteraemia that occurs after primary vaccination of cattle using the T_1 strain. In addition, the persistence of the M. mycoides

organism and associated antigens will be described and the development of antibodies in cattle vaccinated with the T_1 broth culture vaccine via the tail tip route will be discussed.

MATERIALS AND METHODS

ORGANISM

The T_1 strain of M. mycoides maintained at the East African Veterinary Research Organization was used for vaccinating cattle by the subcutaneous and tail tip routes. This vaccine strain has been used throughout East Africa for the control of CBPP. In both cases, the conventional dose employed in East Africa was used; that is 0.5 ml. of the T_1 broth culture vaccine (BROWN, GOURLAY and MACLEOD, 1965), containing approximately 10^9 organisms.

CATTLE

Seventeen zebu or zebu-exotic cross bred animals were vaccinated subcutaneously for studies on resultant bacteraemia. Ten other cattle were vaccinated by the tail tip route for investigations on the persistence of the organism and associated antigens in tissues following primary vaccination. Forty five cattle were used for the primary serological response study.

All the animals were obtained from an area of Kenya known to be free from CBPP and they were serologically negative before the experiment. All cattle weighed between 150-200 Kg. and were between 1-2 years of age.

RECOVERY OF T_1 STRAIN OF M. MYCOIDES FROM BLOOD OF VACCINATED CATTLE

Various blood samples were obtained from the jugular vein immediately before and at frequent intervals after subcutaneous vaccination. Samples were collected at timed intervals up to 72 hours post primary vaccination for 5 animals and up to 147 hours for the remaining 12 cattle. On each occasion 100 ml. of blood was collected into 100 ml. of Newing's tryptose broth (GOURLAY, 1964) (see Table I).

TABLE I

TIME INTERVALS FOR COLLECTION OF BLOOD SAMPLES

Animal No.	TIME IN HOURS																												
	0	¼	½	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
C526	B	B	B	B	B	B	B	B	B	B	B		B														B.		
C520	B	B	B	B	B	B	B	B	B	B	B		B														B		
C507	B	B	B	B	B	B	B	B	B	B	B		B		B		B		B							B	B		B
C528	B										B		B		B		B		B						B		B		B
C506	B										B		B		B		B		B								B		B
C498	B							B																B					
C505	B							B																B					
C515	B							B																B					
C522	B							B																B					
C523	B							B																B					
C529	B							B																B					
C531	B							B																B					
C525	B							B																B					
C530	B							B																B					
D947				B							B														B				
D754				B							B														B				
D976				B							B														B				

..../2.

TABLE I (CONTINUED)

(2)

ANIMAL NO.	TIME IN HOURS																											
	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54
C507		B		B		B		B		B												B						
C528		B		B		B		B		B										B		B		B		B		B
C506		B		B		B		B		B												B		B		B		B
C498			B																B								B	
C505			B																B								B	
C515			B																B								B	
C522			B																B								B	
C523			B																B								B	
C529			B																B								B	
C531			B																B								B	
C525			B																B								B	
C530			B																B								B	
D947				B														B								B		
D754				B														B								B		
D976				B														B								B		

..../3.

ANIMAL NO.	TIME IN HOURS																											
	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82
C 528		B		B		B										B		B										
C506		B		B		B												B										
C 498															B								B					
C 505															B								B					
C 522															B								B					
C 523															B								B					
C 529															B								B					
C 531															B								B					
C 530															B								B					
D947												B								B								
D754												B								B								
D976												B								B								

...../4.

ANIMAL NO.	TIME IN HOURS																								
	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108
C498											B								B							
c505											B								B							
C515											B								B							
c522											B								B							
c523											B								B							
c529											B								B							
c531											B								B							
c525											B								B							
c530											B								B							
D947						B								B												
D754						B								B												
D976						B								B												

..../5.

TABLE I (CONTINUED)

ANIMAL NO.	TIME IN HOURS																						
	109	110	111	112	113	114	115	116	117	118	119	120	121	122	123	124	125	126	127	128	129	130	131
C 498									B								B						
C 505									B								B						
C 515									B								B						
C 515									B								B						
C 522									B								B						
C 523									B								B						
C 529									B								B						
C 531									B								B						
C 525									B								B						
C 530									B								B						
D947		B								B													B
D754		B								B													B
D976		B								B													B

..../6.

ANIMAL NO.	TIME IN HOURS														
	132	133	134	136	137	138	139	140	141	142	143	144	145	146	147
C 498									B						B
C 505									B						B
C 513									B						B
C 515									B						B
C 522									B						B
C 523									B						B
C 529									B						B
C 531									B						B
C 525									B						B
C 530									B						B
D947								B							B
D734								B							B
D976								B							B

B = Time of Bleeding

ANIMAL NO.	TIME IN HOURS														
	132	133	135	136	137	138	139	140	141	142	143	144	145	146	147
C 498									B						B
C 505									B						B
C 513									B						B
C 515									B						B
C 522									B						B
C 523									B						B
C 529									B						B
C 531									B						B
C 535									B						B
C 530									B						B
D947								B							B
D754								B							B
D976								B							B

B = Time of Bleeding

All incubation was carried out at 37°C. Subcultures were made every 2 days up to 14 days. Cultures showing no growth by this time were discarded. Blood was diluted 10-fold up to 10^{12} in 3 rows, and incubated for 7 days and the titre of the organisms was calculated by the REED and MUENCH (1938) method. The identity of the organisms isolated was confirmed by the growth inhibition test; described by DAVIES and READ (1968): briefly a wire loopfull of the cultures was applied on one sector of a well dried tryptose serum agar plate which was then allowed to dry before a drop of a specific rabbit anti-M. mycoides serum was applied to the centre of the culture. After drying, the plates were wrapped in polythene bags and incubated for 4 - 7 days. To facilitate reading, the plates were fixed in phosphate buffer for three minutes, then flooded with neutral red stain for 3 minutes. The plates were then washed in the buffer until the Mycoplasma colonies were pale in appearance.

Blood collected at the same time for serological tests was allowed to clot and serum for serological tests separated by centrifugation at 3,000 r.p.m.

For the recovery of M. mycoides with associated antigens from other tissues six animals were slaughtered at 1, 2, 3, 71, 116 and 204 days, respectively, after primary vaccination. The remaining 4 animals were slaughtered at various stages during the course of complement fixing (CF) antibody development, at the onset, peak and decline of CF activity and 2 weeks after

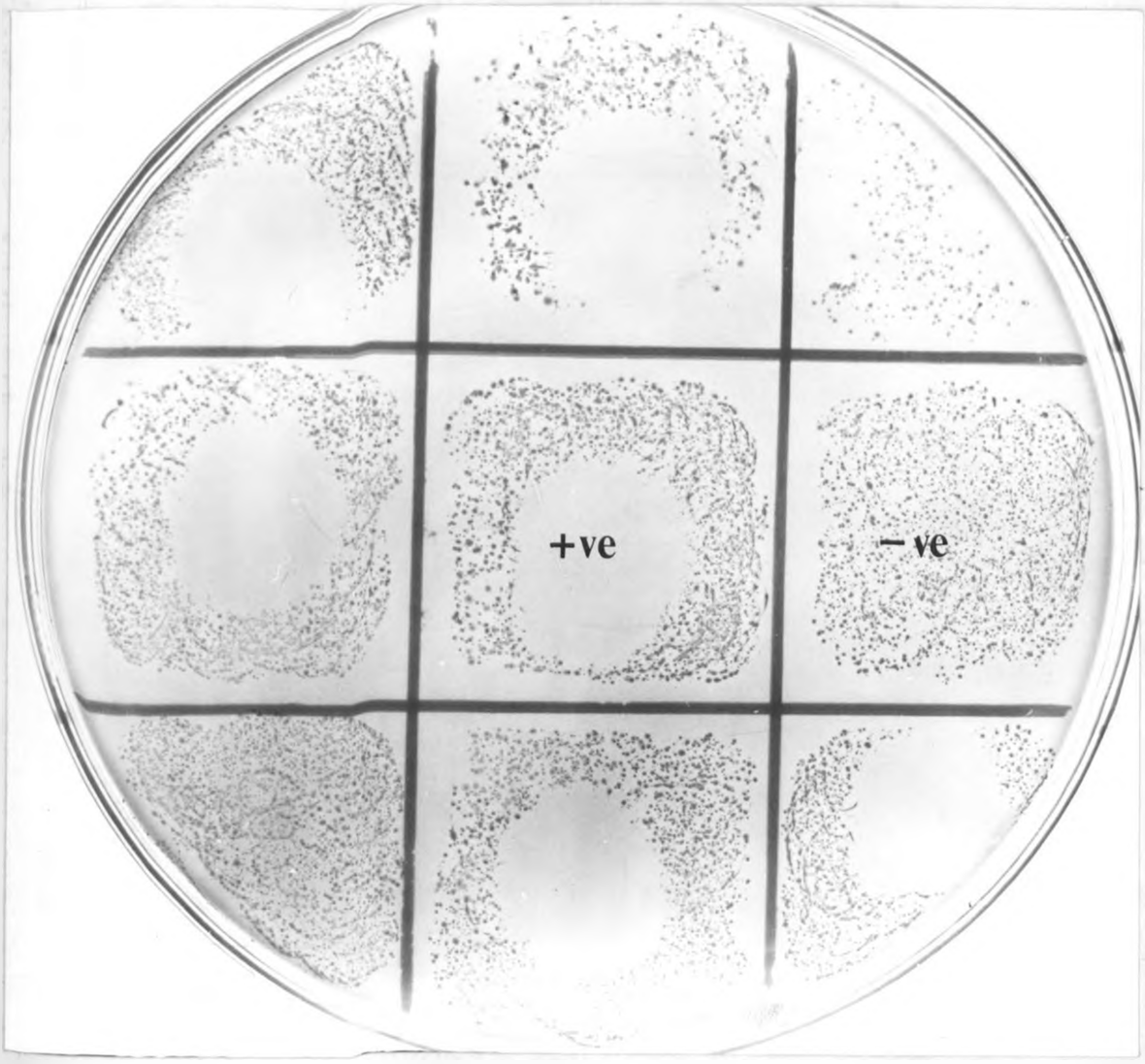

Fig. 1: Growth Inhibition Test using rabbit anti-M. mycoides serum.

The Agar Gel Diffusion Precipitin Test Pattern

KEY: 1 = Tissue

2 = Galactan or Whole M. mycoides antigen

3 = Tissue

4 = Galactan or Whole M. mycoides antigen

5 = Tissue

6 = Galactan or Whole M. mycoides antigen

7 = Pig Anti-M. mycoides serum

The agar gel plates were incubated overnight at room temperature in a moist chamber.

PREPARATION OF ANTI-M. MYCOIDES SERUM FOR AGAR GEL DIFFUSION TEST:

Large white or landrace pigs were inoculated with 3 ml. of whole M. mycoides antigen emulsified in complete Freund's adjuvant on 3 occasions at weekly intervals. Before each ino-

culation the pigs were bled and the sera tested by the AGT and immunoelectrophoresis. When strong precipitin lines were obtained on both tests, the pigs were bled out, serum separated, lyophilized in 2 ml. amounts and stored at -20°C. In addition, some whole serum was divided up into 2 ml. amounts and stored at -20°C.

PREPARATION OF THE ANTISERA FOR THE GROWTH INHIBITION TEST:

ANTIGEN:

M. mycoides was grown at 37°C. for 48 hours in tryptose broth containing normal rabbit serum in place of pig serum usually employed in Newing's Tryptose broth. The broth cultures were centrifuged at 18,000 g. harvested and washed 3 times in 0.8% saline. The pellets were resuspended in normal saline and adjusted to 10 times Brown's opacity tube No. 6. Some of the organisms were then mixed in complete Freund's adjuvant in equal volumes.

ANTISERA:

The rabbits were inoculated with 2 ml.of the antigen in saline by the intravenous route. Three days later, they were given 2 ml. of antigen emulsified in complete Freund's adjuvant and administered into four intramuscular sites

After a further 3 days the inoculation of antigen/adjuvant mixture was repeated, using a single subcutaneous site. At this stage the rabbits were test bled and also clinically examined. The serum was tested by the growth inhibition test and the slide agglutination serum test. If the serum gave strong positive results the animals were bled out and the serum stored at -20°C. If the serum at this stage was not strong enough, depending, on the clinical condition of the animals, a final inoculation of the antigen was carried out either by the subcutaneous route or intravenous route. On each occasion of inoculation the rabbits were given penicillin to decrease other bacterial contaminations.

The serum was inactivated for 30 minutes at 56°C. before use. Using this method of antisera production, it was possible to obtain antisera for growth inhibition test within 2 weeks.

SEROLOGICAL TESTS

"COMPLEMENT FIXATION TEST"

Guinea pig complement was used at 2.5 haemolytic units per tube. The complement titre of the normal guinea pig serum averaged 1:100. Antigen for the complement fixation test was prepared using the T_1 strain of *M. mycoides*. Broth cultures

were separated in the lister milk separator, resuspended in distilled water, autoclaved and diluted in distilled water to 10 times Brown's opacity tube No. 2. On titration the antigen batches had titres ranging from 1:45 to 1:60. Phenol was added as a preservative at a concentration of 0.5 gms/100 ml. The antigen was stored for six weeks with weekly shaking: this stabilised the antigen.

TESTS:

These were carried out using a modified Campbell and Turner (1953) method.

REAGENTS

1. Antigen suspension
2. Standard positive serum which had been freeze dried and stored at -20°C and which had a titre of 1/640.
3. Complement - 2.5 minimum haemolytic units.
4. 3% Sensitised sheep red blood cells.
5. Veronal buffer diluent.

TITRATION OF ANTIGEN:

METHOD

1. Doubling dilutions of the antigen were carried out in veronal buffer from 1/10 to 1/320.

2. The standard positive serum from 1/10 to 1/640 was diluted in doubling dilutions in venoral buffer.

TEST

One volume of antigen, 1 vol. of test serum and 1 vol. of complement were mixed:- (1 vol. = 0.25 ml.). The mixtures were then arranged as a chequer-board titration and incubated in a 37°C water bath for 30 minutes. One volume of sensitized. Sheep red blood cells (RBC) were added. The mixtures were shaken to mix and then incubated in the same water bath for the same time.

The test was read off depending on the degree of haemolysis:-

4 = Complete haemolysis

3 = 3/4 haemolysis

2 = 1/2 haemolysis

1 = 1/4 haemolysis

0 = No haemolysis

SLIDE AGGLUTINATION SERUM TEST:

ANTIGEN:

The antigen was prepared from 60 hour old T_1 broth culture,

it was separated in the lister separator as for the CF-antigen, autoclaved and resuspended in saline. The antigen was diluted to 10 times Brown's opacity tubes between Nos. 5-8 before alcian blue dye was added by drops until the colour matched a standard batch stored in the laboratory.

TEST:

One drop of the test serum was placed in a slide well and an equal volume of the antigen was added. The two drops were thoroughly hand mixed and further mixing was carried out by the automatic rotator. The test was read and graded as follows:-

++++ = Strong positive with large agglutination particles

+++ = Positive with smaller agglutination particles

++ = Weak positive

+ = Very weak positive

(See Fig. 2)

RESULTS

ISOLATION OF M. MYCOIDES FROM BLOOD

Table II shows the times post primary vaccination, which M. mycoides was isolated from the blood of cattle. The organisms were recovered from 6 of the 18 animals at 8, 10, 12, 16, 24, 30, 32 and 50 hours. No isolation of M. mycoides was made from blood of the other animals. Thus M. mycoides was

recovered from blood of these subcutaneously vaccinated cattle only rarely and at irregular intervals. No isolation of M. mycoides was made from the blood of animals vaccinated by the tail tip route.

ISOLATION OF M. MYCOIDES AND DETECTION OF ASSOCIATED ANTIGENS AND TISSUE ANTIBODY FROM TISSUES FOLLOWING TAIL TIP VACCINATION OF CATTLE

Table III shows the tissues from which M. mycoides was isolated. Organisms were recovered from the tail tip of all the animals killed during the 24 days following primary vaccination, but could not be detected at the site of inoculation in those killed from the 36th day onward. The only other tissues from which M. mycoides was isolated were lymph nodes of the iliac inguinal and sacral regions, which drain the site of vaccination. Organisms were recovered from these regional lymph nodes up to 71 days post vaccination. The tissues in which precipitating antigens were detected by the AGT are also shown in Table III. Antigens were first detected in the liver and the right iliac lymph node 72 hours after vaccination. M. mycoides or related antigens were found to have wider distribution in the body tissues of animals killed from the 9th day onward. In addition, antigens were detected periodically in kidney, spleen and lung (Fig. 3), as well as

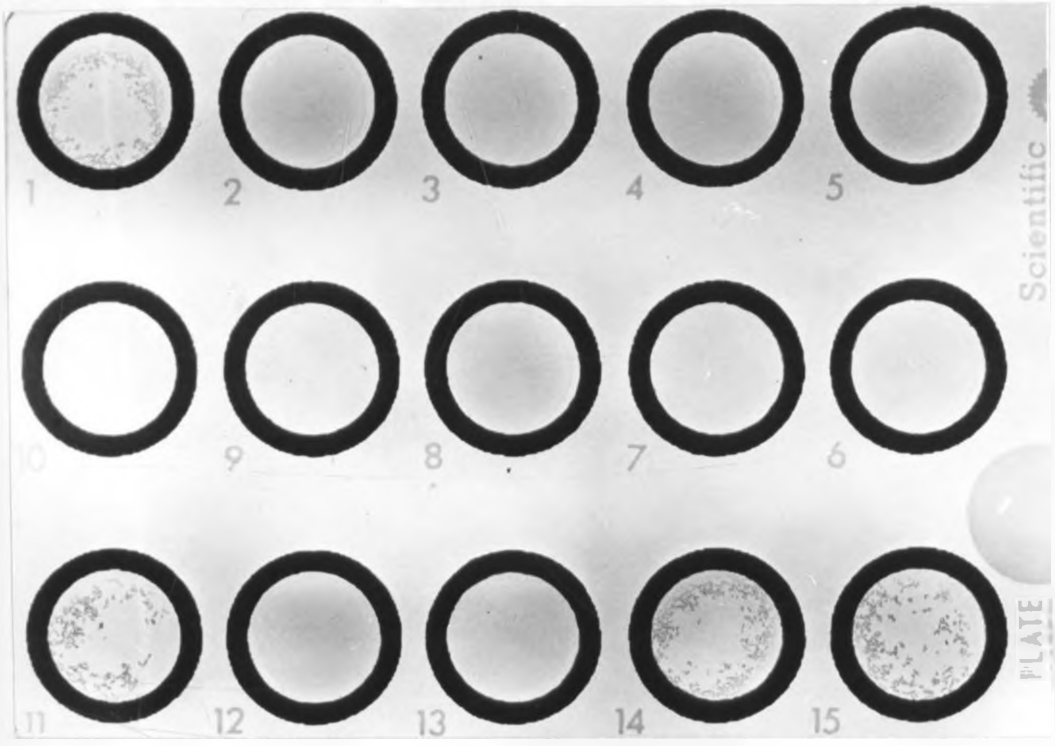

Fig. 2: Slide Agglutination Serum Test showing agglutination in wells, 1, 11, 14 and 15 with negative reaction in the remaining wells.

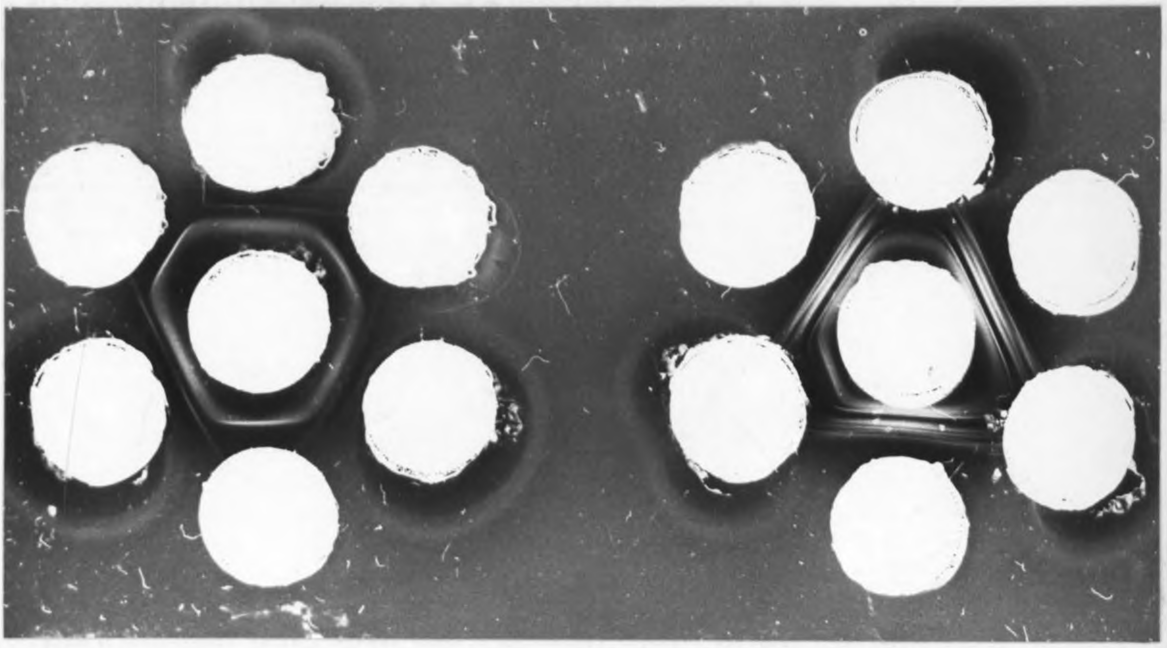

Fig. 3: Detection of M. mycoides antigen using Agar Gel Precipitin Diffusion Test. Left: wells at 2, 6 and 10 o'clock contain galactan and wells at 4, 8 and 12 o'clock contain lung tissue from cattle vaccinated with the T_1 strain of M. mycoides. Right: wells at 12, 4 and 8 o'clock contain lung tissue from vaccinated cattle and wells at 2, 6 and 10 o'clock contain whole M. mycoides antigen.

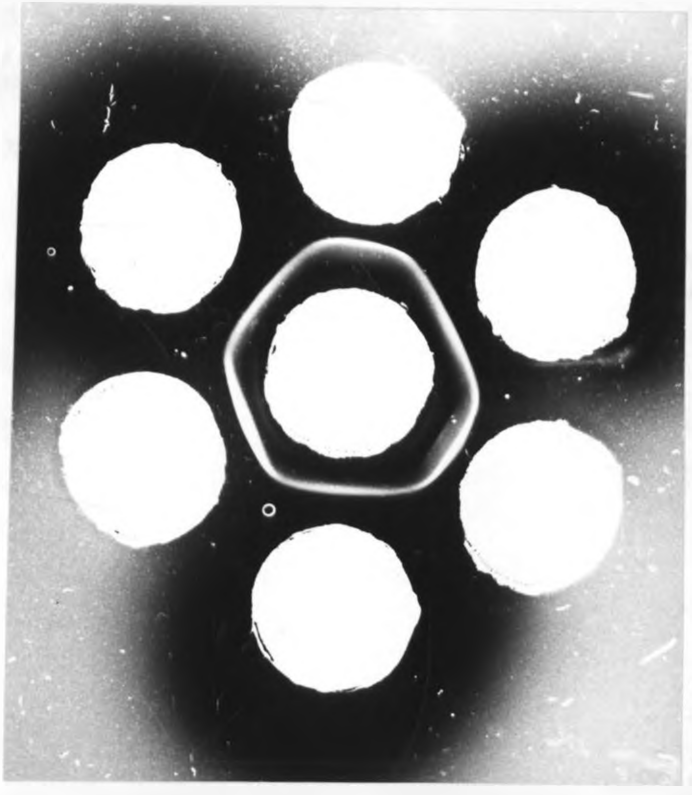

Fig. 4: Detection of M. mycoides antigen using Agar Gel Precipitin Diffusion Test: wells at 12, 4 and 8 o'clock contain galactan and wells at 2, 6 and 10 o'clock contain lymph node material from cattle vaccinated with the T_1 strain of M. mycoides.

in lymph nodes (Fig. 4) draining regions other than the tail. In none of the animals were antigens detected in the upper respiratory tract. Gel diffusion tests for the presence of M. mycoides specific galactan were positive for kidney and liver (animal 4), spleen and liver (animal 5) and for the liver, left cardiac lung lobe and regional lymph nodes (animal 7). These animals were killed 9, 11, and 36 days after vaccination, respectively. Galactan was more widely distributed in 2 animals killed at 116 and 204 days of primary vaccination: the antigen was found in left cardiac and left diaphragmatic lobes, in the kidney, liver and spleen in the anterior mediastinal, right bronchial, left popliteal, left prefemoral, right medial sacral, right and left submandibular and left parotid lymph nodes.

Antibody to M. mycoides was detected in the left diaphragmatic lung lobe and the right prescapular lymph node only in the animal killed 204 days after primary inoculation.

SEROLOGY:

The results in Table IVa and IVb show the serological response of cattle vaccinated with T_1 broth culture vaccine. The earliest complement fixing antibody was recorded after

5 days (animal 9). Three animals were killed before onset of CF-antibody. Three other animals were killed during the onset, height and decline of the CFT response, and hence the duration of CF-antibody could not be determined. Of the remaining 4 animals, 2 had CFT titres for 23 days, one for 12 days, and the remaining animal for 10 days (see Table III). All the animals that lived beyond 72 hours recorded a CF titre of more than 160, the highest titre being 2560 (animal 9). After the initial appearance of CF antibodies, the development of antibody titres was rapid, for instance one animal (animal 5) had a negative titre in the morning but had a titre of 20 by the evening and was up to 80 the following day, and another (animal 9) had a titre of 40 in the morning which rose to 160 in the evening. In general, the majority of the animals had doubled CFT titres within 8 hours.

The development of the agglutinating antibodies is also recorded in Tables IVa and IVb. All animals had agglutinating antibodies detectable by the fifth day after primary vaccination and persisting up to 24 days (animal 9).

DISCUSSION

The isolation of M. mycoides from the lymph nodes of

cattle for periods ranging from 14 days to 3 months after inoculation has been recorded by HUDSON (1965), DAVIES (1969a), LINDLEY and PEDERSEN (1968) and MASIGA and MUGERA (1973). However, the recovery of organisms from the blood seems to be possible only for a relatively short period after inoculation; for example TURNER et al. (1961) found a bacteraemia which appeared 5 minutes after vaccination of cattle with the V_5 strain and which persisted for only 2 hours. Similarly in the present in vivo experiments no bacteraemia was detected later than 50 hours after vaccination of cattle by the subcutaneous route (see Table II). A notable difference between the findings of TURNER et al (1961) and the results of the present experiment was the intermittent nature of the bacteraemia demonstrated by this study. It should be noted that TURNER et al. (1961) used the V_5 strain of M. mycoides and not the T_1 strain and that they used the tail tip route of vaccination.

Many factors play a part in governing the number of Mycoplasma organisms in the blood at any one time after vaccination, among these are the rate of entry of injected Mycoplasma into blood and or lymph channels, the extent to which they may be phagocytosed by leucocytes both at the site of vaccination and in the blood by the circulating macrophages, the bactericidal activity of the complement (Chapter 6) and the rate of multiplication of organisms in blood and other tissues.

With all these factors involved it is probable that the titre of circulating Mycoplasma can vary considerably in the period following their injection; and this may account for the fact that they were detected only at irregular intervals. The ingestion and subsequent release of Mycoplasma by leucocytes *in vitro* (Chapter 7) suggested that the intermittent bacteraemia which occurred *in vivo* may have been due to the release of large numbers of organisms into the blood by the disruption of leucocytes due to the toxic activity of *M. mycoides*.

M. mycoides was recovered from the regional lymph nodes of all except three of the ten animals vaccinated with T_1 broth culture vaccine.

Whereas *M. mycoides* was recovered from the tails (site of inoculation) of six animals and the regional lymph nodes of 7 animals post primary vaccination, it was not recovered from any of the other tissues examined. This finding is similar to that of LINDLEY and PEDERSEN (1968) who, using a subcutaneous route of inoculation at the side of the neck, were unable to recover the KH_5J strain from sites other than the regional lymph nodes. DAVIES (1969) however, recovered *M. Mycoides* from the trachea of one of 24 animals vaccinated at the tail tip with the T_1 strain.

Whereas LINDLEY and PEDERSEN (1968) suggested that *M. mycoides* antigens persisted in the tissues of vaccinated

animals for as long as the organism, the results of the present experiment suggest that the antigens can persist in animal tissues for a period longer than that for which the organism is detectable by traditional means.

DAVIES (1969) recovered the T_1 strain of M. mycoides from regional lymph nodes draining the tail of non-stressed and stressed animals 14 and 42 days respectively after primary vaccination. The present findings show that the T_1 strain of M. mycoides can persist in animal tissues for longer periods.

DAVIES (1969) concluded that because M. mycoides was not recovered from any tissue other than the regional lymph nodes (draining the tail), the immunological message was transported from the local lymph nodes to other parts of the body. However, it is interesting to note that on this occasion M. mycoides antigens or antigenically related antigens became wide spread throughout the body within 10 days of vaccination (see Table III). Common antigens are known to exist between M. mycoides and bovine tissues (SHIFRINE and GOURLAY, 1965). The antigens detected in various tissues of the vaccinated animals might have been "altered bovine tissue antigens". It has been hypothesised by WEISSMAN, KEISER and BERNHEIMER (1963) that in auto-immune diseases such as rheumatoid arthritis, degrading enzymes released from subcellular particles may denature native

body tissues to render them to be auto-antigens. This hypothesis was further supported by HIRSHORN, SCHREIBMAN, VERBO and GRUNAKIN (1964), who advanced the theory that the release of lytic enzymes alters native proteins which are no longer recognized as "self". KAKOMA, MASIGA and WINDSOR, (Res. Vet. Sci. - in Press) have suggested that CBPP is an auto-immune disease. If this is the case, then the antigens observed in these experiments may be "altered host tissue antigens".

At 204 days both M. mycoides galactan, or related host antigens, and antibody were detected by the AGT in the left diaphragmatic lobe and the right prescapular lymph node of animal 10. This detection of galactan or related antigens 204 days after primary vaccination seems to be significant. GOURLAY (1965) demonstrated that galactan could be excreted in urine. It would be expected that most, if not all, the galactan from the original organism in the vaccine dose (approximately 10^8 organisms) would be excreted by 204 days. It is therefore reasonable to suggest that the galactan which was detected at this time had probably been either tissue bound, or that its nature had been changed to resemble galactan of the host or vice versa.

LINDLEY and PEDERSEN (1968) found no evidence of multiplication of the KH_3J strain of M. mycoides in animal tissues following vaccination. In the present experiments

the T_1 strain of M. mycoides persisted in tissues up to 71 days and at 36 days the sum titre of approximately 2.11×10^7 was obtained. Although the findings in these experiments did not suggest that the organism multiplied, it would be difficult to explain how the organism could persist in tissues for 71 days without multiplication. It is reasonable to suggest that to stimulate immunity against CBPP, the organism must lodge in the animals tissue for sometime and also that it must multiply in the tissues. This may be the reason why dead vaccines against CBPP are thought to be useless. GILBERT and WINDSOR (1971) suggested that animals which had been vaccinated with 10^5 organisms were found to be more susceptible to CBPP than the control animals. It appears therefore that to elicit immunity against CBPP a minimum number of organisms is required. If less than this minimum number of organisms is inoculated into the animals then the animals will be sensitized rather than immunized. It has been demonstrated in vitro that phagocytosis occurs with the T_1 strain of M. mycoides and that complement has a bactericidal action on the organism (see Chapter 7). It seems probable that when animals are vaccinated a large number of the organisms are destroyed by the normal body defence mechanisms. Thus, if the vaccine dose is below a certain minimum number (GILBERT and WINDSOR (1971) suggested 10^7) then insufficient numbers survive to stimulate immunity and instead hyper-sensitivity results.

PROVOST, PERREAU and QUEVAL (1963) found lysed M. mycoides when used as a vaccine not to be efficacious against CBPP, and SHIFRINE and BEACH (1968) demonstrated that dead vaccines did not protect cattle against CBPP. It is therefore difficult to explain what part in immunity against CBPP the persisting antigens play. However, it might be suggested that M. mycoides vaccine stimulates the immune mechanism of the animal but that the persisting antigens maintain the immunity at a local level. The antigens become wide spread within 10 days of vaccination (see Table III). GILBERT (personal communication) also found galactan in the skin of vaccinated and infected animals: thus galactan could play the role of maintaining immity at a local level as it is found wide spread in the animals that have had experience of M. mycoides. Indeed, in these experiments antibody to galactan has been demonstrated in the lungs and lymph nodes of cattle vaccinated with the T_1 broth culture vaccine.

LINDLEY and PEDERSEN (1968) and DAVIES (1969) suggested that the duration of CF antibody coincided with the persistence of M. mycoides in the vaccinated animals. The results in Tables II, VIa and VIb show that this may not be necessarily true for the T_1 strain. The longest period for which the CF antibodies were detected was 23 days, but the organism was isolated as late as 71 days. The experience gained in this laboratory

shows that CF antibody does not persist in the animal after primary vaccination with T_1 strain for periods longer than 8 weeks. Similarly, HUDSON (1965) recovered the KH_3J strain 3 months after primary vaccination, and LINDLEY and PEDERSEN (1968) after 2 months. Thus, M. mycoides seems to persist for much longer periods than the CF antibody, and the antigens persist for even longer periods. The results in Table IV a show that the appearance and duration of both the agglutinating and CF antibodies seem to coincide, in that all animals had agglutinating and CF antibodies within 5 days and both types of antibody persisted up to 24 and 23 days respectively.

It is difficult to explain why M. mycoides or its associated antigens persists in the animal body in the absence of a measurable CF and SAST response. It is equally difficult to explain why a serological response is stimulated by the organism for only a short period. A possible explanation is that the animal develops immune paralysis to certain antigens of M. mycoides after prolonged exposure to organism. This type of paralysis could be achieved in a similar manner as paralysis caused by immunogens which resist degradation. It has been found that some bacterial capsular polysaccharides such as those of pneumococcus Type III are very resistant to digestion by the enzymes present in the bodies of mice and rabbits. In these experiments M. mycoides galactan has been found in animals vaccinated 3-7 months previously. These resistant antigens

may mop up antibodies as they may be produced. Despite the absence of circulating antibodies, cell producing antibodies may be demonstrated in vitro, (HUMPHREY and WHITE, 1970, Chapter 6). Another possible explanation is that the animals develop "high zone" paralysis in which quantities of immunogens are substratially greater than those normally required to elicit a positive immunological response. The latter stands in contrast to another mode of inducing immunological paralysis, namely "low zone" paralysis which may result from continuous experience over a long time, of amounts of an immunogen smaller than those which elicit a detectable immune response. It may be added that M. mycoides galactan may be a weak immmunogen in cattle as it is not too distinct from bovine galactan (SHIFRINE and GOURLAY, 1965). This type of antigen easily causes paralysis (HUMPHREY and WHITE, 1970).

LLOYD (1967) failed to passively immunize susceptible cattle against CBPP, using immune sera. He consequently suggested that humoral antibodies do not play a part in the immunity of CBPP, but that the immunity may be due to some local factor. DAVIES (1969) also suggested that the immunity in CBPP could be vested in cell bound antibodies. The detection of antibody activity in the tissues of one animal 204 days after vaccination may have some significance.

Although bacteraemia has been demonstrated in this experiment after subcutaneous vaccination with the T_1 strain no bacteraemia has been observed when the tail tip route was used. The role played by the bacteraemia in immunity against CBPP following vaccination with the T_1 strain is difficult to explain. However, animals which had either been vaccinated by the subcutaneous route behind the shoulder (GILBERT and WINDSOR, 1971, WINDSOR and MASIGA , 1973) or the tail tip route (DAVIES, MASIGA, SHIFRINE and READ, 1968) were found solidly immune on challenge. It appears therefore that bacteraemia is neither essential nor detrimental for initiation and development of immunity against CBPP. Nevertheless, viable organisms are essential for the stimulation of immunity (PROVOST et al. 1963, and SHIFRINE and BEACH, 1968) and the minimum period for which they must persist in animal tissues to elicit the immunity is a subject of Chapter 5.

TABLE II

THE ISOLATION OF *MYCOPLASMA MYCOIDES* FROM THE BLOOD OF CATTLE VACCINATED BY THE SUBCUTANEOUS ROUTE USING THE T_1 BROTH CULTURE VACCINE

CATTLE NO.	TIME* OF ISOLATION OF *M. MYCOIDES* AFTER VACCINATION
C 520	10 and 24
C 507	10 and 16
C 528	10, 12, 30, 32 and 50
D 754	8
D 497	32
D 976	8 and 32
11 Others	NO ISOLATION MADE

* = Hours

TABLE III

ISOLATION OF M. MYCOIDES AND DETECTION OF MYCOPLASMA ANTIGENS IN TISSUES OF CATTLE VACCINATED WITH T_1 STRAIN OF M. MYCOIDES BY THE TAIL TIP ROUTE

No. of Animal	1	2	3	4	5	6	7	8	9	10
Days killed after vaccination	1	2	3	9	11	24	36	71	116	204
TISSUE										
L.C.	-	-	-	-	-	-	Ag	-	Ag	Ag
L.D.	-	-	-	-	-	-	-	-	Ag	Ab^{Ag}
Kidney	-	-	-	Ag	Ag	-	-	-	Ag	Ag
Liver	-	-	Ag	Ag	Ag	Ag	Ag	-	Ag	-
Spleen	-	-	-	-	Ag	-	-	-	Ag	Ag
Post. med. L.N.	-	-	-	-	-	Ag	Ag	-	-	-
Ant. med. L.N.	-	-	-	-	WAg	Ag	Ag	WAg	-	Ag
L. medial sac. L.N.	$+(10^2)$	-	-	$+(10^6)$	$+(10^2)$Ag	Ag	$+(10^7)$	-	-	-
R. medial sac. L.N.	+	-	-	-	$+(10^2)$	-	$+(10^7)$Ag	+Ag	Ag	-
L. submand. L.N.	-	-	-	WAg	-	-	-	WAg	Ag	-
R. submand. L.N.	-	-	-	-	WAg	-	Ag	-	Ag	Ag
L. Parotid L.N.	-	-	-	-	-	-	WAg	-	Ag	Ag
L. prescap. L.N.	-	-	-	-	-	-	-	-	Ag	Ag
R. prescap. L.N.	-	-	-	-	-	-	-	Ag	Ag	Ab^{Ag}
L. prefem. L.N.	-	-	-	-	WAg	-	-	-	Ag	Ag
R. prefem. L.N.	-	-	-	-	Ag	-	-	-	-	-
L. lateral sac. L.N.	-	-	-	+Ag	-	Ag	-	-	-	-
R. lateral sac. L.N.	+	-	-	(10^4)	-	-	-	-	-	-
L. sup. ing. L.N.	-	-	-	-	Ag	Ag	$+(10^2)$	WAg	-	-
R. sup. ing. L.N.	-	-	-	-	Ag	-	$+(10^6)$	-	-	-
L. deep. ing. L.N.	-	-	-	-	-	WAg	+	-	WAg	Ag
R. deep. ing. L.N.	-	-	-	-	WAg	$+10^6$	Ag	-	-	-
L. bronchial L.N.	-	-	-	-	WAg	-	-	-	-	-
R. bronchial L.N.	-	-	-	-	WAg	-	-	Ag	WAg	Ag
L. popliteal L.N.	-	-	-	-	-	-	-	-	WAg	Ag
R. popliteal L.N.	-	-	-	-	Ag	-	-	-	-	-
L. int. iliac. L.N.	-	-	$+(10^2)$	-	-	Ag	-	-	-	-
R. int. iliac. L.N.	$+(10^2)$	-	+Ag	Ag	+Ag	$+(10^5)$Ag	$+(10^5)$Ag	-	-	-
Tail	$+(10^5)$	$+(10^1)$	$+(10^6)$	$+(10^3)$	$+(10^7)$	+	-	-	-	-

Key: Ag = Antigen detected in AGT
Ab = Antibody detected in AGT
(10^2) etc. = approximate titres

WAg = Weak antigen detected in AGT
+ = M. mycoides isolated from tissues
+ approximate titres

L.C. = Left cardiac lung lobe
L.D. = Left diaphragmatic lung lobe
L.N. = Lymph node
SUF. ING. = Superficial inguinal

Seven other tissues (turbinate, trachae, left and right apical, right cardiac, right diaphragmatic lung lobes and right parotid lymph node) were examined and found negative.

TABLE IV a

THE SEROLOGICAL RESPONSE OF CATTLE VACCINATED WITH THE T_1 STRAIN OF M. MYCOIDES

ANIMAL NO.	DAY 1		DAY 2		DAY 3		DAY 4		DAY 5		DAY 6		DAY 7		DAY 8		DAY 9		DAY 10		DAY 11		DAY 12		DAY 13		DAY 14	
	AM	PM	AM	PM	AM	PM	AM	PM	AM	PM	AM	PM	AM	PM	AM	PM	AM	PM	AM	PM	AM	PM	AM	PM	AM	PM	AM	PM
1	0 −	0 −	0 −	0 −																								
2	0 −	0 −	0 −	0 −	0 −																							
3	0 −	0 −	0 −	0 −	0 −	0 −	0 −																					
4	0 −	0 −	0 −	0 −	0 −	0 −	0 −	0 −	0 −	0 −	0 −	0 −	0 −	10 +	20 ++	160 +++	80 ++++	160 +	40 ++									
5	0 −	0 −	0 −	0 −	0 −	0 −	0 −	0 −	0 −	0 −	0 +	20 +	40 ++	80 ++++	160 ++++	160 ++++	320 ++++	320 ++++	320 ++++	320 ++++	160 ++++	40 ++++	40 ++++					
6	0 −	0 −	0 −	0 −	0 −	0 −	0 −	0 −	0 −	0 −	0 −	0 −	20 +	20 +++	80 ++++	160 ++++	160 ++++	160 ++++	320 ++++	320 ++++	80 ++++	80 ++++	40 ++++	80 ++++	160 ++++	160 ++++	320 ++++	160 ++++
7	0 −	0 −	0 −	0 −	0 −	0 −	0 −	0 −	0 −	0 −	0 −	20 −	40 +	80 +++	160 ++++	320 ++++	320 ++++	320 ++++	80 ++++	160 ++++	160 ++++	80 +++	40 +++	80 +++	320 +++	320 +++	640 ++	1280 ++++
8	0 −	0 −	0 −	0 −	0 −	0 −	0 −	0 −	0 −	0 −	0 −	0 −	0 +	0 ++	20 ++	20 +++	80 ++++	20 ++++	40 ++++	80 ++++	20 ++++	20 +++	20 ++++	20 +++	40 ++++	40 +++	160 ++++	80 +++
9	0 −	0 −	0 −	0 −	0 −	0 −	0 −	0 −	0 −	0 −	20 −	40 +	40 ++++	160 ++++	320 ++++	640 ++++	1280 ++++	2560 ++++	640 ++++	640 ++++	160 ++++	160 ++++	160 ++++	320 ++++	640 ++++	320 ++++	1280 ++++	640 ++++
10	0 −	0 −	0 −	0 −	0 −	0 −	0 −	0 −	0 −	0 −	0 −	0 −	0 −	0 −	0 −	0 −	0 −	0 −	0 −	0 −	0 −	0 −	0 −	0 +	10 +	10 +	40 +++	20 ++

...../2.

TABLE IVa (CONTINUED)

ANIMAL NO.	DAY 15		DAY 16		DAY 17		DAY 18		DAY 19		DAY 20		DAY 21		DAY 22		DAY 23		DAY 24		DAY 25		DAY 26		DAY 27		DAY 28	
	AM	PM	AM	PM	AM	PM	AM	PM	AM	PM	AM	PM	AM	PM	AM	PM	AM	PM	AM	PM	AM	PM	AM	PM	AM	PM	AM	PM
6	320 ++++	320 ++++	640 ++++	1280 ++++	640 ++++	1280 ++++	+++	++++	++++	++++	160 ++++	80 +++	160 ++++	80 +++	80 +++	80 ++++	160 +++	160 ++++	40 +++	80 +++	40 +++							
7	320 ++++	640 +++	640 +++	1280 ++++	1280 ++++	1280 ++++	+++	+	+	+	40 +	40 -	20 +	80 +	20 +	20 +	80 +	80 +	20 -	40 +	80 -	0 -	0 -	20 -	20 -	0 -	0 -	20 -
8	40 +++	80 +++	80 +++	80 +++	80 +++	40 +++	40 +++	0 +	++	0 +	0 +	20 ++	0 ++	0 +	0 +	10 +	0 +	0 +	0 +	0 +	0 +	0 +	0 +	0 +	0 +	0 +	10 -	0 -
9	320 ++++	1280 ++++	640 ++++	1280 ++++	1280 ++++	640 +++	+++	+++	+++	+++	80 +++	80 +++	80 +++	80 +++	80 +++	80 +++	160 +++	80 +++	40 +++	80 +++	40 +	40 ++	0 +	10 ++	40 ++	10 ++	10 ++	10 +
10	40 ++	80 +++	320 +++	160 +++	160 +++	160 +++	+++	+++	+++	+++	0 +++	0 ++	0 +++	0 ++	0 +	20 ++	0 +	40 +++	40 +	40 +	0 ++	0 +	0 +	0 +	0 +	0 ++	0 -	0 -

....../3.

TABLE IVa (CONTINUED)

ANIMAL NO.	DAY 29		DAY 30		DAY 31		DAY 32		DAY 33		DAY 34		DAY 35		DAY 36		DAY 37		DAY 38		DAY 39		DAY 40		DAY 41	
	AM	PM	AM	PM	AM	PM	AM	PM	AM	PM	AM	PM	AM	PM	AM	PM	AM	PM	AM	PM	AM	PM	AM	PM	AM	PM
7	0 -	0 -	0 -	0 -	0 -	0 -	0 -	0 -	0 -	0 -	0 -	0 -	0 -	0 +	0 -	0 -	0 -	0 -	0 -	0 +	0 -	0 -	0 -	0 -	0 -	0 -
8	0 -	0 -	0 -	0 -	0 -	0 -	0 -	0 -	0 -	0 -	0 -	0 -	0 -	0 -	0 -	0 -	0 -	0 -	0 -	0 -	0 -	0 -	0 -	0 -	0 -	0 -
9	0	0 +	0 +	0	0	0	0	0	0	0	0	0	0	0 +	0 -	0	0	0	0 -	0 +	0	0	0	0	0	0
10	0			0 -										0	0				0	0						

THE SEROLOGICAL RESPONSE OF CATTLE VACCINATED WITH THE T_1 STRAIN

ANIMAL NO.	COMPLEMENT FIXATION TEST				SLIDE AGGLUTINATION SERUM TEST		
	FIRST APPEARANCE OF ANTIBODY (DAYS)	PEAK TITRE (RECIPROCAL)	DAY ON WHICH PEAK TITRE ATTAINED	DISAPPEARANCE OF ANTIBODY (DAYS)	FIRST APPEARANCE OF ANTIBODY (DAYS)	PEAK (DAYS)	DISAPPEARANCE OF ANTIBODY (DAYS)
1	K	K	K	K	K	K	K
2	K	K	K	K	K	K	K
3	K	K	K	K	K	K	K
4	7	160	8	K	7	9	K
5	7	320	9	K	6	7	K
6	7	1280*	16	K	7	8	K
7	6	1280	14	23	7	8	18
8	8	160	14	10	7	9	21
9	5	2560	9	23	5	7	24
10	13	320	16	12	11	19	16

* Reciprocal of CF titre

K = Killed before parameter could be measured

CHAPTER 4

IN VIVO STUDIES WITH T_1 STRAIN OF MYCOPLASMA MYCOIDES VAR. MYCOIDES: SECONDARY VACCINATION

INTRODUCTION

SHIFRINE, STONE and DAVIES (1968) reported an amnestic response following revaccination of 6 cattle which had been vaccinated 3 months previously. These authors however, did not observe an amnestic response when cattle were revaccinated 1 month after primary vaccination. LINDLEY (1971) observed that in animals vaccinated for the first time with the T_1 lyophilized vaccine, about 50% developed CF antibodies after 3 weeks, whereas, after a second vaccination (12 months later) only 12% showed positive results. Experience gained in this laboratory shows that 30 to 100% of animals vaccinated with the T_1 broth culture vaccine respond serologically following vaccination.

LINDLEY and PEDERSEN (1968) recovered the KH_3J strain of M. mycoides from cattle vaccinated with the strain 2 months previously. HUDSON (1965) recovered the V_5 strain of M. mycoides from cattle vaccinated 3 months previously. DAVIES (1969) isolated

the T_1 strain from cattle vaccinated 2 weeks earlier and in the present experiments (Chapter 3) the T_1 strain of M. mycoides was recovered for up to 71 days after primary vaccination. Apparently, no work has been done on the fate of T_1 strain following secondary vaccination.

In this chapter the results of the serological response and the persistence of the organism in the tissues of cattle following revaccination with the T_1 broth culture vaccine are recorded.

MATERIALS AND METHODS

CATTLE:

Fifty eight cattle weighing between 150 - 200 kg. and between 1 and 2 years of age were selected. These animals were vaccinated with the T_1 broth culture vaccine using the conventional dose of 0.5 ml (containing approximately 10^9 organism/ml) administered subcutaneously in the tail tip. The ensuing serological response was studied. Attempts to isolate M. mycoides from blood were also carried out as described in previous chapters.

EXPERIMENT 1:

For serological response studies 10 cattle were revaccinated with the T_1 broth culture vaccine 6 months after primary vaccination, and 10 cattle were vaccinated after 1 year. Two years after primary vaccination these 20 animals were challenged in an "incontact trial" together with another 11 animals which had been vaccinated two years previously but had not been revaccinated. Ten control animals were also used. An incontact trial will be described in detail in Chapter 5.

EXPERIMENT 2:

Eighteen cattle were used to study the persistence of the T_1 strain of M. mycoides following revaccination. The serological response following revaccination was also studied. Six animals were slaughtered within 7 days after revaccination. These animals were revaccinated 166, 175, 185, 573, 579 and 638 days after primary vaccination. Two cattle were slaughtered within 2 weeks following revaccination: these animals had been vaccinated 314, and 594 days previously. One animal was slaughtered 2½ weeks after revaccination. This animal had been vaccinated 312 days previously. Two animals were

killed 3 weeks after revaccination: these animals had been vaccinated 166 and 535 days previously. Four animals were slaughtered four weeks after revaccination and these animals had been vaccinated 221, 244, 315 and 315 days previously. The remaining 3 animals were slaughtered 43, 44 and 49 days after revaccination. These animals had been vaccinated 221, 221 and 244 days before the revaccination. The last animal was revaccinated 1 year after primary vaccination and was slaughtered 2 weeks after secondary vaccination.

EXPERIMENT 3:

The remaining 10 animals were also used to study the persistence of M. mycoides in tissues following revaccination. All the animals were revaccinated 15 months after primary vaccination. As no recovery of M. mycoides from tissues following revaccination had been achieved beyond 3 weeks following revaccination (See Results Table IX) the slaughter programme was designed to start 20 days after revaccination. Three animals (D 837, E 90 and E 975) were slaughtered at 20 days; two animals (D 894 and E 92) were slaughtered at 30 days; two animals(D 877 and D 971) at 36 days; two (D 896 and D906) at 42 days and of the remaining 2 animals, one was slaughtered

at 50 days and the other 52 days after revaccination.

At slaughter the tissues as described in Chapter 3 were dissected and inoculated into the isolation medium described in Chapter 3 in an attempt to isolate M. mycoides. The tissues were also examined for antigen using the AGT as described in Chapter 3.

EXPERIMENT 4:

To study further the serological secondary response following revaccination of cattle with the T_1 broth culture vaccine, 20 more cattle were selected. These animals were vaccinated with 0.5 ml. of T_1 broth culture vaccine via the tail tip route. Four months later the animals were revaccinated via different routes using increased amounts of the vaccine: 4 animals were given 50 ml. of T_1 broth culture vaccine intravenously; 8 animals were vaccinated subcutaneously; 4 with 10 ml, and 4 animals with 50 ml of the vaccine; 4 animals were inoculated with 20 ml. of the vaccine intramuscularly and the remaining 4 animals were revaccinated with 2 ml. of the T_1 broth culture vaccine in the tail tip. The animals were bled at weekly intervals and the CFT was carried out on all the sera.

SEROLOGY:

The serological tests employed were the CFT, the SAST, the AGT and the Immunoelectrophoresis (IEP).

The serum samples which had positive CFT reactions were fractionated on Sephadex G 200 and the optical density of the various fractions determined on a Beckman D.B. Spectrophotometer* using wave length of 280 nm. Three peaks were obtained (see Fig. 5a, b, c, d, e, f and g). CFT was carried out on each of the fractions.

Each of these 3 fractions was tested using immunoelectrophoresis against rabbit anti-bovine IgM and IgG. To each of the fractions, 2-mercaptoethanol (Koch-Light, Ltd.x) (2-ME) was added, in order to destroy the activity of IgM. Before use, the stock solution of 2-ME was diluted 1:2 in saline. To each 1 ml. of the fractions 0.1 ml. of 2-ME was added and the mixture was left at room temperature for 12 hours. This was then dialysed overnight at room temperature against saline. The dialysates were then subjected to the CFT using M. mycoides antigen.

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* Beckman Instruments, Inc. Fullerton, California, U.S.A.

x Koch-Light Laboratories Ltd., Colnbrook, Bucks, England.

PREPARATION OF RABBIT-ANTI-BOVINE IgG and IgM:

Pure bovine IgM and IgG (kindly prepared by Dr. S.S. Stone) in complete Freund's adjuvant were inoculated into rabbits. 1.5 ml. of the immunoglobulins in complete Freund's adjuvant was given to each rabbit on each occasion. The rabbits were simultaneously inoculated through the foot pads (0.2 ml.) and intramuscularly (1.3 ml.). The inoculation was repeated 3 times at weekly intervals. Test bleedings were carried out between the inoculations. Strong lines were obtained on IEP after the third inoculation, with rabbit serum being developed against IgM and IgG.

R E S U L T S

EXPERIMENT 1:

The CFT and the SAST reaction results on the sera of 20 animals are recorded in Table V. With the exception of two animals (C702 and C679) all cattle developed CF antibodies following primary vaccination. The duration

of the CF titres ranged between 1 and 2 weeks. The lowest CF titre registered was 20 (animal C831) and the highest was 320 (animal C682 and C834). All animals had agglutinating antibodies which persisted up to 8 weeks (animal C702). The majority of the animals were negative to the SAST by the SAST by the 6th week (see Table V).

Following revaccination only 6 animals (C859, C374, C514, C709, C850 and C831) had CF antibody titres (Table V). Two of the animals had titres of 40 (C859 and C374) two of 20 (C514 and C709) and 2 of 10 (C850 and C831). None of these animals had CF titres beyond two weeks following revaccination. Three animals were revaccinated 6 months after primary vaccination and the remaining 3 after one year. Three animals had CF titres 2 weeks after revaccination and the other 3 were positive by the 10th week after revaccination. All animals in this group had weak SAST reactions which in many cases persisted for longer periods than the response observed during the perimary vaccination.

CLINICAL AND POST-MORTEM FINDINGS AFTER CHALLENGE OF REVACCINATED CATTLE:

The results of challenging revaccinated cattle are shown in Table VI. Two control animals died of CBPP and the remaining 9 animals were killed 6 weeks after the first

appearance of CFT reaction. Five animals had temperature reactions higher than 39.5°C for more than 3 days. At post-mortem examination eight animals showed CBPP lesions; 4 animals with acute or subacute lesions; 3 had sequestra and one had old pleural adhesions. Three had no lesions despite the prolonged exposure.

M. mycoides was isolated from 8 of the 11 control animals; of these 8, seven were those with lesions of CBPP; the 8th animal (C778) had lung lesions which were not typical CBPP and M. mycoides was not isolated. Of the 3 animals without respiratory tract lesions, the organism was only isolated from one (C843) and the isolations were only made from the upper respiratory tract.

None of the vaccinated animals had lesions of CBPP and no isolates of M. mycoides were made from these animals.

EXPERIMENTS 2 and 3:

In Table VII the serological results of Experiments 2 and 3 are recorded. The general picture of response was similar to that recorded in Table V except that the peak CF titre, following primary vaccination, was 2560 in 7 animals and in

fractionated are shown in Fig. 5a, b, c, d, e, f and g. Only fraction I fixed complement in the presence of M. mycoides antigen following either primary or secondary vaccination. The complement fixing activity was abolished when fraction I was treated with 2-ME.

RECOVERY OF M. MYCOIDES AND ASSOCIATED ANTIGENS FROM TISSUES FOLLOWING REVACCINATION OF CATTLE WITH THE T_1 BROTH CULTURE VACCINE:

In Table IX the results of the isolation of M. mycoides and associated antigens are recorded. M. mycoides was isolated from the tail (site of inoculation) of 5 animals following revaccination. These animals had been vaccinated with the T_1 broth culture vaccine 185, 579, 175, 166 and 166 days previously and were killed, 1, 3, 4, 7 and 22 days respectively after revaccination. The organism was not isolated from tissues of the animals revaccinated between 6 and 18 months unless the animals were slaughtered within 22 days of revaccination. The organism was isolated from regional lymph nodes of 5 animals following revaccination. The organism was recovered, only from animals which were slaughtered within one week of revaccination, with the exception of 1 animal. The organism

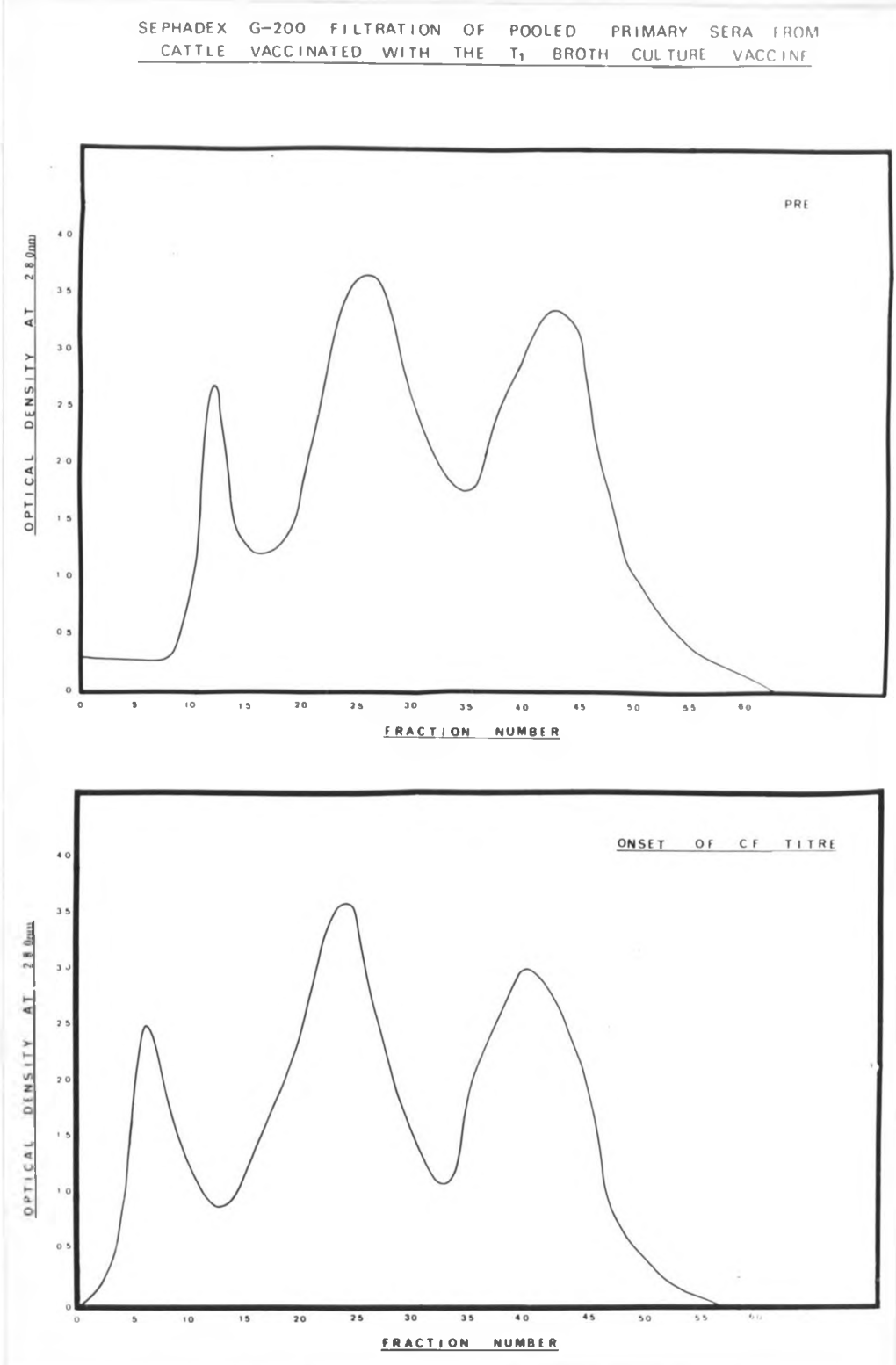
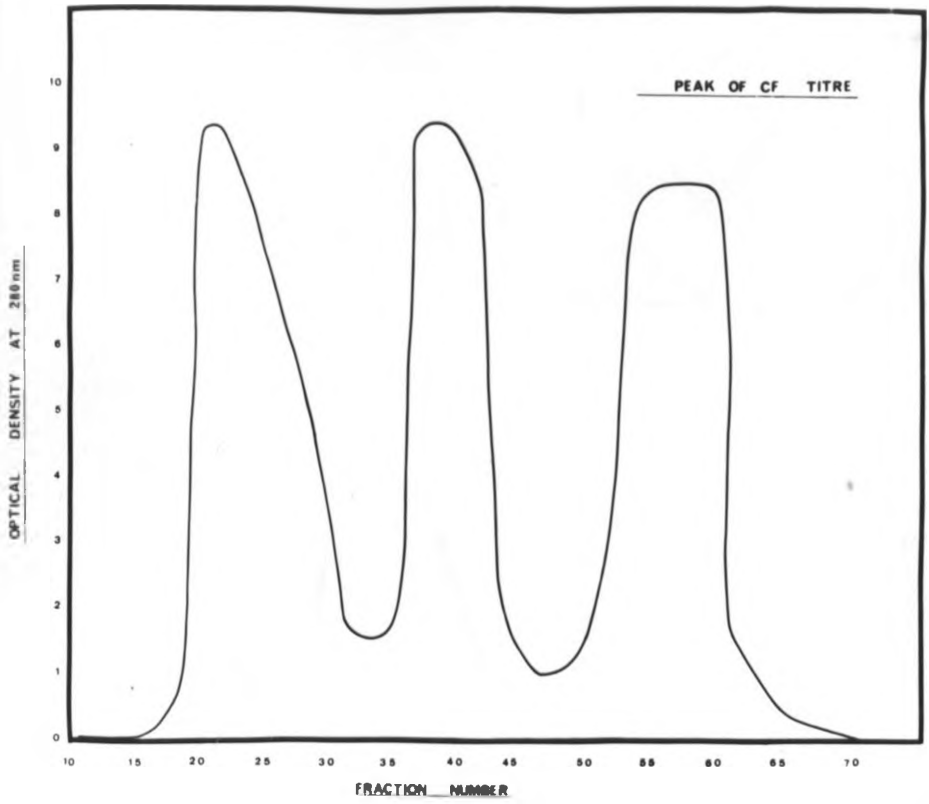


Fig. 5a and b: Sephadex G 200 filtration charts:
(a) pooled pre-vaccination serum and
(b) pooled primary serum collected when complement fixing antibody first detected.

SEPHADEX G 200 FILTRATION OF POOLED PRIMARY SERA FROM CATTLE VACCINATED WITH THE T_1 BROTH CULTURE VACCINE

c



d

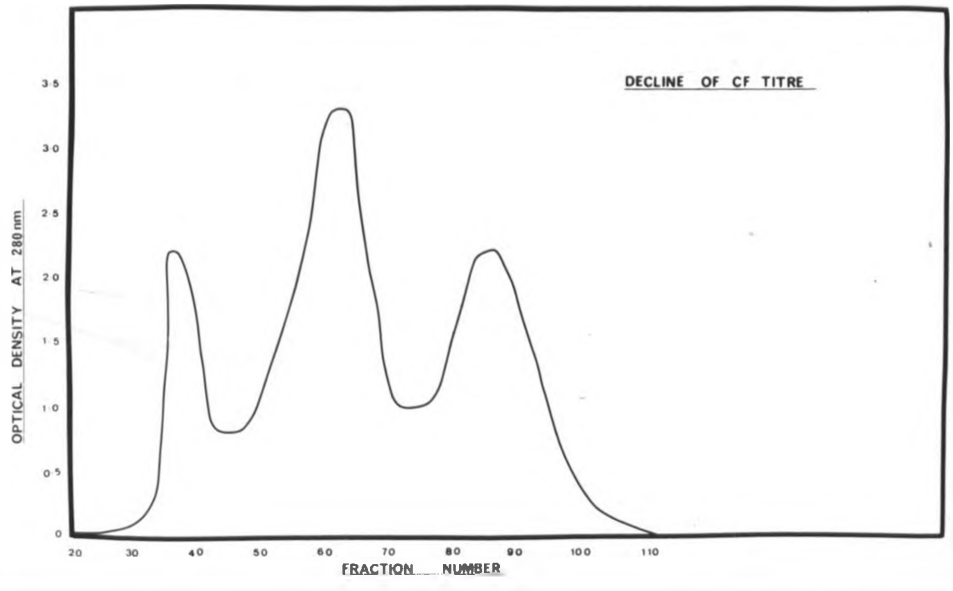
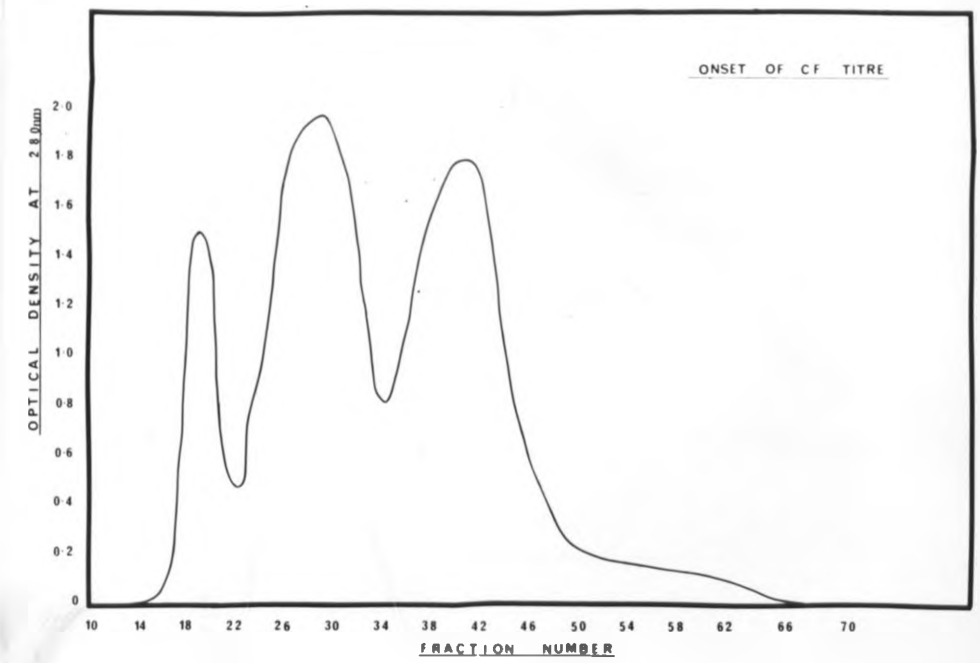


Fig. 5c and d: Sephadex G 200 filtration charts:
(c) pooled primary serum collected at peak of complement fixing antibody and
(d) pooled primary serum collected at the decline of complement fixing antibody.

e



f

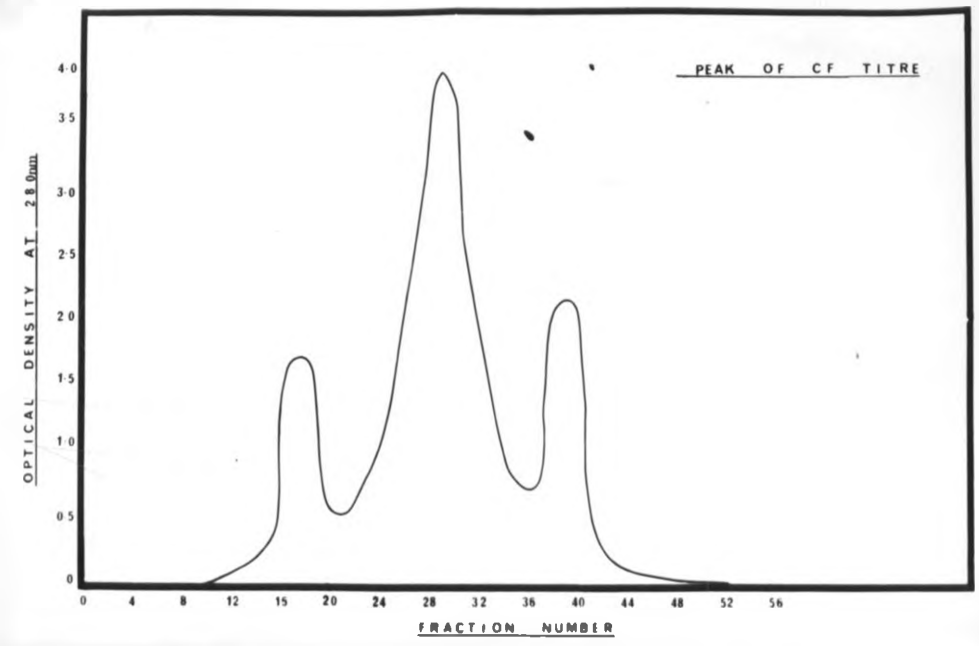


Fig. 5e and f: Sephadex G 200 filtration charts:
(e) pooled secondary serum collected when complement-fixing antibody was first detected and
(f) pooled secondary serum obtained at peak of complement-fixing antibody.

g

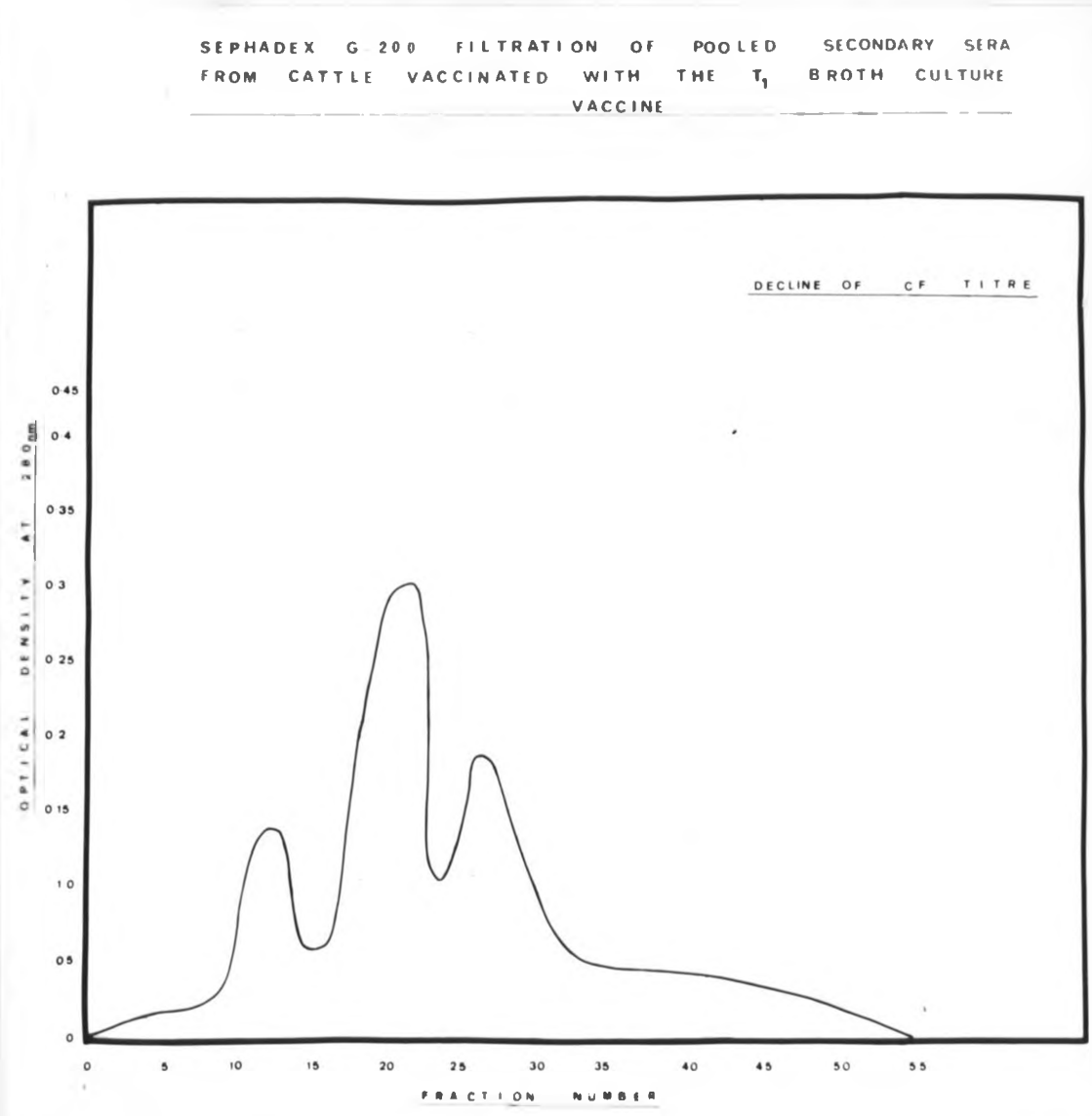


Fig. 5g: Sephadex G 200 filtration chart: pooled serum collected at decline of complement-fixing antibody.

was isolated from the tail (site of inoculation) of 1 animal 22 days following revaccination. With the exception of 3 animals (D74, D78 and D894) M. mycoides antigens were detected in tissues of all animals. The antigens were found in many more tissues as compared to those detected following primary vaccination (see Tables III and IX) of cattle with the T_1 broth culture vaccine.

D I S C U S S I O N

The T_1 strain of M. mycoides has been isolated from tissues of one animal up to 71 days following primary vaccination (see Chapter 3). The isolation of the organism from tissues has been recorded following primary vaccination 3 months, (V_5 HUDSON, 1965), 2 months (KH_5J LINDLEY and PEDERSEN, 1968) and 14 days (T_1 DAVIES, 1969). In the present experiments the latest date of isolation of M. mycoides from tissues of revaccinated animals was 22 days. This was considerably less than that recorded after primary vaccination in the same series of experiments. The organism was not isolated from the tissues of 18 other animals slaughtered more than 22 days after revaccination. The period between primary vaccination had no bearing on these results. Thus, the

organism persisted in tissues for a very short period of time following revaccination. In the majority of cattle from whose tissues M. mycoides was isolated following revaccination, this was within the first 7 days of re-vaccination. This finding tends to support PRIESTLEY and KARIB's (1954) hypothesis that when animals were inoculated once, they would be immunised and that organisms introduced into the animals on revaccination would be destroyed.

It has already been shown (see Chapter 3) that during primary vaccination, M. mycoides antigens were present 3 days after vaccination and became widespread by 9 days whereas on revaccination the antigens became widespread by the 4th day (see Tables III and IX). The rapid spread of M. mycoides antigens suggests that the organisms were rapidly destroyed by the body defence mechanism: this destruction of the organisms would lead to the release of M. mycoides antigens: conversely the organism might have multiplied, thereby producing a greater antigenic mass which then spread through the body. The latter suggestion is less likely since the organism itself was isolated once after 7 days of revaccination. That some of these antigens could be auto-antigens has been discussed in Chapter 3, and if they are, it would be difficult to explain whether the antigens observed on revaccination were

of those organisms which had been introduced into the animal during primary vaccination or those introduced during secondary vaccination. However, the organisms could not be recovered from tissues, in the majority of cases 7 days after revaccination. It is reasonable to assume therefore that some of the antigens detected following revaccination were antigens which were present as a result of the rapid destruction of M. mycoides by the already primed body defence mechanisms.

CAMPBELL (1938) stated that immunity to CBPP lasted for more than 12 months, whether single or double vaccination was employed; and that after the second vaccination the antibody response was rapid and the titre stayed high for a long time. The present findings have confirmed that some animals are protected two years after vaccination. All animals which had been revaccinated with T_1 broth culture vaccine either 6 or 12 months after primary vaccination were resistant to CBPP on challenge. Another group of animals which had received the primary and not secondary vaccination were also found to be immune 2 years after primary vaccination. However, it is interesting to note that immunity at 2 years is not absolute. WINDSOR, MASIGA and READ (1972) had shown that 5 of 16 vaccinated cattle developed small CBPP lesions on challenge 2 years after primary vaccination.

In the present experiments, the serological response

following revaccination was rare and erratic: of 48 animals only 6 responded following revaccination. The highest CF titre recorded was 20 and the SAST reaction was ++ as compared to a CF titre of 2560 and an SAST reaction of ++++ which were typical of those recorded following primary vaccination. Thus the findings of these experiments are at variance with those of CAMPBELL (1938). One reason for this difference may be that CAMPBELL used an M. mycoides strain other than T_1. SHIFRINE, STONE and DAVIES (1968) showed an amnestic response in 6 cattle revaccinated 3 months after primary vaccination using the CFT, Indirect haemagglutination test (IHA) and the SAST. They, however, failed to show an amnestic response in cattle revaccinated one month following vaccination. No evidence of amnestic response has been observed in the present study. The majority of cattle did not respond to a second vaccination with the T_1 broth culture vaccine as measured by the CFT and SAST. Some agglutinating antibodies were produced by majority of cattle on revaccination but these were not quantitatively more than those seen after primary vaccination although they persisted for longer times. None of the animals that responded serologically to revaccination had CF titres above those recorded during primary vaccination. The duration of CF titres averaged 7 days as compared to 2 3days observed

during the primary vaccination (see Tables IVa, IVb, V and VII).

In another experiment, 4 routes of administration were used to revaccinate cattle and amounts of the T_1 broth culture vaccine were increased from 4 to 100 fold. Nevertheless, only 6 of 20 animals responded serologically following revaccination, and only 2 (A751 and B561) had CF titres higher than those recorded during primary vaccination. These animals had been inoculated intravenously with 50 ml. of the vaccine during the 2nd vaccination. The results of this experiment, have, thus confirmed that the conventional secondary serological response, following vaccination of cattle with the T_1 broth culture vaccine as measured by the CFT does not occur.

PEARSON and LLOYD (1972) demonstrated both IgG and IgM antibodies in sera from cattle in hyperacute, acute, chronic and recovered stages of CBPP. Specific CF antibody appeared in fractions from the IgM peak and persisted there throughout the course of the disease. CF antibody was present in fraction from the IgG peak in most, but not all affected animals. Agglutinating antibodies occurred early in the disease in all the sera of all animals but its presence was transitory and confined to IgM fractions. The CF and agglutinating antibodies were shown

by absorption studies to be different from each other. Only IgM fixed complement in sera following both primary and secondary vaccination and because IgM is a primary antibody, these experiments have confirmed that there was no secondary response in cattle vaccinated with the T_1 broth culture vaccine after 6, 9, 12, 15, 17 and 18 months.

It is difficult to explain the failure of most animals to respond serologically to the secondary vaccination with the T_1 broth culture vaccine. Immune paralysis has been suggested in Chapter 3 to be a possible explanation for the failure of some animals to respond serologically to primary vaccination. If this reasoning is accepted for the failure of some animals to respond to secondary vaccination then the "high zone" paralysis which is necessary to overcome the "memory" cells would be a probable explanation for this failure.

TABLE V

SEROLOGICAL RESPONSE OF CATTLE FOLLOWING PRIMARY AND SECONDARY VACCINATION WITH T_1 STRAIN OF *M. MYCOIDES*

ANIMAL NUMBER		COMPLEMENT FIXATION TEST (CFT) POST-VACCINATION								SLIDE AGGLUTINATION SERUM TEST (SAST) POST-VACCINATION					
		FIRST APPEARANCE OF ANTIBODY (DAYS)		PEAK* TITRE		DAY OF PEAK TITRE		DISAPPEARANCE OF ANTIBODY (DAYS)		FIRST APPEARANCE OF ANTIBODY (DAYS)		PEAK DAY		DISAPPEARANCE OF ANTIBODY (DAYS)	
		1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd
VACCINATED 6 MONTHS AFTER PRIMARY VACCINATION	C438	14	-	160	-	14	-	28	-	7	-	7	-	70	-
	C514	14	7	40	20	14	7	28	21	7	7	7	7	49	28
	C682	7	-	320*	-	14	-	35	-	7	7	7	7	49	49
	C702	-	-	-	-	-	-	-	-	7	7	7	28	63	49
	C709	7	7	160	20	7	7	42	21	7	14	21	14	42	35
	C842	14	-	80	-	14	-	21	-	7	7	14	7	42	42
	C846	7	-	80	-	21	-	28	-	7	7	14	7	70	35
	C850	14	7	40	10	14	7	21	14	7	14	7	14	49	49
	C852	7	-	40	-	21	-	28	-	7	7	7	7	42	21
	C859	7	-	80	-	14	-	28	-	7	28	7	28	49	28
VACCINATED 12 MONTHS AFTER PRIMARY VACCINATION	C374	7	258	40	40	7	258	21	258	7	-	7	-	28	-
	C449	14	-	40	-	14	-	28	-	7	-	21	-	49	-
	C463	7	-	160	-	14	-	35	-	7	-	7	-	63	-
	C679	-	-	-	-	-	-	-	-	7	-	14	-	49	-
	C687	7	289	160	10	7	289	28	289	7	-	7	-	49	-
	C831	14	-	20	-	14	-	21	-	14	-	14	-	42	-
	C834	7	-	320	-	7	-	42	-	7	-	7	-	49	-
	C844	7	-	40	-	7	-	28	-	7	-	14	-	49	-
	C848	7	-	160	-	14	-	28	-	7	-	7	-	49	-
	C855	7	-	160	-	7	-	42	-	7	-	7	-	49	-

* Reciprocal of CF Titre.

T A B L E V I

CLINICAL AND POST MORTEM FINDINGS AFTER CHALLENGE OF REVACCINATED CATTLE

Experimental Animals	(1) Maximum CF Titres	(2) Clinical	Post-mortem	M. mycoides isolated	(3) H. and T. Score Pathology	Total
			VACCINATED TWO YEARS			
C478	-	-	No lesions	-	0	0
C481	-	-	No lesions	-	0	0
C684	-	-	No lesions	-	0	0
C696	-	-	No lesions	-	0	0
C697	-	-	No lesions	-	0	0
C836	-	-	No lesions	-	0	0
C840	-	-	No lesions		0	0
C857	-	-	No lesions	-	0	0
				TOTAL	0	0
				MEAN	0	0
			REVACCINATED 6 MONTHS AFTER PRIMARY VACCINATION			
C438	-	-	No lesions	-	0	0
C514	-	-	No lesions	-	0	0
C682	-	-	No lesions	-	0	0
C702	-	-	No lesions	-	0	0
C709	-	-	No lesions	-	0	0
C842	-	-	No lesions	-	0	0
C846	-	-	No lesions	-	0	0
C850	-	-	No lesions	-	0	0
				TOTAL	0	0
				MEAN	0	0
			REVACCINATED 1 YEAR AFTER PRIMARY VACCINATION			
C374	-	-	No lesions	-	0	0
C463	-	-	No lesions	-	0	0
C679	-	-	No lesions	-	0	0
C687	-	-	No lesions	-	0	0
C831	-	-	No lesions	-	0	0
C834	-	-	No lesions	-	0	0
C844	-	-	No lesions	-	0	0
C848	-	-	No lesions	-	0	0
				TOTAL	0	0
				MEAN	0	0
			CONTROLS			
C510	640	Died	Acute and sub-acute lesions whole of right lung	+	6	18
C701	20	-	Sequestrum 2 x 2 cm. in R.D.	+	2	6
C708	1280	Temp.>7 days	Sequestrum 2 x 10 cm. and 10 x 8 cm. in R.D.	+	4	13
C841	640	-	No lesions	-	0	3
C843	20	-	No lesions	+	0	4
C851	640	-	Multilocular sequestra 6 x 5 cm. in R.D.	+	4	11
C856	160	-	No lesions	-	0	3
C858	2560	Temp. 5-7 days	Sub-acute lesions L.D. 3 x 5 cm.	+	2	8
C632	640	Died	Hyperacute lesion 20 cm.	+	6	18
C777	2560	Temp.>7 days	Massive acute lesion in R.D.	+	6	17
C778	160	-	Some pleural adhesions. Doubtful C.B.P.P.	-	1	4
				TOTAL	31	105
				MEAN	2.82	9.54

Key:

(1) = Reciprocal of Complement Fixation Test Titre
(2) = Temp. = Temperature of 103°C or higher
(3) = H.& T Score = Hudson and Turner Score
Ep and Et = Efficacy of vaccine

Ep and Et of all groups of vaccinates were 100%.

TABLE VII

THE SEROLOGICAL RESPONSE FOLLOWING PRIMARY AND SECONDARY VACCINATION OF CATTLE WITH T_3 BOTH CULTURE VACCINE

ANIMAL NO.	COMPLEMENT FIXATION TEST (CFT) POST-VACCINATION								SLIDE AGGLUTINATION SERUM TEST (SAST) POST-VACCINATION					
	FIRST APPEARANCE OF ANTIBODY (DAYS)		PEAK* TITRE		DAY OF PEAK TITRE		DISAPPEARANCE OF ANTIBODY (DAYS)		FIRST APPEARANCE OF ANTIBODY (DAYS)		PEAK DAY		DISAPPEARANCE OF ANTIBODY (DAYS)	
	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd
D396	7	-	2560	-	7	-	21	-	0	-	14	-	78	-
D403	7	10	2560	20	7	10	7	10	0	-	14	-	78	-
D632	7	-	2560	-	7	-	21	-	0	0	7	1	78	7
D692	7	-	2560	-	14	-	14	-	0	0	7	1	64	2
D693	7	-	2560	-	14	-	14	-	0	0	7	1	36	2
D725	7	-	1280	-	14	-	14	-	0	0	14	1	64	17
D735	7	-	2560*	-	14	-	21	-	0	4	14	10	78	17
D737	7	-	2560	-	14	-	14	-	0	0	7	1	64	17
D804	7	-	80	-	14	-	14	-	0	1	7	1	36	3
D806	7	-	80	-	14	-	14	-	0	0	7	1	36	17
D813	7	-	160	-	14	-	21	-	0	0	7	1	64	17
D816	7	-	160	-	7	-	14	-	3	2	7	3	29	9
D837	-	-	-	-	-	-	-	-	14	1	14	8	15	15
D877	-	-	-	-	-	-	-	-	14	1	14	8	71	18
D880	-	8	-	20	-	8	-	11	0	1	14	7	85	14
D882	14	6	40	320	14	8	28	22	14	1	14	7	42	34
D894	14	-	20	-	14	-	28	-	0	1	14	7	42	10
D896	14	-	40	-	14	-	28	-	0	1	14	7	71	10
D904	14	-	10	-	14	-	21	-	14	1	14	7	63	21
D906	14	-	10	-	14	-	21	-	0	1	14	8	42	17
D971	-	-	-	-	-	-	-	-	0	1	14	8	71	22
E90	14	-	10	-	14	-	21	-	14	1	28	7	63	10
E92	-	-	-	-	-	-	-	-	0	1	14	8	63	10

* Reciprocal of CF Titre

TABLE VIII

SEROLOGICAL RESPONSE FOLLOWING PRIMARY AND SECONDARY VACCINATION OF CATTLE WITH INCREASED AMOUNTS OF T_1 BROTH CULTURE VACCINE USING DIFFERENT ROUTES OF ADMINISTRATION

ANIMAL NUMBER	COMPLEMENT FIXATION TEST (CFT) POST-VACCINATION											
	FIRST APPEARANCE OF ANTIBODY (DAYS)		PEAK TITRE		DAY OF PEAK TITRE		DURATION OF TITRE		AMOUNT OF VACCINE GIVEN (MLS)		ROUTE OF ADMINISTRATION	
	1	2	1	2	1	2	1	2	1	2	1	2
A751	14	7	160	320	14	28	14	28	0.5 ml.	50 ml.	T/T	I/V
A756	-	-	-	-	-	-	-	-	"	20	T/T	I/M
A761	7	14	40	20	14	14	28	7	"	10	T/T	SUB/CUT
A802	-	-	-	-	-	-	-	-	"	50	T/T	SUB/CUT
B374	7	7	320	80	14	14	28	14	"	2	T/T	T/T
D743	21	21	160	20	21	21	14	14	"	50	T/T	I/V
B422	7	-	40	-	21	-	14	-	"	20	T/T	I/M
C754	21	-	160	-	21	-	14	-	"	10	T/T	SUB/CUT
B405	7	-	640	-	14	-	56	-	"	50	T/T	SUB/CUT
B559	-	-	-	-	-	-	-	-	"	2	T/T	T/T
B561	7	7	40	160	7	28	21	28	"	50	T/T	I/V
C66	14	-	20	-	14	-	7	-	"	20	T/T	I/M
B852	14	-	20	-	14	-	7	-	"	10	T/T	SUB/CUT
B681	14	-	320	-	14	-	14	-	"	50	T/T	SUB/CUT
C277	14	-	80	-	14	-	7	-	"	2	T/T	T/T
C918	42	14	10	20	42	14	7	7	"	50	T/T	I/V
D834	-	-	-	-	-	-	-	-	"	20	T/T	I/M
D799	7	-	640	-	7	-	21	-	"	10	T/T	SUB/CUT
D138	14	-	40	-	7	-	14	-	"	50	T/T	SUB/CUT
B414	7	-	20	-	7	-	21	-	"	2	T/T	T/T

Key:

1 = First vaccination on the 6th June, 1972
2 = Second vaccination on the 9th October, 1972
T/T = Tail tip
I/M = Intramuscular
I/V = Intravenous
SUB/CUT = Subcutaneous

TABLE IX

ISOLATION OF M. MYCOIDES AND DETECTION OF MYCOPLASMA ANTIGENS IN TISSUES OF CATTLE FOLLOWING REVACCINATION WITH T, STRAIN OF M. MYCOIDES BY THE TAIL TIP ROUTE

ANIMAL NUMBER	C581	C861	D74	D390	C865	D633	C863	D804	D816	D403	D78	D692	D737	D693	D806	D813	D725	D735	D837	D877	D880	D894	D896	D904	D906	D971	E90	E92
DAYS INTERVAL BETWEEN 1ST AND 2ND VACCINATION	185	573	579	175	636	166	594	314	312	166	535	315	221	315	244	244	221	221	459	459	459	459	459	459	459	459	459	459
DAY OF SLAUGHTER POST REVACCINATION	1	3	3	4	6	7	14	15	17	22	22	29	29	29	30	43	44	49	20	36	52	30	42	50	42	36	20	30
TISSUES																												
TURB.								Ag		Ag															Ag	Ag		
TRAC.				Ag					Ag	Ag								Ag		Ag						Ag		
L.A.				Ag									Ag			Ag									WAg	Ag		
L.C.							Ag	Ag		Ab			WAg							Ag						WAg		
L.D.									Ag						Ag									Ag	Ag	LaM		
R.A.									Ag								Ag									WAg		
R.C.				Ag					AbAg						WAg					WAg								
R.D.								Ag	Ag									Ag							Ag			WAg
KIDNEY				Ag			Ag		Ag				Ag							WAg						WAg		Ag
LIVER							Ag								Ag	Ag		Ag						Wag	Ag	Ag		Ag
SPLEEN				Ag					Ag			Ag				Ag										LaM		
POST. MED. L.N.									Ag			Ag		Ag												Ag		
ANT. MED. L.N.									Ag	Ag					Ag		Ag	Ag										
L. MEDIAL SAC. L.N.				Ag	+				Ag								Ag				WAg				Ag			
R. MEDIAL SAC. L.N.		WAg		+Ag				AbAg	Ag							Ab				Ag					Ag			
L. SUBMAND. L.N.								AbAg	Ag						WAg		Ag								Ag			
R. SUBMAND. L.N.				Ag				AbAg				Ag				AbAg				WAg					Ag			
L. PAROTID. L.N.															Ag		Ab								Ag	WAg		Ag
R. PAROTID. L.N.												Ag					AbAg		Ag							WAg		
L. PRESCAP. L.N.												Ag					Ag								WAg			
R. PRESCAP. L.N.								WAg					Ag			Ag	Ag								Ab			
L. PREFEM. L.N.								WAg				Ag			Ag			Ab		WAg					Ag			
R. PREFEM. L.N.																	WAg			WAg					Ag			
L. LATERAL SAC. L.N.	+Ag			Ag																					Ag			
R. LATERAL SAC. L.N.				+Ag													Ag									WAg		
L. SUP. ING. L.N.									Ag									Ab		WAg					Ag			
R. SUP. ING. L.N.		WAg	+			Ag	WAg		Ag				Ag					Ab										
L. DEEP. ING. L.N.									Ag			Ag					AgAb			WAg			WAg		Ag	WAg		
R. DEEP ING. L.N.									Ag								WAg						WAg		Ag	LaM		WAg
L. BRONCHIAL L.N.							Ag		Ag							AbAg												
R. BRONCHIAL L.N.				Ag					Ag								WAg								Ag		Ag	
L. POPLITEAL L.N.								AbAg	Ag					Ag	WAg		AbAg						WAg	WAg		WAg		
R. POPLITEAL L.N.						Ag	Ag		Ag								Ab			Ag								
L. INT. ILIAC L.N.		+				Ag	Ag	Ag	Ag			Ag	WAg												Ag	WAg		
R. INT. ILIAC L.N.									Ag				WAg							WAg	WAg				Ag			
TAIL	+		+	+	Ag	+			Ag	+										WAg								
URINE		WAg								Ag				Ag												Ag		Ag
JOINT FLUID				Ag					Ag																Ag	LaM		
BLOOD					Ab				Ag											Ag				Ag		WAg		

Key:

Ag = Antigen detected in AGT
Ab = Antibody detected in ACT
WAg = Weak Antigen detected in AGT
WAb = Weak antibody detected in AGT
\+ = M. mycoides isolated from tissues
LaM = Line of Similarity

L.C. = Left Cardiac lung lobe
L.D. = Left diaphragmatic lung lobe
L.A. = Left apical lung lobe
R.C. = Right cardiac lung lobe
R.D. = Right diaphragmatic lung lobe
R.A. = Right apical lung lobe

L.N. = Lymph node
SUP. ING. = Superficial inguinal
SAC. = Sacral
TURB. = Turbinate
TRAC. = Trachea

C H A P T E R 5

THE EFFECT OF POST-VACCINAL TREATMENT WITH THE ANTIBIOTIC TYLOSIN ON THE IMMUNITY PRODUCED BY THE T_1 STRAIN OF MYCOPLASMA MYCOIDES VAR. MYCOIDES

INTRODUCTION

There has been considerable debate about the role of inactivated vaccines in control of CBPP. POTAPOV, quoted by GRYAZIN and SHCHERBAKOV (1954) was of the opinion that formalin - inactivated cultures of infected tissues conferred immunity not inferior to that produced by living vaccines. However, GRYAZIN and SHCHERBAKOV (1954) found that inactivated vaccines, even those containing tissue extracts, did not protect against subcutaneous challenge with virulent M. mycoides. These workers explained this discrepancy by assuming that their vaccinating strains did not have sufficiently "pronounced antigenic and immunizing qualities". The French workers CURASSON (1936) and, MOULIS (1938) were of the opinion that inactivated vaccines conferred

immunity sufficient to withstand natural infection but not sufficient to withstand subcutaneous injection of the virulent organism. More recently, workers in this laboratory have suggested that inactivated culture vaccines of M. mycoides when inoculated with an adjuvant sensitized rather than protected cattle; for when cattle were challenged by subcutaneous inoculation a "Willems reaction" occurred in the vaccinated cattle before it appeared in the control animals (SHIFRINE and BEACH, 1968). Fifty Mg inactivated culture injected subcutaneously (equivalent to 10^{11} - 10^{12} organisms) did not confer solid immunity (SHIFRINE and BEACH, 1968) whereas GILBERT and WINDSOR (1971) demonstrated that 10^{7} viable organisms of the T_1 strain of M. mycoides were sufficient to produce immunity. The latter workers suggested that a vaccination dose of 10^{5} organisms was not only insufficient to protect cattle against natural challenge, but may instead sensitize them to the state that they would be more susceptible than the control cattle.

Many antibiotics and other drugs have been tested for their activity against M. mycoides, more for the treatment of vaccinal reaction than for treatment of clinical disease. TURNER (1960) tested the in vitro bacteriostatic effects of different antibiotics and other chemotherapeutic drugs to M. mycoides. As a result of such studies Tylosin* was selected

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* "Tylan" Eli Lily and Co.

by HUDSON and ETHERIDGE (1965) for treating cattle in a controlled experiment. From their studies HUDSON and ETHERIDGE recommended that 7.5 mg/kg. of Tylosin when administered twice daily for 5 treatments was sufficient to control CBPP vaccine reactions providing the lesions had not been for a long period of time. In the nomadic areas of East Africa where the T_1 broth culture vaccine is widely used the cattle are regularly bombarded with drugs; antibiotics are used for the treatment of bacterial infections and anaplasmosis and proticidal drugs are used for the treatment of babesiosis and the treatment and prophylaxis of trypanosome infections. It is more than possible that cattle could be vaccinated against CBPP on one day and treated with a potentially Mycoplasmacidal drug the next day. The evidence tends to indicate that viable Mycoplasmas are essential for the production of immunity but there is no work to suggest the period required for the organism to exist in the animal tissues to elicit solid immmity. It was therefore decided to investigate the effects of treating vaccinated cattle with a Mycoplasmacidal drug. As a result of the studies of HUDSON and ETHERIDGE (1965) the drug "Tylosin" was chosen for this work.

MATERIALS AND METHODS

CATTLE:

Sixty high grade dairy type cattle (57 steers, 2 bulls and 1 heifer) ranging in age from 15 months to 3 years and in weight from 200 to 300 kg. were obtained from areas of Kenya free from CBPP. Before selection their sera were tested and found negative to the CFT (CAMPBELL and TURNER, 1953). The cattle were assigned to groups of 5 animals of similar age, type and weight. By a random method 2 of

these 5 were selected as controls and the others were selected for 3 vaccinated groups. Three groups of 12 cattle each were vaccinated in the tail tip with 0.5 ml. of T_1 broth culture vaccine.

TREATMENT:

One week later one group of the vaccinated cattle began a therapeutic course of Tylosin. On the recommendation of Mr. M.J. Fussell the animals were given 3 intramuscular treatments at 24 hour intervals with 'Tylan 200' at a dosage rate of 10 mg/kg.

Four weeks after vaccination the second group of vaccinated cattle commenced a therapeutic course of 'Tylan 200' and again 3 daily intramuscular treatment at a rate of 10 mg/kg. were given. No adverse reactions to the treatment were noticed. The third vaccinated group were given no therapeutic treatments. One animal in each of the vaccinated and treated groups died from anaplasmosis. It was decided that to have treated these animals might have affected the results of the trial.

SEROLOGICAL TESTS:

The animals were bled weekly from the week before vaccination until the termination of the trial. All sera were examined by the CF test of CAMPBELL and TURNER (1953) using antigen prepared from the T_1 strain of M. mycoides.

CHALLENGE:

The immune status of the vaccinated cattle was determined by challenging them 12 weeks after vaccination by mixing them with 'donor' cattle artificially infected with the Gladysdale strain of M. mycoides by the endobronchial route, (BROWN, 1964). In this method animals are anaesthetised with

40% chloral hydrate and then CBPP lung lesion extract is poured down the throat. In this way, the majority of cattle become artificially infected with CBPP. As is now the standard practice in this laboratory, cattle to be challenged are mixed with 'donor' cattle between 10 and 14 days after the 'donors' have been infected. Initially, the experiment was conducted in a building measuring 45 x 28 feet with 10 feet eaves and 16 feet ridge. At night the animals were confined closely to one part of this shed measuring 29 x 19 feet, but despite severe disease in the 'donor' cattle, little evidence of disease appeared in the control animals. Eight weeks after the commencement of this trial the animals were transferred to another building consisting of 2 rooms measuring 10m x 5.5m x 3m. The walls and roof of this building are insulated. Twenty four 'donor' cattle were infected at the start and during the course of the trial a further 24 'donor' animals were added, 10 at the time of transfer to the new building.

ASSESSMENT OF IMMUNE STATUS:

Unless they died, vaccinated and control cattle were killed 6 weeks after the first development of CF antibodies in their sera, so that the scoring system of HUDSON and

TURNER (1963) could be applied. This scoring system is carried out as follows:-

(a) Serological response to challenge:

Reciprocal of CF titre	Score
<10	0
10	1
20 - 80	2
160 or higher	3

(b) Clinical response to challenge

Pyrexia (at least 103°F, 39.5°C)

for >3 but < 7 days	1
Pyrexia for >7 days	2
Animal died or was destroyed in extremis	3

(c) Autopsy results (TURNER, 1961)

If only unencapsulated resolving or fibrous lesions, or pleural adhesions are present	1
If other lesions are present (consolidated), acute, necrotic or sequestrated)	2

	Score
In addition, if *M. mycoides* be isolated, add	2
Multiply the sum by a factor depending on average diameter of lesion	
If < 5 cm.,	Factor = 1
If > 5 but< 20 cm,	Factor = 2
If> 20 cm.,	Factor = 3

Thus the maximum total score (T) is:-

Serology	3
Clinical	3
Autopsy (2 + 2)3	12
	18

For some purposes, it is useful to consider only the pathological evidence, disregarding the bacteriological, clinical and serological evidence which may be missing or incomplete.

Post-mortem examinations and tissue culturing were carried out in a similar manner to that described in previous chapters with the exception that only the mediastinal lymph

nodes were cultured. All Mycoplasmas isolated were subjected to the growth inhibition test of DAVIES and READ (1968). This test has already been described in previous chapters. Once it became evident that no further serological reactions were appearing in either control or vaccinated cattle, the experiment was terminated.

R E S U L T S

VACCINATIONS:

Each animal received a vaccine dose of 1.65×10^9 viable organisms. There were no clinical reactions to the vaccination. Fourteen animals developed CF antibodies in their sera by one week, the maximum titre at this time being 20. Overall 26 of 36 vaccinated animals developed CF antibodies but by 6 weeks after vaccination these antibodies were no longer detectable (see Table X). The maximum titre recorded was 320 but only 6 animals (3 in the group treated at 1 week and 3 in the group treated at 1 month) developed a CF titre in excess of 20. Seven days after treatment in the group given antibiotics, at 1 week, all sera were negative to the CFT, but a week later

6 were positive. Finally, five weeks after vaccination the sera of all the vaccinated cattle were negative to the CF test. The week following treatment in the group treated at 1 month none of the sera had demonstrable CF antibody. However, none of the animals in the vaccinated groups possessed CF antibody at a comparable time. No further CF antibody developed as a result of vaccination. The CF reactions were irregular and intermittent; 8 of 26 animals had CF antibody in their sera once with no re-appearance, but 4 cattle had periods of 2 or more weeks between positive CF reactions.

CHALLENGE:

The results of exposure of 36 vaccinated and 24 control animals to 24 infected donor animals are summarized in Table XI. Six of the 24 control animals died from acute CBPP and one animal died from chronic disease with 6 large sequestra in the right lung. Three animals had febrile reaction and showed evidence of the disease on post-mortem examination. Six animals showed no clinical evidence of disease but had lesions of CBPP when examined post-mortem, two of which (E952 and F91) had extensive lesions

in the lungs. One animal (E863) had a typical or old CBPP lesion in its lung; one had a high CF titre (320) but no lung lesion and 6 animals did not respond to the challenge in any way. M. mycoides was isolated from those animals with lesions but not from those without lesions.

Two of the vaccinated cattle developed CF antibody in their sera, but neither showed evidence of CBPP on post-mortem examination. One animal had fibrous adhesions between the cardiac and diaphragmatic lobes of the right lung. M. mycoides was not isolated from any animal in this group.

In the group of animals treated with 'Tylan 200' one week after vaccination, one animal died from anaplasmosis before the time of the challenge, 4 animals developed moderate to high levels of CF antibody and one animal died from unidentified causes during the trial. On post-mortem examination one animal had typical CBPP lesions. Four animals, however, had lesions, 3 were fibrotic lesions in the lung tissue and the fourth was fibrous adhesions between lobes of the lung. M. mycoides was not isolated from any of these animals.

One animal developed a moderate titre and 4 developed high titres of CF antibodies in the group of animals treated with 'Tylan 200' one month after vaccination. One animal died from

anaplasmosis before the commencement of the challenge. On post-mortem examination 3 of 11 animals had lesions typical of CBPP and M. mycoides was isolated from these lesions. M. mycoides was not isolated from any of the animals with no CBPP lesion.

STATISTICAL ANALYSIS:

All 3 groups of vaccinated animals had a greater resistance to the disease than the control animals during the challenge. The mean pathology scores of the 4 groups of cattle were as follows:-

Control animals 4.08;

Vaccinates 0.08;

Vaccinates treated one week after vaccination 0.45;

and Vaccinates treated one month 1.23.

Despite this difference in Mean Pathology Scores there was no significant difference between the 3 vaccinated groups of cattle.

The following was the summary of the statistical data:-

1. SUMMARY OF DATA:

	Controls (C)	Vaccinates (V)	1 Week (W)	1 Month (M)
Pathology Score:				
Mean	4.08	0.08	0.45	1.23
S.E.	0.46	0.08	0.20	0.61
Total Score:				
Mean	11.54	0.58	1.55	3.73
S.E.	1.31	0.34	0.59	1.48
No. of Animals	24	12	11	11

2. ANALYSIS:

For a preliminary analysis the animals were divided into two groups, those with score = 0 and those with scores greater than 0.

	Pathology Score		Total	Total Score	
	0	> 0		0	> 0
C	4	20	24	3	21
V	11	1	12	9	3
W	7	4	11	4	7
M	6	5	11	4	7
TOTAL	28	30	58	20	38

$X^2 = 19.89^{***}$ (0.1%)

$X^2 = 13.89^{**}$ (1.0%)

CHI squared tests showed highly significant differences between the proportion of animals showing disease symptoms. Inspection of the table suggests that this is due mainly to the differences of the controls from the three treatment groups.

To examine this hypothesis further, the controls were excluded from the table and CHI squared tests done on the treatment groups alone. The values obtained were not significant at the 5% level, supporting the hypothesis.

In order to compare the severity of the disease attacks, the mean disease scores were compared using t-tests.

(i) CONTROL V TREATMENTS:

The hypothesis that there were no decreases in disease scores due to the treatments were all rejected (one tail t-tests) at the 0.1% level.

	V	W	M
Pathology Score	4.00***	3.63***	2.85***
Total Score	10.96***	9.99***	7.81***

Differences between treatments and control

(ii) AMONG TREATMENTS

None of the differences was significant at the 5% level (2-tail t-test).

	V - W	V - M	W - M
Pathology Score	-0.37 NS	-1.15 NS	-0.78 NS
Total Score	-0.97 NS	-3.15 NS	-2.18 NS

Differences among treatments

CONCLUSION:

All three treatments lowered the incidence and severity of disease significantly, but there were no significant differences among the treatments.

DISCUSSION

A considerable variation in the CF antibody response to vaccination with the T_1 broth culture vaccine has been recorded. Following vaccination 7 of 27 cattle developed CF antibody, to a titre in excess of 10 and the maximum titre of 40 was recorded by DAVIES et al. (1968). MASIGA and READ (1972) showed however that 16 animals gave a CF antibody response to vaccination with titres ranging from 10 to 1280. The findings of the present study fall in between. Twenty six of 36 vaccinated animals developed CF antibody, the titres mostly in the range of 10 to 40 with only 4 being in excess of 80. This adds weight to the suggestion of GILBERT and WINDSOR (1971) that the effect of vaccination can not be judged by the serological response.

The mean pathology score of the control animals was 4.08 and this is well above the 2.5 score suggested by WINDSOR,

MASIGA and READ (1972) as being the minimum score required for an adequate challenge. It was interesting to note that the change of housing made for a very much more severe challenge. The difference between the 2 houses is that one had the height of the roof 4.5 m. as opposed to 3 m. of the other. The second house also responded less to changes in the external environment as both walls and ceiling were insulated. MASIGA and WINDSOR (1973) suggested that the ratio of control to vaccinated cattle is important when comparing trials, for with widely divergent ratios the opportunity for an animal to encounter M. mycoides varies in proportion. In the present work there were 2 control to 3 vaccinated animals.

Cattle which had been vaccinated for 3 months were solidly immune to the challenge. This was expected, for GILBERT et al. (1970) had shown that 6 and 12 months after vaccination cattle were immune to challenge. Furthermore, WINDSOR and MASIGA (1973 in Press) had shown that cattle vaccinated by the tail tip and by the subcutaneous route were resistant to a severe challenge 15 months after primary vaccination.

Animals treated with a therapeutic course of 'Tylan 200' starting one week after vaccination were all immune when challenged and yet in the group of cattle in which the treatment with 'Tylan 200' commenced one month after vacci-

nation, 3 of 11 developed typical CBPP lesions. These results lend weight to the suggestion that live vaccines are essential for the development of immunity to M. mycoides. They also suggest that persistence of the organism for more than one month is vital for development of the immune state.

GRIFFIN and LAING (1966) noted that successful vaccination against CBPP may depend upon a prolonged stimulus from living organisms, but that even then different responses might be expected from animals at different stages of infection and susceptible animals possessed degrees of natural resistance. They also stated that the latent period before immunity developed appeared to vary indirectly with the degree of attenuation of the vaccine. The organism has been isolated from animals vaccinated with T_1 strain for up to 71 days (see Chapter 3). But why should all the animals treated one week after vaccination be solidly immune when those treated at one month were not? One explanation is centred round the fact that Tylosin has a bacteriostatic rather than a bacteriocidal effect on Mycoplasmas. Over 10^9 organisms were given in the vaccinating doses. There will be less viable organisms present one month after vaccination than there will be at one week. It is possible to argue that in some of the animals treated with Tylosin one month after vaccination, the number of organisms had fallen so low that

during the bacteriostatic action of the 'Tylan 200' the defence mechanisms of the body were able to eliminate all the remaining organisms. The number of viable organisms present one week after vaccination were so great that the body defence mechanisms were unable to eliminate them all before the bacteriostat effects waned. Those remaining then continued multiplying. This finding suggests that by one month the immunity to CBPP has not fully developed. This suggestion is well supported by the decreased number of plaques observed in the spleens of all the treated animals (see Chapter 6). This hypothesis might be proven by repeating the trial and giving the drug a much longer period of time.

An alternative hypothesis is that following vaccination the organism is taken up by leucocytes of the blood and removed to lymph nodes. During this phase the organism would be inaccessible to the drug. If this is the case it is difficult to explain how the drug gains access to the organism at one month. Both explanations of this phenomenon depend upon the postulate that viable organisms are required for the initiation and development of immunity.

All 3 vaccinated groups differed significantly in their response to challenge to the control group. The group treated with 'Tylan 200' at one month was different

from the other two vaccinated groups as the pathology score for this group was 1.23 as compared to 0.45 for the animals treated after one week and 0.08 for the vaccinates alone. However, these differences were not significant.

It would seem that these results have significance for field use of the T_1 broth vaccine. Until more is known of the mechanisms of immunity to CBPP produced by T_1 vaccine it must be assumed that the use of any chemotherapeutic and antibiotic agent can result in the loss of immunity to M. mycoides. It would therefore seem advisable at the present time to insist that any vaccinated animal treated with such an agent cshould be revaccinated when the effect of that agent has dropped. PROVOST and DUEVAL (1971) have shown that in vitro 'Berenil'* (a commonly used tryponocidal drug) has no activity against M. mycoides. It is however, suggested that such studies should be repeated with all tryponocidal drugs. It would also seem that in vitro studies with these drugs is indicated.

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* Berenil Hoechst Ltd.

TABLE X

SEROLOGICAL RESPONSE OF CATTLE VACCINATED WITH THE T_1 BROTH CULTURE VACCINE AND TREATED WITH "TYLAN 200"

TOTAL NO. OF ANIMALS EXAMINED	NUMBER SHOWING ANTIBODIES IN SERA AND MAXIMUM TIME									
	Vaccination 1 week	Time of Vaccination	1 Week	2 Weeks	3 Weeks	4 Weeks	5 Weeks	6 Weeks	7 Weeks	Total Number in group that Develop CF Antibodies
Controls (24)	0	0	0	0	0	0	0	0	0	0
Vaccinated Cattle (12)	0	0	6: 4/20	3: 4/20	2: 4/20	2: 2/20	6: 4/20	0	0	9
Vaccinated Cattle Treated at 1 Week with "Tylan 200" (12)	0	0	3: 4/20	0	6: 2/80	4: 2/160	6: 2/80	0	0	8
Vaccinated cattle Treated at 1 Months with "Tylan 200" (12)	0	0	5: 4/20	5: 4/20	5: 3/320	5: 2/160	5: 2/80	0	0	9

TABLE II

CLINICAL AND POST MORTEM FINDINGS AFTER CHALLENGE OF CATTLE TREATED WITH "TYLAN 200"

Experimental Animals	Maximum CF Titres	Clinical	Post-mortem	M. mycoides isolated	M. and T. Score	
					Pathology	Total
			CONTROLS			
E1	2560	-	Sequestrum 10 x 10 cm. R.A. + Fibrosis	+	6	15
E3	2560	Died	Acute C.B.P.P. massive	+	6	18
E439	2560*	Died	6 Large sequestra	+	6	18
E441	-	-	Consolidation ?C.B.P.P.	+	2	4
E473	-	-	No lesions	-	0	0
E657	1280	Died	Acute C.B.P.P. massive	+	6	18
E727	640	-	2 Sequestra 6 cm. x 10.8 cm.	+	4	11
E751	2560	Temp. > 39.5°C. for > 7 days	Sequestra 6 cm. x 4 cm.	+	4	13
E836	-	-	Sequestrum 6 cm. x 4 cm.	+	4	11
E854	2560	-	Sequestrum 8 cm. x 6 cm.	+	4	11
E863	40	-	Fibrosis 8 x 6 cm.	-	2	4
E875	320	Died	Acute C.B.P.P. massive	+	6	18
E876	2560	Temp. > 39.5°C. for > 3 days	Sub-acute lesion 25 x 18 cm.	+	6	16
E952	2560	-	2 Sequestra 1 x 15 cm.; 1 x 20 cm.	+	6	15
E955	2560	Died	Acute C.B.P.P. massive	+	6	18
E956	-	-	No lesions	-	0	0
E965	2560	-	2 Sequestra: 1 x 8 cm.; 1 x 3 cm.	-	4	11
E967	320	-	No lesions	-	0	3
E977	-	-	----------	-	0	0
E994	2560	Died	Massive C.B.P.P.	+	6	18
F55	640	Temp. > 39.5°C. for 3 > 7 days	Sequestrum 6 x 5 cm.	+	4	12
F91	1260	-	Sequestrum massive	+	6	15
F93	80	-	Sequestrum 5 cm.	+	4	10
F99	-	Died	Acute C.B.P.P. massive	+	6	18
					TOTAL 98	277
					MEAN 0.08	0.58
					S.D. 5.03	40.8
			VACCINATES			
E2	-	-	No lesions	-	0	0
E206	-	-	No lesions	-	0	0
E401	-	-	No lesions	-	0	0
E680	1260	-	No lesions	-	0	3
E850	-	-	No lesions	-	0	0
E856	-	-	No lesions	-	0	0
E883	-	-	No lesions	-	0	0
E959	-	-	No lesions	-	0	0
E962	-	-	Adhesion	-	1	1
E963	-	-	No lesions	-	0	0
E968	-	-	No lesions	-	0	0
E978	640	-	No lesions	-	0	3
					TOTAL 1	7
					MEAN 0.08	0.58
			TREAT AT 1 MONTH			
E5	-	-	No lesions	-	0	0
E194	320	-	Multiple sequestra	+	6	15
E290	-	-	No lesions	-	0	0
E512	2560	-	Acute C.B.P.P. 6 cm.	+	4	11
E849	40	-	Adhesions	-	1	3
E860	160	-	Adhesions	-	1	4
E877	-		No lesions	-	0	0
E953	-	-	Sequestrum 1 cm.	-	2	2
E954	640	-	No lesions	-	0	3
E965	Died					
E974	1280	-	No lesions	-	0	3
E979	-	-	No lesions	-	0	0
					TOTAL 14	44
					MEAN 1.23	3.73
			TREAT AT 1 WEEK			
E9	-	-	No lesions	-	0	0
E106	-	-	Consolidation 0.5 cm.	-	1	1
E660	2560	-	No lesions	-	0	3
E795	-	-	Adhesions	-	1	1
E840	1280	-	2 Areas Consolidation 1 x 3cm; 1 x 2 cm.	-	2	5
E847	160	-	No lesions	-	0	3
E969	-	Died Not C.B.P.P.	Lesion 4 cm.	-	1	1
E975	Died					
E983	80	-	No lesions	-	0	3
E984	-	-	No lesions	-	0	0
E985	-	-	No lesions	-	0	0
E996	-	-	No lesions	-	0	0
					TOTAL 5	17
					MEAN 0.45	1.55

* Reciprocal of CFT Titre

CHAPTER 6

OBSERVATIONS ON THE IMMUNITY TO CONTAGIOUS BOVINE PLEUROPNEUMONIA

INTRODUCTION

Cattle vaccinated with the T_1 broth culture vaccine have been found on challenge to be solidly immune to CBPP (DAVIES, MASIGA, SHIFRINE and READ, 1968; GILBERT, DAVIES, READ and TURNER, 1970; WINDSOR, MASIGA and READ, 1972; MASIGA and READ, 1972). LLOYD (1967) failed to transfer immunity by injecting susceptible cattle with bovine immune sera prior to challenge and consequently suggested that immunity was probably cell mediated. ROBERTS, WINDSOR, MASIGA and KARIAVU (1973) and ROBERTS and WINDSOR (1973) came to the conclusion that immunity was not completely of the cellular type. This section reports the observation on the type of immunity involved following vaccination with the T_1 broth culture vaccine.

MATERIALS AND METHODS

CATTLE:

Cattle used in this investigation varied from 1 to 1½ years

of age and were obtained from an area of Kenya free from CBPP; the sera of all cattle were negative in the CFT. Three groups of cattle were used.

Seven cattle in the first group were vaccinated twice with the T_1 broth culture vaccine, first when the animals were 12 months of age, and a second time between 6 and 18 months later. All these animals were slaughtered between 3 and 7 weeks after second vaccination.

In the second group, 8 animals were vaccinated once. One of the animals was killed 7 months after vaccination and the remaining animals were killed 18 months after vaccination. Five of these animals had been challenged and found to be resistant to CBPP using the incontact method of DAVIES et al., (1968).

The third group of cattle, consisting of 12 animals, were vaccinated in the tail tip with 0.5 ml. of T_1 broth culture vaccine. One week later 3 of the vaccinates were started on a therapeutic course of Tylosin. The animals were given 3 intramuscular injections at 24 hour intervals with 'Tylan 200' at a dose rate of 10 mg/kg. Four weeks after vaccination another 3 vaccinates commenced a therapeutic course of 'Tylan 200' and again received 3 daily intramuscular treatment at a rate of 10 mg/kg. The last 3 vaccinated cattle were left untreated.

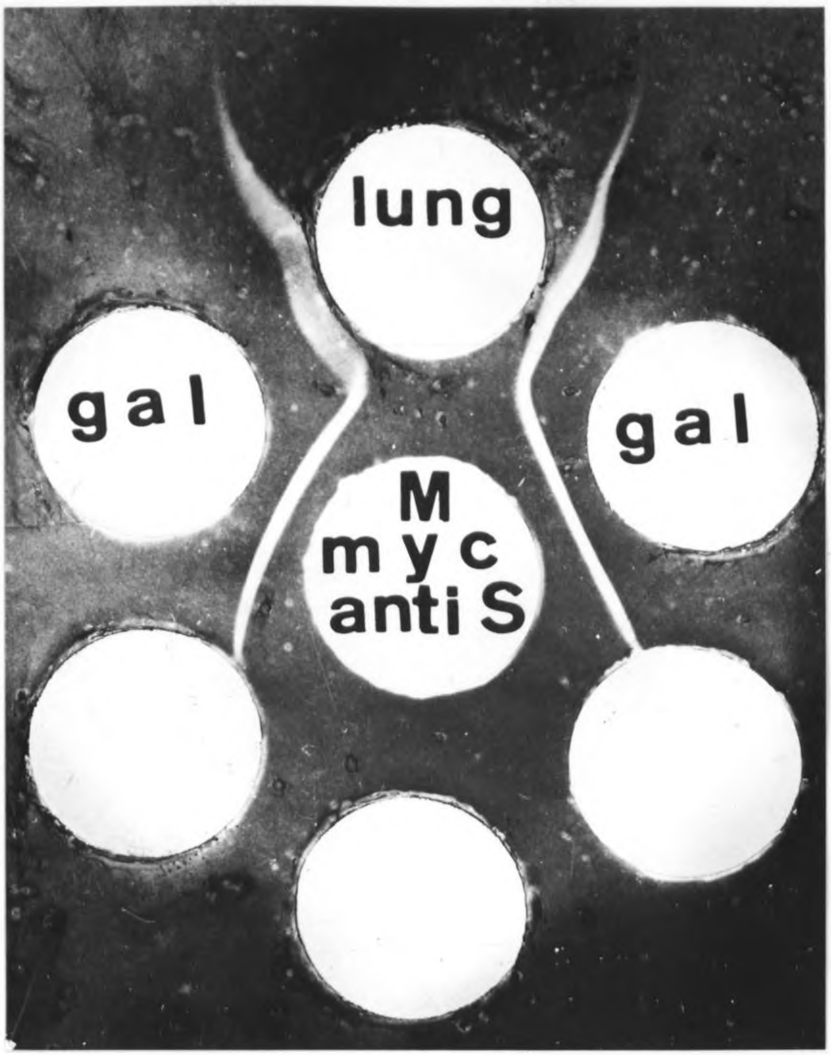

Fig. 6a: Agar Gel Precipitation reaction between lung tissue of cattle vaccinated with T_1 strain of M. mycoides and galactan. Centre well contains pig-anti-M. mycoides serum. Well at 12 o'clock contains lung tissue and wells at 2 and 10 o'clock contain galactan.

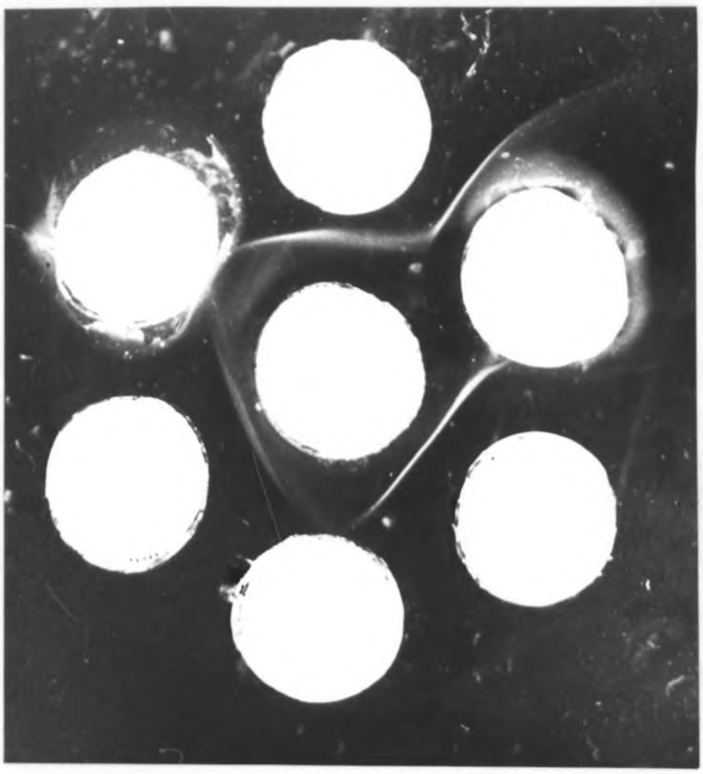

Fig. 6b: Agar gel precipitation reaction between lymph node material from cattle vaccinated with the T_1 strain of *M. mycoides*. Centre well contains pig anti-*M. mycoides* serum. Wells at 12, 4 and 8 o'clock contain galactan and wells at 2, 6 and 10 o'clock contain lymph node material from cattle vaccinated with the T_1 strain of *M. mycoides*.

plaques varied from 3 to over 200 in the lymph nodes and from 0 - 200 in spleens.

DISCUSSION

Following primary vaccination with the M. mycoides T_1 broth culture vaccine, complement fixing and agglutinating antibodies persist for approximately 60 days, whereas a secondary antibody response is invariably undetectable following a second inoculation of vaccine (see Chapter 4). Using the complement fixation test and slide agglutination serum test, cell mediated tests such as lymphocyte transformation, inhibition of leucocyte migration and the intradermal allergic tests, it is not possible to distinguish immune cattle from susceptible cattle (ROBERTS, WINDSOR, MASIGA and KARIAVU, 1973; ROBERTS and WINDSOR, 1973). It was therefore of interest to detect precipitating antibodies to galactan in tissues of lungs and lymph nodes of those cattle vaccinated twice and in 3 of the cattle vaccinated once at 6, 7 and 18 months previously respectively. Precipitating antibodies to galactan were not detected in the serum obtained at slaughter in any of these vaccinated cattle.

In 20 of the cattle vaccinated once, precipitating

antibodies to galactan were not detected in the lungs or mediastinal lymph nodes but galactan antibody producing cells were detected in the spleens and mediastinal lymph nodes. The agar gel diffusion test may not, however, be sensitive enough to detect the precipitating antibodies in the majority of animals vaccinated once. Galactan producing cells were more prevelant in the spleens of cattle which had not been treated with 'Tylan 200' as compared to those treated with the drug at 1 week and at 4 weeks. The significance of this difference is difficult to explain. However, 3 of the animals which were treated with 'Tylan 200' at one month after vaccination had typical CBPP lesions. This difference could therefore be an indication of the state of immunity in these cattle following vaccination with or without treatment: it may be suggested therefore that the animals which had less galactan antibody producing cells had not developed the same degree of immunity as those which had more galactan producing cells in their spleens.

Galactan isolated from M. mycoides and from the bovine lung are serologically related (SHIFRINE and GOURLAY, 1965). The anti-galactan antibodies found in the lung and in other tissues are possibly auto-antibodies. Indeed, one hypothesis on the formation of CBPP lesions (SHIFRINE and MOULTON, 1968)

is that M. mycoides organisms enter the respiratory tract and localize temporarily in the regional lymph nodes where they multiply and elicit an antibody response. The circulating antibodies, which are anti-lung antibodies, attach to certain sites of the lung where they cause tissue damage. During the ensuing bacteraemia, M. mycoides is attached to the damaged site by the antibody and multiplies there. On the other hand circulating antibody is sometimes found in vaccinated cattle which on challenge are solidly immune to CBPP (HUDSON, 1971).

In recognised auto-immune diseases, such as experimental tyroditis and glomerulonephritis, auto-antibodies are detectable in the serum, only when the target organ is removed (LENNON and FELDMANN, 1972). It is not surprising therefore that precipitating antibodies to galactan were not found in the serum of cattle vaccinated 15 months previously.

DAVIES (1969) confirmed the fact that serological responses are no measure of immunity but rather that a serological response was more an indication of the viable organisms. DAVIES (1969) suggested that a cell-bound antibody which was widespread in the body could be responsible for immunity against CBPP. The author also suggested that because of the localization of M. mycoides in regional lymph nodes, the immunological message was transported from there. DAVIES (1969) looked for IgA in

mucus of CBPP infected animals without success. He concluded that IgA did not play part as local protective antibody in CBPP.

The mechanism by which protection is acquired following vaccination is not clear. The anti-galactan antibodies may protect the lung by coating the cells and preventing them coming into intimate contact with the M. mycoides organism. The anti-galactan antibodies may protect the lung in a way similar to the way enhancing antibodies can protect tumours from cell-mediated immune response (TURK, 1972). LLOYD (1966) has reported a toxin like substance in the virulent Gladysdale strain of M. mycoides. He inserted chambers containing culture of the organism and closed by filter membranes into the peritoneal cavity of rabbits and was able to demonstrate that products diffuse through the filter membrane which stimulate connective tissue formation, attract leucocytes, induce deposition of fibrin and later cause necrosis of the cells and connective tissue and changes in the fibrin. The anti-galactan antibodies might prevent these M. mycoides products from coming into contact with the lung cells. The part played by galactan in the disease process is not fully understood but HUDSON, BUTTERY and COTTEW (1967) found that the administration of galactan before inoculating M. mycoides had the effect of

increasing the invasiveness of M. mycoides but did not appear to enhance its virulence.

TABLE XII

DETECTION OF GALACTAN ANTIBODY IN TISSUES OF VACCINATED CATTLE

ANIMAL NO.	TIMES VACCINATED	INTERVAL BETWEEN VACCINATION	PERIOD AFTER VACCINATION WHEN KILLED	TISSUES SHOWING ANTIBODY REACTION
1	2	18 months	6 weeks	Prescapular LN (L)
2	2	11 months	3 weeks	Lung (RC)
3	2	11 months	3 weeks	Submandibular LN (L) Popliteal LN (L) Prescapular LN (R) Medial Sacral LN (R)
4	2	10 months	6 weeks	Parotid LN (R and L) Popliteal LN (R and L) Deep Inguinal LN (R)
5	2	10 months	6 weeks	Submandibular LN (R) Medial Sacral LN (R)
6	2	10 months	7 weeks	Prescapular LN (R and L) Popliteal LN (R) Superficial Inguinal LN (L) Submandibular LN (R)
7	2	6 months	3 weeks	Lung (LC)
8	1	-	18 months	Lung (RD)
9	1	-	7 months	Lung (LD) Prescapular LN (L)

Key: LN = Lymph node
RC = Right Cardiac lobe
RD = Right Diaphragmatic lobe
LD = Left Diaphragmatic lobe
L = Left
R = Right
LC = Left Cardiac lobe

CHAPTER 7

IN VITRO STUDIES WITH THE T_1 STRAIN OF MYCOPLASMA MYCOIDES VAR. MYCOIDES

INTRODUCTION

TURNER and TRETHEWIE (1961) observed a bacteraemia soon after primary vaccination of cattle with the V_5 strain of M. mycoides. HUDSON and LEAVER (1965) demonstrated a bacteraemia in two cattle following the tail tip inoculation of a V_5 egg-vaccine. MASIGA and BOARER (1973) observed irregular bacteraemia following the vaccination of cattle with the T_1 broth culture vaccine.

This chapter records the results of further investigation on the irregular bacteraemia that occurred after primary inoculation of cattle using the T_1 strain of M. mycoides.

MATERIALS AND METHODS

ORGANISM:

The T_1 broth culture vaccine produced at the East

African Veterinary Research Organization was used to vaccinate cattle. Cattle were vaccinated by the subcutaneous route behind the shoulder using 0.5 ml. (approximately 10^9 organisms/ml.) of the T_1 broth culture vaccine (BROWN, GOURLAY and MACLEOD, 1965).

CATTLE:

Zebu or zebu-cross bred animals were obtained from an area of Kenya known to be free from CBPP. The cattle had live-weights ranging from 150 - 200 kg. and gave negative results in the complement fixation test already described in Chapter 3.

CULTURE ENUMERATION AND ISOLATION PROCEDURES FOR THE T_1 STRAIN OF M. MYCOIDES:

MEDIUM:

Newing's tryptose broth as modified by GOURLAY (1964) was used for the dilution of the T_1 broth culture vaccine for plate counts (MILES and MISRA, 1938); for the estimation of the number of the organisms in culture by the method of REED and MUENCH (1938) and as the culture medium when

labelling the organism with tritiated thymidine. Tryptose serum agar as described by GOURLAY (1964) was used for the estimation of the number of colony forming units (CFU) by a modified method of MILES and MISRA (1938).

INVESTIGATION OF MIXING T_1 STRAIN OF M. MYCOIDES WITH WHOLE BLOOD FROM NORMAL CATTLE:

Blood samples were collected, into heparinized bottles, from each of 5 CBPP susceptible cattle. These blood samples were divided into 9 ml. amounts and ten-fold dilutions of the T_1 broth culture vaccine were carried out using this blood. The mixtures of the T_1 strain and blood were left on the bench at room temperature for one hour and afterwards incubated at 37°C. Plate counts and dilution titres were estimated on each of these dilutions at 1 hour and 24 hours for plate counts and daily for 5 days for the dilution counts. Ten-fold dilutions of T_1 broth culture vaccine were made in tryptose broth and the titres of each dilution were determined and compared concurrently with the blood T_1 broth culture vaccine mixture.

PLATE COUNTS METHOD:

The modified MILES and MISRA (1938) method employed was as follows:-

Tryptose serum agar plates were dried at 37°C for between 40 minutes and 60 minutes. The T_1 broth culture vaccine or T_1 broth culture vaccine/blood mixture was diluted in Tryptose broth up to the 10th tube. After preliminary work it was established that optimal colonies for counting were found in dilutions 10^6, 10^7 and 10^8 in the T_1 broth culture vaccine. All the dilutions had to be plated out in the T_1 broth culture vaccine/blood mixture. In either case 10 drops from all the dilutions to be counted were dropped on one tryptose serum agar plate. Care was taken to observe the vertical angle of the pipette and the height of the drop pipette from the plate (2 - 3 cm.). A fifty dropper pipette which discharged a drop of 0.02 ml. was used throughout the experiment. The plates were allowed to dry and incubated at 37°C in polythene bags. The colonies were counted after 7 days incubation. The average number of colonies per plate was obtained and the viable count estimated by the method of MILES and MISRA (1938) viz. n x 50 x dil. factor, where n = number of colonies.

DILUTION TITRES:

In the case of dilution titres the method of REED and MUENCH (1938) was used to calculate the final titres. Three rows of tubes were employed. This method was carried out by 10-fold dilutions of the T_1 broth culture vaccine or the T_1 broth culture vaccine/blood mixture, in tryptose broth. The mixtures were incubated for 7 days and the last tubes, in which there was growth, were recorded. The calculation of the titres was as the following example; the titre calculated being the 50% end-point.

DILUTION	NO. OF TUBES (+) GROWTH	NO. OF TUBES (-) NO GROWTH	CUMULATIVE TOTALS (+)	(-)	GROWTH RATIO
10^7	5	0	11(5 + 4 + 2)	0	11/11=100%
10^8	4	1	6(4 + 2)	1	6/7 = 86%
10^9	2	3	2	4(3 + 1)	2/6 = 33%
10^{10}	0	5	0	9(5 +3+ 1)	0/9 = 0%

The 50% end point will be between 10^8 (86%) and 10^9 (33%).

To calculate the exact position:

$$\frac{\text{Growth above 50\% - 50}}{\text{Growth above 50\% - Growth below 50\%}} = \frac{86 - 50}{86 - 33} = \frac{36}{53}$$

$$\frac{36}{53} = 0.68 \qquad \therefore \text{ Titre } 10^{8.68}$$

RECOVERY OF T_1 STRAIN OF M. MYCOIDES FROM PLASMA AND SERUM

Blood was collected into sterile bottles for serum and into heparinized bottles for plasma from the same 5-CBPP susceptible animals. Both serum and plasma were separated by centrifugation of clotted blood and heparinized blood respectively. The spinning was carried out in the M.S.E. 50 centrifuge which was refrigerated. Some of the plasma and serum was inactivated at 56°C for 30 minutes. Blood cells separated from plasma were added to inactivated plasma and tryptose broth to give the same cell concentration as in the whole blood. Ten-fold dilutions of the T_1 broth culture vaccine were subsequently made, using fresh non-inactivated plasma and serum, inactivated plasma + cells tryptose broth as diluents. The mixtures were allowed to stand at room temperature for 48 hours and afterwards incubated at 37°C. Viable counts using the method described above were made at 24, 48 and 72 hours.

LABELLING OF THE T_1 STRAIN OF M. MYCOIDES WITH TRITIATED THYMIDINE

Ten ml. of tryptose broth were seeded with approximately 10^7 organisms of a T_1 culture, which was in the logarithmic phase of growth. One ml. of sterile tritiated thymidine (Thymidine-6-T)* containing 1 mCi of tritium was then added. The specific activity of the tritiated thymidine was 22 Ci/mM. One half of a millilitre of the culture was kept to assess the concentration of the tritiated thymidine, which was added to the broth. The culture containing tritiated thymidine was incubated, at 37°C, for 48 hours, by which time a titre of approximately 10^9 organisms/ml. was obtained. A control culture, without tritiated thymidine, was grown under similar conditions.

The amount of tritiated thymidine taken up by the organism was calculated from measurements of the activity of the labelled broth culture and of its supernatant after harvest of the organisms by centrifugation. The labelled medium and the supernatant were diluted with distilled water (1:2,000) and 0.4 ml. of this diluted medium was added to 15 ml. of a liquid scintillator, which was composed

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* Radiochemical Centre, Amersham, United Kingdom.

of toluene, ethanol, P.P.O. and dimethl-P.O.P.O.P. (GLICK, 1969). The activity of the samples was measured using a liquid Scintillation Spectrometer* and the count rates were used to calculate the uptake of tritiated thymidine by M. mycoides.

The organisms were recovered from the medium by centrifugation at 18,000 x g and they were washed 3 times with 0.2M Sodium chloride solution. The pellets of the washed organisms were stored at 4°C for 15 hours in 0.5 ml. of broth diluted 1:10 with 0.2M Sodium chloride before use.

THE PREPARATION OF BLOOD/T_1 ORGANISM MIXTURE FOR MICROSCOPY:

The tritiated thymidine labelled organisms were mixed with 5 ml. of freshly collected heparinized blood, which had been obtained from a CBPP-susceptible steer. Unlabelled organisms were similarly mixed with blood to act as a control. Blood smears were made at timed intervals of 15 to 30 minutes, and thence hourly to 10 hours and 24 hours and these were fixed in absolute ethanol. This was done for both blood/

* Packard, Liquid Scintillation Spectrometer.

organism mixtures. In addition the unlabelled organism/blood mixture was added to 5% gelatin, which as soon as it solidified was fixed in buffered formalin. Paraffin sections were prepared from the "buttons" and stained by Giemsa stain.

The fixed blood smears were subsequently "dipped" in Ilford 14 nuclear emulsion* and were then developed after 3 weeks' exposure, at 4°C. Developed autoradiographs were stained by Giemsa to demonstrate blood cells and Mycoplasma .

MEASUREMENT OF BOVINE COMPLEMENT IN SERUM LEFT AT ROOM TEMPERATURE

Bovine blood was obtained from cattle by vene-puncture. The bottles containing the blood were chilled in ice and the separation of the serum carried out in a refrigerated centrifuge (MSE 50). Bovine complement was measured soon after serum separation and the serum was left on the bench. Complement titres were again measured after 48 hours of incubation at room temperature.

The titration of bovine complement was carried out as described in Chapter 3. The only difference was that rabbit erythrocytes were used as the indicator system in the place of

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* Ilford Ltd., Ilford, Essex, United Kingdom.

sheep erythrocytes. The test was read as follows:-

4 = 100% haemolysis

3 = 75% haemolysis

2 = 50% haemolysis

1 = 25% haemolysis

0 = No haemolysis

R E S U L T S

The recovery of M. mycoides from serial dilution of the T_1 broth culture vaccine in blood and broth are shown in Table XIII. One hour after mixing the organisms with blood, M. mycoides was recovered in a dilution of 10^{-8} from culture diluted in tryptose broth but in a dilution of 10^{-5} when normal bovine blood was used as the diluent. After 24 hours of incubation at 37°C, the organisms were detected in the 10^{-8} dilution of broth + T_1 mixture and 10^{-7} in the blood + T_1 mixture.

The addition of blood to the T_1 broth culture vaccine reduced the number of viable organisms as shown by the dilution titre (Table XIV). The titre dropped from $10^{9.3}$ when diluted (10^{-1}) in broth to $10^{6.0}$ when diluted in blood and allowed to stand for 1 hour at room temperature after mixing. The dilution titre in the blood/organism mixture had risen by 48 hours and

was approximately at the original titre. There was no rise in titre in the dilution factors 10^{-2} to 10^{-9} during the course of the experiment.

The results shown in Table XV indicate that the addition of filtered non-inactivated plasma to the T_1 strain of M. mycoides reduced the titres of all the dilutions. This drop was evident after 1 hour and persisted up to 72 hours when the experiment was ended. The eddition of freshly prepared serum also reduced the titres of the T_1 strain of M. mycoides (Table XV). No colony forming units were detected in any dilution at 72 hours, which included 24 hours of incubation at 37°C, in the M. mycoides/ serum mixture.

There was a 50% loss of viability of organisms in all dilution of the broth culture when it was diluted with inactivated plasma mixed with blood cells and tryptose broth. At 48 hours, however, there was a 100% increase of organisms in the dilutions of 10^{-1} to 10^{-3} but there was none in the remaining dilutions from either cells + broth or cells + plasma mixtures.

Scintillation counts showed that a 10% uptake of tritiated thymidine occurred with the growth of organisms from 10^7 to 10^9/ml. Autoradiography confirmed that the organisms were labelled. Figure 7 shows an autoradiograph of M. mycoides labelled with tritiated thymidine and stained with Giemsa, as seen microscopically with transmitted light.

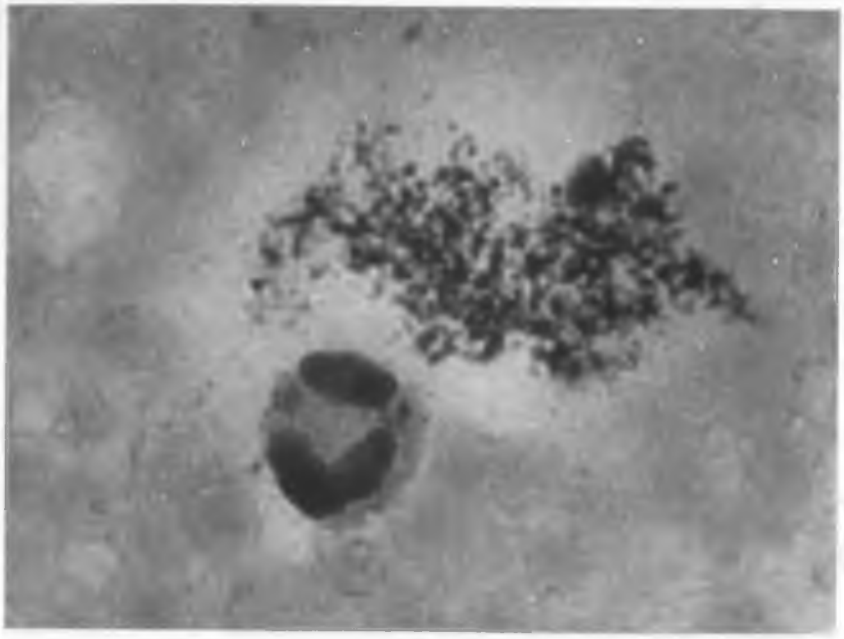

Fig. 7: Clumps of tritiated thymidine labelled M. mycoides.

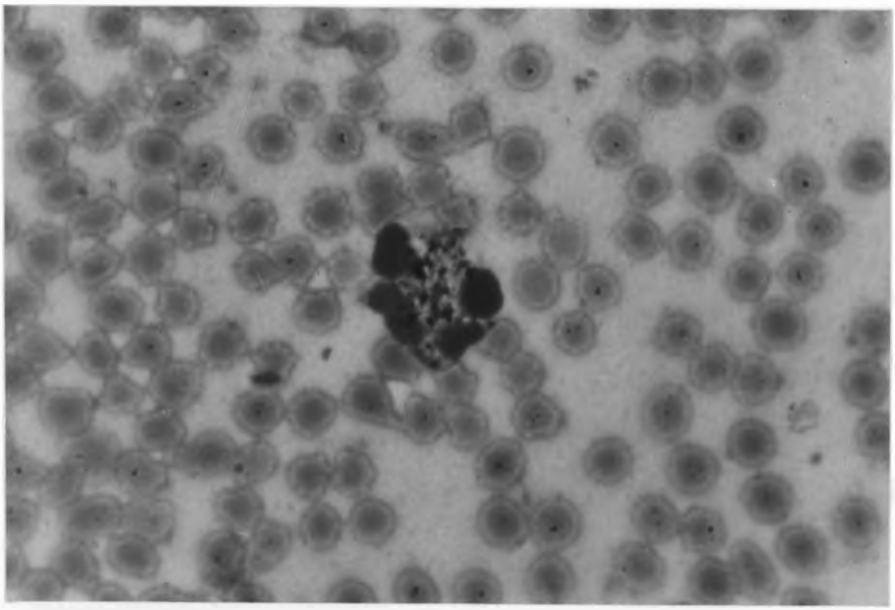

Fig. 8: Neutrophil with ingested tritiated thymidine labelled M. mycoides.

Autoradiography also showed blackening of neutrophils which was caused by phagocytosed labelled M. mycoides. The neutrophil in Figure 8 was found in a smear prepared after 7 hours contact of the blood with the organisms. Phagocytosis of the T_1 strain by leucocytes was observed from approximately 30 minutes to 24 hours (final sample). In many cases part of the clump of M. mycoides could be seen adjacent to the leucocyte cell membrane and the remainder appeared to be within the cell. Presumably the clump of M. mycoides, in Figure 9, was in the process of being phagocytosed. Neutrophils showed evidence of degranulation. Lymphocytes (Figure 10), with ingested Mycoplasma, were not observed as frequently as were neutrophils. Leucocytes were seen on the periphery of clumps of M. mycoides by 30 minutes (Figure 11). However, this could have been taken as an artifact produced in preparing the blood smears. Five to 7 hours later, however, clumps of M. mycoides were seen where the white blood cells had migrated into the central areas of the clumps of organisms (Figure 12) and it is unlikely that this could have been the result of making the smears. Examination of the paraffin sections prepared from the gelatin embedded blood/organism mixture gave results similar to the smear examination. Numbers of leucocytes were visible on the edge and within clumps of organisms. No evidence of blackening was observed in

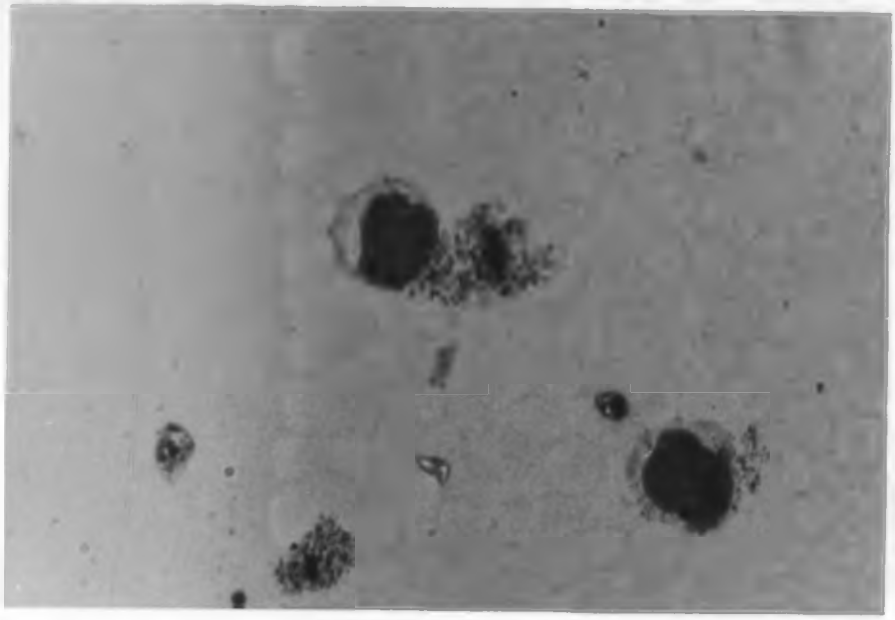

Fig. 9: Clumps of M. mycoides in close association with phagocytes.

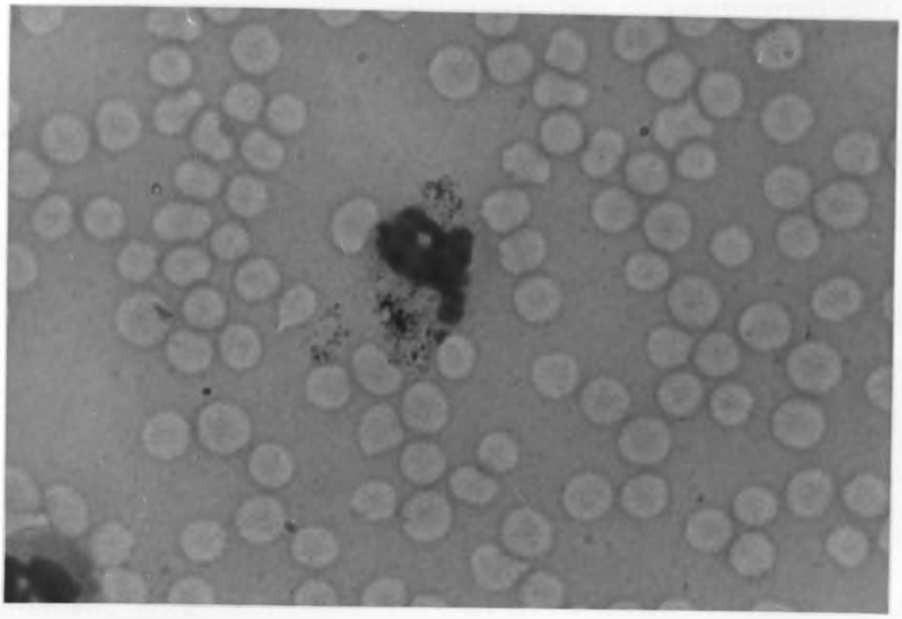

Fig. 10: Lymphocyte with ingested labelled M. mycoides.

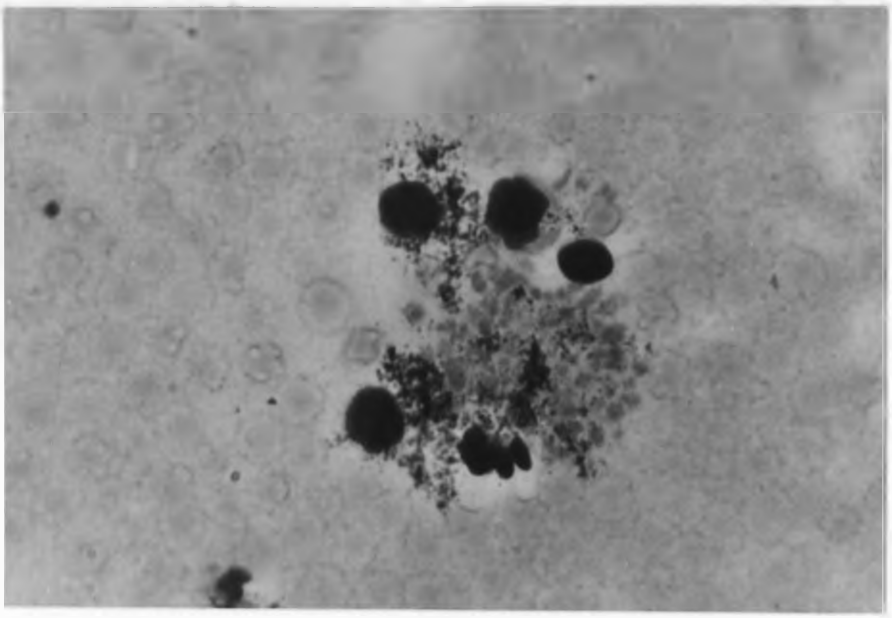

Fig. 11: Leucocytes at the periphery of clumps of M. mycoides 30 minutes after mixing Mycoplasma with blood from normal cattle.

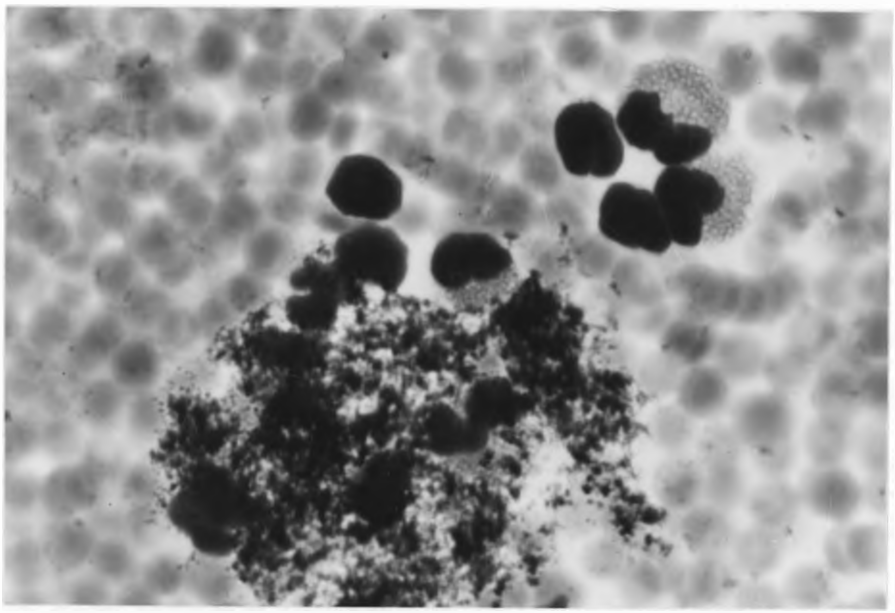

Fig. 12: Leucocytes in the midst of clumps of M. mycoides 7 hours after mixing Mycoplasma with blood from normal cattle.

autoradiography of control smears of blood containing unlabelled organisms, which were prepared simultaneously.

D I S C U S S I O N

A marked decrease in number of viable organisms was clearly demonstrated *in vitro* when the T_1 broth culture vaccine was mixed with normal bovine blood, plasma and serum. There was no decrease in viable counts of the organism when it was diluted in broth, and only a small loss of viability, after 48 hours, when diluted in inactivated serum or plasma. Thus the decrease in viable counts in plasma and serum was presumably due to a heat labile factor present in plasma and serum possibly complement. This finding is in agreement with that of PRIESTLEY (1952) who found that plasma and serum were bactericidal to Mycoplasma.

The examination of stained blood films and autoradiographic plates suggested that part of the decrease of titre in whole blood was due to the phagocytosis of organisms by leucocytes quite apart from the complement effect. There was also a marked increase in the number of organisms in mixed broth culture and blood after 48 hours incubation. This may

have been the multiplication of organisms which had not been phagocytosed or killed by the complement. However, DAVIES (1969b) and WINDSOR (personal communication) have shown that, in broth culture, the doubling time of the T_1 strain of M. mycoides is between 2 - 5 hours depending on the size of the initial inoculum of the organisms, and that the logarithmic phase of growth is complete in 48 hours; thus if the rate of increase of the number of organisms in blood/broth mixture was similar to that in broth alone, it might be expected that the rise in titre, if due only to this process, would have been observed much earlier. The length of time before such increase occurred, and in particular the rapidity of its appearance strongly suggests that large number of viable organisms were at that time released from the leucocytes, presumably following their disruption. This increase in titre was not observed during the course of the experiment when M. mycoides was mixed with either non-inactivated plasma or serum which suggests that the increase in titre in the blood/T_1 mixture after 48 hours of incubation was due to the release of the organisms from the phagocytes. Presumably phagocytosis protects the organisms from the effects of the complement and indeed by 48 hours the level of the complement in serum left at room temperature was found to be very low (KAKOMA and MASIGA,

unpublished data). This may be the reason why the titre remained high after it increased at 48 hours.

The reduction in numbers of M. mycoides when they were mixed with either blood cells - tryptose broth or blood cells + inactivated plasma was not as marked as that observed when M. mycoides was mixed with whole blood. This difference may have been due to insufficient complement being available, in the former two mixtures, for the enhancement of phagocytosis. An alternative explanation could be that many leucocytes may have been killed during the process of separating the cells from the plasma.

WILSON (1953) has reported that the release of viable bacteria from phagocytes may occur in certain circumstances. Also LLOYD (1966) has reported that M. mycoides contains a toxin that causes necrosis of tissues. This necrosis has been observed when a culture of pathogenic strains of M. mycoides come in contact with bovine leucocytes (PROVOST, 1964). It is possible that some of the ingested M. mycoides released toxin which subsequently killed the phagocytes and this led to the release of viable organisms.

The marked rise in the titre of the mixture of blood and T_1 broth culture occurred after 48 hours incubation, whereas an increase in the number of organisms circulating

in the blood of cattle was first indicated by the bacteraemia detected 8 hours after vaccination (see Chapter 3). Despite the considerable difference between these intervals, the observable fate of the organisms *in vitro* offers a possible explanation for the intermittent bacteraemia which was shown by 4 of the 6 animals in which bacteraemia was detected (see Chapter 3).

Many factors play a part in governing the number of Mycoplasma organisms in the blood at any one time after vaccination; among these will be the rate of entry of injected Mycoplasma into blood or lymph channels; the extent to which they may be phagocytosed by leucocytes, both at the site of vaccination and in the blood by the circulating macrophages; the bactericidal activity of the complement; and the rate of multiplication of organisms in blood and other tissues. With all these factors involved, it is probable that the titre of circulating Mycoplasma will vary considerably in the period following their injection; and this may account for the fact that they were detected only at irregular intervals. The ingestion and subsequent release of Mycoplasma by leucocytes *in vitro* suggest that the intermittent bacteraemia which occurred *in vivo* may have been due to the release of large numbers of organisms into the blood by the disruption of leucocytes due to the toxic activity of *M. mycoides*.

TABLE XII

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6	2	10 months	7 weeks	Prescapular LN (R and L) Popliteal LN (R) Superficial Inguinal LN (L) Submandibular LN (R)
7	2	6 months	3 weeks	Lung (LC)
8	1	-	18 months	Lung (RD)
9	1	-	7 months	Lung (LD) Prescapular LN (L)

Key:
LN = Lymph node
RC = Right Cardiac lobe
RD = Right Diaphragmatic lobe
LD = Left Diaphragmatic lobe
L = Left
R = Right
LC = Left Cardiac lobe

MEAN CULTURE PLATE RECOVERIES OF THE T_1 VACCINE STRAIN OF *M. MYCOIDES* AFTER DILUTION

IN BLOOD OF CBPP-SUSCEPTIBLE CATTLE OR BROTH

TIME LAPSE	DILUENT	DILUTION FACTOR											
		10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	10^{-11}
1 hour	BROTH	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	NCFU	NCFU	NCFU
1 hour	BLOOD	N.T.	+ve	+ve	+ve	+ve	+ve	NCFU	NCFU	NCFU	NCFU	NCFU	NCFU
24 hours	BLOOD	N.T.	+ve	+ve	+ve	+ve	+ve	+ve	+ve	NCFU	NCFU	NCFU	NCFU
24 hours	BROTH	N.T.	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	NCFU	NCFU	NCFU

NCFU = No Colony Forming Units found. N.T. = Not Tested

GROWTH DILUTION TITRES OF THE T. STRAIN OF M. MYCOIDES AFTER DILUTION IN BLOOD OF CBPP SUSCEPTIBLE CATTLE OR BROTH

TIME LAPSE	DILUENT	DILUTION FACTOR											
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	10^{-11}	10^{-12}
1 Hour	BROTH	$10^{9.3}$	$10^{9.3}$	$10^{7.7}$	$10^{7.0}$	$10^{5.3}$	$10^{4.3}$	$10^{4.0}$	$10^{3.7}$	$10^{2.0}$	$10^{1.0}$	-	-
	BLOOD	$10^{6.0}$	$10^{6.0}$	$10^{3.3}$	$10^{3.3}$	$10^{3.0}$	-	-	-	-	-	-	-
24 Hours	BROTH	$10^{9.3}$	$10^{8.3}$	$10^{7.3}$	$10^{6.3}$	$10^{5.7}$	$10^{4.7}$	$10^{3.7}$	$10^{2.0}$	$10^{2.0}$	-	-	-
	BLOOD	$10^{5.3}$	$10^{3.7}$	-	-	-	-	-	-	-	-	-	-
48 Hours	BROTH	$10^{10.0}$	$10^{9.0}$	$10^{9.7}$	$10^{8.3}$	$10^{8.0}$	$10^{5.0}$	$10^{4.7}$	$10^{3.3}$	$10^{4.0}$	$10^{3.0}$	-	-
	BLOOD	$10^{9.7}$	$10^{4.5}$	-	-	-	-	-	-	-	-	-	-
72 Hours	BROTH	$10^{9.7}$	$10^{9.4}$	$10^{9.0}$	$10^{9.0}$	$10^{9.0}$	$10^{8.3}$	$10^{9.7}$	$10^{9.0}$	$10^{10.5}$	$10^{9.3}$	-	-
	BLOOD	$10^{8.3}$	$10^{5.6}$	-	-	-	-	-	-	-	-	-	-
96 Hours	BROTH	$10^{8.7}$	$10^{9.5}$	$10^{8.3}$	$10^{9.0}$	$10^{9.0}$	$10^{8.3}$	$10^{9.7}$	$10^{9.0}$	$10^{10.5}$	$10^{9.3}$	-	-
	BLOOD	$10^{9.0}$	$10^{4.0}$	-	-	-	-	-	-	-	-	-	-

MEAN DILUTION TITRE RECORDED BY 50% END-POINT OF REED and MUENCH (1938)

TABLE XV

CULTURE PLATE TITRES OF THE T_1 STRAIN OF *M. MYCOIDES* AFTER DILUTION IN SERUM OR PLASMA FROM CBPP SUSCEPTIBLE CATTLE OR BROTH

TIME LAPSE	DILUENT	DILUTION FACTOR											
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	10^{-11}	10^{-12}
1 Hour	BROTH	2.8×10^{8}	2.7×10^{7}	3.4×10^{6}	5.8×10^{5}	3.3×10^{4}	3.9×10^{3}	2.3×10^{2}	-	-	-	-	-
	PLASMA NON-INACT.	2.7×10^{4}	-	-	-	-	-	-	-	-	-	-	-
	PLASMA 56°C	2.2×10^{8}	1.6×10^{7}	2.6×10^{6}	7.8×10^{4}	2.6×10^{3}	1.1×10^{3}	1.5×10^{2}	-	-	-	-	-
	SERUM NON-INACT.	3.5×10^{3}	-	-	-	-	-	-	-	-	-	-	-
	SERUM 56°C	1.6×10^{8}	2.8×10^{7}	3.9×10^{6}	2.3×10^{5}	3.0×10^{4}	2.7×10^{3}	2.0×10^{2}	-	-	-	-	-
24 Hours	BROTH	5.6×10^{7}	5.1×10^{7}	5.0×10^{7}	5.1×10^{6}	1.4×10^{5}	4.7×10^{3}	1.0×10^{2}	5.0×10^{1}	-	-	-	-
	PLASMA NON-INACT.	3.5×10^{4}	-	-	-	-	-	-	-	-	-	-	-
	PLASMA 56°C	3.1×10^{8}	2.8×10^{7}	2.5×10^{6}	4.2×10^{4}	1.3×10^{4}	2.0×10^{3}	3.2×10^{3}	-	-	-	-	-
	SERUM NON-INACT.	3.3×10^{4}	-	-	-	-	-	-	-	-	-	-	-
	SERUM 56°C	3.2×10^{8}	3.9×10^{7}	2.1×10^{6}	3.0×10^{5}	2.4×10^{4}	3.1×10^{3}	4.0×10^{2}	-	-	-	-	-
48 Hours	BROTH	4.2×10^{8}	2.9×10^{8}	3.0×10^{8}	2.3×10^{8}	2.5×10^{6}	2.8×10^{4}	5.5×10^{3}	1.0×10^{2}	-	-	-	-
	PLASMA NON-INACT.	-	2.9×10^{4}	-	-	-	-	-	-	-	-	-	-
	PLASMA 56°C	1.9×10^{8}	1.4×10^{7}	1.4×10^{6}	1.5×10^{5}	1.4×10^{4}	2.7×10^{3}	-	-	-	-	-	-
	SERUM NON-INACT.	5.0×10^{4}	-	-	-	-	-	-	-	-	-	-	-
	SERUM 56°C	3.8×10^{8}	3.0×10^{7}	2.2×10^{6}	3.3×10^{5}	2.9×10^{4}	2.3×10^{3}	4.0×10^{2}	-	-	-	-	-
72 Hours	BROTH	2.4×10^{8}	2.0×10^{8}	3.3×10^{8}	3.5×10^{8}	TOO MANY TO COUNT	TOO MANY TO COUNT	TOO MANY TO COUNT	TOO MANY TO COUNT	-	-	-	-
	PLASMA NON-INAC.	1.5×10^{6}	-	-	-	-	-	-	-	-	-	-	-
	PLASMA	4.9×10^{8}	7.4×10^{7}	1.2×10^{7}	3.7×10^{6}	8.6×10^{3}	8.0×10^{3}	-	-	-	-	-	-
	SERUM NON-INACT.	-	-	-	-	-	-	-	-	-	-	-	-
	SERUM 56°C	5.8×10^{8}	4.7×10^{7}	3.0×10^{6}	2.1×10^{5}	2.3×10^{4}	2.3×10^{3}	-	-	-	-	-	-

Titre recorded as CFU/ml.

CHAPTER 8

GENERAL DISCUSSION AND CONCLUSIONS

Following vaccination of cattle with the T_1 strain of *M. mycoides*, many factors probably influence the survival of the organisms in animal tissues. These are:- the rate of entry of injected Mycoplasma into the blood and lymph channels; the extent to which they may be phagocytosed by leucocytes, both at the site of vaccination and in other tissues including blood; the bactericidal activity of the complement; and the rate of their multiplication in blood and other tissues. The recovery of the T_1 strain from blood was possible for only a relatively short period of time after inoculation and only if vaccination was carried out subcutaneously. Under these conditions a bacteraemia could not be detected for longer than 50 hours, and was never apparent when the tail-tip route of inoculation was employed.

Whereas *M. mycoides* was recovered following primary vaccination from the tails (site of inoculation) of 6 animals and from the regional lymph nodes of 7 of a total of 10 animals; it was not recovered from any of the other tissues examined. The latest recovery of the organism from the tissues of any

animal was 71 days after primary vaccination.

It was established that M. mycoides antigens persisted in the body of vaccinated cattle for as long as 204 days. That some of the antigens detected might have been auto-antigens or antigens due to other bacterial infections has been discussed in Chapter 3. The antigens and the organisms persisted in the animal tissues for longer than CF and SAST antibodies following primary vaccination. The antigens were present 3 days after primary vaccination and became widespread by 9 days. The reason for the apparent lack of appearance of the antibodies or its detection for only short periods, even when the organism and associated antigens persist in the body tissues, cannot be satisfactorily explained without further study of the immune reactions involved.

Although the T_1 strain was isolated for up to 71 days from tissues of animals vaccinated once, the latest date of isolation of M. mycoides from the tissues of any of the revaccinated cattle was 22 days, and in the majority of revaccinates organisms could not be isolated even after 7 days. The period between primary and secondary vaccination had no bearing on these findings. Whereas the antigens became widespread by the 4th day following secondary vaccination, they became widespread by the 9th day following primary vaccination. From the above findings it is clear that, during the period when an animal is immunized against CBPP, on introduction of the vaccine strain, the organism will quickly be destroyed.

All animals which had been revaccinated with the T_1 broth culture vaccine either 6 or 12 months after primary vaccination were resistant to a CBPP challenge. Another group of animals which had received the primary but not the secondary vaccination was also found to be immune 2 years after primary vaccination.

The serological response following revaccination was rare and erratic: of 48 animals only 6 responded following revaccination. The highest CF titre recorded was 20 and the SAST reaction was ++ as compared to a CF titre of 2560 and an SAST reaction of ++++ which were typical of those recorded following primary vaccination. There was no evidence therefore of an anamnestic response following secondary vaccination. This finding was also seen when four routes of administration were used to revaccinate cattle, and on these occasions amounts of the T_1 broth culture vaccine were increased from 4 to 100 fold. Despite this, only 6 of 20 animals responded serologically following revaccination and only 2 had CF titres higher than those recorded during primary vaccination. The animals which had higher CF titres had been inoculated intravenously with 50 ml. of the vaccine at secondary vaccination.

When the serum was fractionated on Sephadex G 200, only the IgM peak fixed complement in the presence of M. mycoides antigen. As IgM is a primary antibody these experiments have further confirmed the absence of a secondary response in cattle

revaccinated with the T_1 broth culture vaccine after 6, 9, 12, 15, 17 and 18 months. The reason for this lack of a secondary response needs investigation.

Cattle which had been vaccinated 3 months earlier were solidly immune to challenge. Animals treated with a therapeutic course of "Tylan 200" starting one week after vaccination were all immune when challenged and yet in the group in which the treatment with "Tylan 200" commencing one month after vaccination, 3 of 11 animals developed typical CBPP lesions. These results support the observation that live vaccines are essential for the development of immunity to CBPP. They also suggest that persistence of the organism for more than one month following vaccination is vital for the development of immunity against CBPP. That all the animals treated with "Tylan 200" one week after primary vaccination were found to be solidly immune to CBPP challenge and yet 3 of 11 animals similarly treated one month after vaccination developed CBPP lesions on challenge, strongly supports the hypothesis that the organisms multiply in the body tissues following primary vaccination. It can be argued that following the therapeutic course of "Tylan 200" one week after vaccination, the surviving organisms were in sufficient numbers to multiply and reach the minimum number required to elicit solid immunity. But at one month after primary vaccination, "Tylan 200" either killed all

the organisms or the organisms that survived the effect of the drug were not in sufficient numbers to multiply to reach the minimum number required to elicit solid immunity. It is therefore assumed that vaccinated and treated animals which developed CBPP lesions during the challenge had not developed solid immunity at the time of treatment.

All the 3 vaccinated groups of animals differed significantly in their response to challenge from the control group. The group treated with "Tylan 200" at one month was different from the other 2 vaccinated groups of cattle as the pathology score for this group was 1.23 as compared to 0.45 for the group treated at one week after primary vaccination and 0.08 for the animals which were vaccinated and which were not treated with the drug. However, the differences between the vaccinated cattle were not significant.

As this investigation has been carried out using only one drug, it is essential that similar experiments should be conducted with tetracyclines which are very commonly used in veterinary practice in East Africa and other broad spectrum antibiotics. After these studies then anti-trypanosomal drugs should also be similarly studied.

Many serological tests including CFT and SAST have failed to measure immunity to CBPP. In the present experiments, animals were challenged when the CF and SAST reactions were negative and yet the animals were resistant to a severe CBPP challenge.

Although the protective factor against CBPP, may, in part, be a serum factor, the present evidence suggests a local factor to be responsible for protection against CBPP. Precipitating antibodies to galactan were detected in tissues of lungs and lymph nodes of cattle vaccinated twice and in 3 of the cattle vaccinated once at 6, 7 and 18 months previously, respectively. In 20 of the cattle vaccinated once precipitating antibodies to galactan were not detected in the lung or mediastinal lymph nodes but galactan antibody producing cells were detected in the spleens and mediastinal lymph nodes. It has been suggested that the antibody to galactan protects the tissues in the same way as anti-tumor antibody protects tumor cells from the effects of cell-mediated immunity.

The implications of the present findings have practical field applications. Until more is understood of the mechanisms of immunity to CBPP induced by the T_1 vaccine, it must be assumed that the use of any chemotherapeutic agent or broad spectrum antibiotic may result in the loss or failure of immunity to CBPP. It is therefore, advisable at the present time to insist that any animals vaccinated against CBPP should not be treated with Mycoplasmacidal or Mycoplasmastatic drugs for at least one month after vaccination. But if treatment must be administered, then the animals receiving such treatment must be revaccinated. It is also recommended that animals which have been vaccinated against CBPP should not freely mix with CBPP suspicious

herds for at least one month. Apparently by one month some of the animals have not developed solid immunity to the disease following primary vaccination.

As many animals do not respond serologically to primary vaccination and virtually none to secondary vaccination, the CFT cannot be used to determine the efficiency of vaccinating teams or indeed the efficacy of vaccination. It is suggested that the only way of identifying a vaccinated animal is by physical means such as branding and tattooing of the animals: different brand marks or tattoos can be devised to represent primary and subsequent inoculations. With the irregular and variable CFT results it is virtually impossible to make any logical conclusions of field CF tests in so far as vaccination "follow up" campaigns are concerned. It is suggested therefore that the CFT ought to be employed in the field only for the purposes of diagnosing clinical CBPP.

The present work was carried out on mixed breeds of cattle which included Zebu, Zebu-exotic grades and exotic animals. These results therefore will be applicable in areas where all these breeds of cattle are found in East Africa.

The experiments under report have confirmed that 2 years after primary vaccination some cattle are still protected against CBPP. However, in a recent experiment, carried out in this laboratory 5 of 16 animals vaccinated 2 years earlier developed

CBPP lesions on challenge and M. mycoides was isolated from these lesions. Thus the animal vaccination programmes must be continued in all CBPP endemic areas.

Animals which had been revaccinated 6 months and one year after primary vaccination have all been resistant to a CBPP challenge. The findings of this study have thus confirmed that revaccination did not interfere with the immunity developed after primary vaccination. But as all the animals vaccinated at the same time 2 years previously have withstood the same challenge, it has been impossible to judge the difference in degree of immunity between vaccinated and revaccinated animals in this study. To be on the safe side it must be stressed that all animals must be vaccinated annually to control CBPP in endemic areas of East Africa.

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isolated was confirmed as *M. mycoides* by the growth inhibition test (Davies & Read, 1968).

All incubation was carried out at 37°C. Subcultures were made every 2 days up to 14 days. Cultures showing no growth by this time were discarded.

In Vitro. Blood samples were collected into heparinized bottles from each of 5 CBPP susceptible cattle. These blood samples were divided into 9 ml amounts, and tenfold dilutions of the T_1 broth culture vaccine were carried out using this blood. The mixtures of the T_1 strain and blood were then left on the bench at room temperature for 1 h and afterwards incubated at 37°C. Plate counts and dilution titres were estimated on each of these dilutions at 1 and 24 h for plate counts and daily for 5 days for the dilution counts. Tenfold dilutions of T_1 broth culture vaccine were made in tryptose broth, and the titres of each dilution were determined and compared concurrently with the blood/T_1 broth culture vaccine mixture.

Recovery of T_1 Strain in M. mycoides *from Plasma and Serum*

Blood was collected into sterile bottles for serum and into heparinized bottles for plasma, from the same 5 CBPP-susceptible animals. Some of the plasma and serum was inactivated at 56°C for 30 min. Blood cells separated from plasma were added to inactivated plasma and to tryptose broth to give the same cell concentration as in whole blood. Tenfold dilutions of the T_1 broth culture vaccine were subsequently made, using non-inactivated and inactivated plasma and serum, inactivated plasma + cells, and cells + tryptose broth as diluents. The mixtures were allowed to stand at room temperature for 48 h and afterwards incubated at 37°C. Viable counts were made at 24, 48 and 72 h.

Labelling of the T_1 Strain of M. mycoides *with Tritiated Thymidine*

Ten ml of tryptose broth were seeded with approximately 10^7 organisms of a T_1 culture, which was in the logarithmic phase of growth. One ml of sterile tritiated thymidine (thymidine-6-T)* containing 1 mCi of tritium was then added. The specific activity of the tritiated thymidine was 22Ci/mM. One half of a ml of the culture was kept to assess the concentration of the tritiated thymidine, which was added to the broth. The culture containing tritiated thymidine was incubated, at 37°C, for 48 h, by which time a titre of approximately 10^9 organisms/ml was obtained. A control culture, without tritiated thymidine, was grown under similar conditions.

The amount of tritiated thymidine taken up by the organisms was calculated from measurements of the activity of the labelled broth culture and of its supernatant after harvest of the organisms by centrifugation. The labelled medium and the supernatant were diluted with distilled water (1 : 2,000) and 0·4 ml of this diluted medium was added to 15 ml of a liquid scintillator, which was composed of toluene, ethanol, 2, 5-diphenyloxazolone (P.P.O.) and 1, 4-di [2-(5-phenyloxazolone)] benzene (dimethyl-P.O.P.O.P.)(Methods of Biochemical Analysis, 1969). The activity of the samples was measured using a liquid scintillation spectrometer† and the count rates were used to calculate the uptake of tritiated thymidine by *M. mycoides*.

The organisms were recovered from the medium by centrifugation at 18,000 *g* and they were washed 3 times with 0·2 M sodium chloride solution. The pellets of the washed organisms were stored at 4°C for 15 h in 0·1 ml of broth diluted 1 : 10 with 0·2 M sodium chloride before use.

The Preparation of Blood/T_1 Organism Mixture for Microscopy

The tritiated thymidine labelled organisms were mixed with 5 ml of freshly collected heparinized blood, which had been obtained from a CBPP-susceptible steer. Unlabelled organisms were similarly mixed with blood to act as a control. Blood smears were made at timed intervals up to 24 h and these were fixed in absolute ethanol. This was done for both blood/organism mixtures. In addition the unlabelled organism/blood mixture was added to 5% gelatin, which as soon as it solidified was fixed in buffered formalin, and paraffin sections were prepared from the 'buttons' and stained by Giemsa stain.

The fixed blood smears were subsequently 'dipped' in Ilford L4 nuclear emulsion‡ and were then developed, after 3 weeks' exposure, at 4°C. Developed autoradiographs were stained by Giemsa to demonstrate blood cells and mycoplasma.

RESULTS

Table I shows the times, after primary vaccination, at which *M. mycoides* was isolated from the blood of cattle vaccinated with T_1 broth culture vaccine. The organisms were

* Radiochemical Centre, Amersham, England.

† Packard, Liquid Scintillation Spectrometer.

‡ Ilford Ltd, Ilford, Essex.

tail, other lymph nodes separately identified in Table 1, lung lobes, trachea, turbinate, liver, spleen, kidney and the tail-tip (site of inoculation). One g. of each tissue was weighed, inoculated into 9 ml. of the isolation medium of Davies and Read (1968) and titrated. The last tube showing growth was noted. Subcultures of the tissues were

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In Vivo and *in Vitro* Studies with the T_1 Strain of *Mycoplasma mycoides* in Cattle

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SUMMARY. Mycoplasma mycoides var. mycoides (M. mycoides) *was recovered only intermittently from the blood of 4 cattle after primary vaccination. Results of* in vitro *experiments suggest that the intermittent recovery of* M. mycoides *from the blood of cattle, vaccinated by the subcutaneous route behind the shoulder, could have been due to phagocytosis and subsequent release of the organism by leucocytes.*

VARIOUS *Mycoplasma mycoides var. mycoides* (*M. mycoides*) strains have been used as vaccines to control contagious bovine pleuropneumonia (CBPP) in many countries. Investigations of the persistence of some of these strains have been carried out following primary vaccination of cattle.

Turner & Trethewie (1961) observed a bacteraemia soon after primary vaccination of cattle with the V_5 strain of *M. mycoides*. Hudson & Leaver (1965) demonstrated a bacteraemia in 2 cattle following the tail tip inoculation of a V_5 egg-vaccine and Hudson (1965) stated that the KH_3J strain persists in regional lymph nodes for up to 3 months after vaccination. Lindley & Pedersen (1968) recovered the latter strain up to 2 months after primary vaccination of cattle; Davies (1969a) and Masiga (unpublished data), using the T_1 strain of *M. mycoides*, recovered the organism from tissues 14 and 71 days, respectively, after vaccination.

This paper records the results of an investigation of the intermittent bacteraemia that occurred after primary vaccination of cattle using the T_1 strain of *M. mycoides*.

MATERIALS AND METHODS

Organism

The T_1 strain of *M. mycoides* maintained at the East African Veterinary Research Organization was used. This is the vaccine strain used throughout East Africa for the control of CBPP.

Cattle

Zebu or Zebu-exotic cross bred animals were obtained from an area of Kenya known to be free from CBPP. The cattle had liveweights ranging from 150 to 200 kg and gave negative results in the complement fixation test (Campbell & Turner, 1936).

Vaccination

Cattle were vaccinated by the subcutaneous route behind the shoulder using 0·5 ml (approx. 10^9 organism/ml) of the T_1 broth culture vaccine (Brown *et al.*, 1965).

Culture, Enumeration and Isolation Procedures for the T_1 strain of M. mycoides

Medium. Newing's tryptose broth as modified by Gourlay (1964) was used for dilution of the T_1 broth culture vaccine for plate counts (Miles & Misra, 1938), for the estimation of the number of the organisms in the cultures by the method of Reed & Muench (1938) and as the culture medium when labelling the organism with tritiated thymidine. Tryptose serum agar, as described by Gourlay (1964) was used for the estimation of the number of colony forming units (c.f.u.) by the method of Miles & Misra (1938) as modified by Davies (1969b), except that 10 drops (0·02 ml) were used for each dilution.

Recovery of T_1 Strain of M. mycoides *from Blood*

In Vivo. Blood samples were obtained from the jugular vein immediately before, and at frequent intervals after, vaccination. Samples were collected at timed intervals up to 72 h after primary vaccination for 5 animals, and up to 147 h for the remaining 12 cattle. On each occasion 20 ml of blood was collected into 80 ml of tryptose broth. The titre of the organism in blood was determined by the Reed & Muench (1938) method. The identity of organisms

* Seconded from the United Kingdom, Ministry of Agriculture, Central Veterinary Laboratory, Weybridge, Surrey, England.

tail, other lymph nodes separately identified in Table 1, lung lobes, trachea, turbinate, liver, spleen, kidney and the tail-tip (site of inoculation). One g. of each tissue was weighed, inoculated into 9 ml. of the isolation medium of Davies and Read (1968) and titrated. The last tube showing growth was noted. Subcultures of the tissues were

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TABLE I
THE ISOLATION OF *Mycoplasma mycoides* FROM THE BLOOD OF CATTLE VACCINATED BY THE SUBCUTANEOUS ROUTE USING THE T_1 BROTH CULTURE VACCINE

Cattle No.	Time (h) of isolation of *M. mycoides* after vaccination
C 520	10 and 24
C 507	10 and 16
C 528	10, 12, 30, 32 and 50
D 754	8
D 497	32
D 976	8 and 32
12 others	No isolation made

recovered from animal C 528 at 10, 12, 30, 32 and 50 h; C 520 at 10 and 24 h; C 507 at 10 and 16 h; D 796 at 8 and 32 h; and D 497 and 754 at 32 and 8 h, respectively. No isolation of *M. mycoides* was made from the blood samples of 12 other animals. Thus *M. mycoides* was recovered from the blood of these vaccinated animals only rarely and at irregular intervals.

The recovery of *M. mycoides* from serial dilutions of the T_1 broth culture vaccine in blood and broth are shown in Table II. One hour after mixing the organisms with blood, *M. mycoides* was recovered in a dilution of 10^{-8} from culture diluted in tryptose broth but in a dilution of 10^{-5} when normal bovine blood was used as the diluent. After 24 h of incubation at 37°C, the organisms were detected in a dilution of 10^{-8} in the broth + T_1 mixture and 10^{-7} in the blood + T_1 mixture.

The addition of blood to the T_1 broth culture vaccine reduced the number of viable organisms as shown by the dilution titre (Table III). The titre dropped from $10^{9 \cdot 3}$ when diluted (10^{-1}) in broth to $10^{6 \cdot 0}$ when diluted in blood and allowed to stand for 1 h at room temperature after mixing. The dilution titre of the blood/organism mixture had risen by 48 h and was approximately its original titre. There was no rise in titre in the dilution factors 10^{-2} to 10^{-9} during the course of the experiment.

The results shown in Table IV indicate that the addition of filtered non-inactivated plasma to the T_1 strain of *M. mycoides* reduced the titres of all the dilutions. This drop was evident after 1 h and persisted up to 72 h when the experiment was ended. The addition of freshly prepared serum also reduced the titres of the T_1 strain of *M. mycoides* (Table IV). No colony forming units were detected in any dilution at 72 h, which included 24 h of incubation at 37°C, in the *M. mycoides*/serum mixture.

There was a 50% loss of viability of organisms in all dilutions of the broth culture when it was diluted with inactivated plasma mixed with blood cells and tryptose broth. At 48 h, however, there was a 100% increase of organisms in the dilutions of 10^{-1} to 10^{-3} but there was none in the remaining dilutions, either in cells + broth or cells + plasma mixtures.

Scintillation counts showed that a 10% uptake of tritiated thymidine occurred with the growth of organisms from 10^7 to 10^9/ml and autoradiography confirmed that the T_1 strain of *M. mycoides* was labelled. Fig. 1 shows an autoradiograph of *M. mycoides* labelled with tritiated thymidine and stained with Giemsa, as seen microscopically with transmitted light.

TABLE II
MEAN CULTURE PLATE RECOVERIES OF THE T_1 VACCINE STRAIN OF *M. mycoides* AFTER DILUTION IN BLOOD OF CBPP-SUSCEPTIBLE CATTLE OR BROTH

Time lapse (h)	Diluent	Dilution factor											
		10^0	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	10^{-11}
1	Broth	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	NCFU	NCFU	NCFU
1	Blood	NT	+ve	+ve	+ve	+ve	+ve	NCFU	NCFU	NCFU	NCFU	NCFU	NCFU
24	Blood	NT	+ve	+ve	+ve	+ve	+ve	+ve	+ve	NCFU	NCFU	NCFU	NCFU
24	Broth	NT	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	NCFU	NCFU	NCFU

NCFU—No colony forming units found. NT—Not tested.

tail, other lymph nodes separately identified in Table 1, lung lobes, trachea, turbinate, liver, spleen, kidney and the tail-tip (site of inoculation). One g. of each tissue was weighed, inoculated into 9 ml. of the isolation medium of Davies and Read (1968) and titrated. The last tube showing growth was noted. Subcultures of the tissues were

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TABLE III

GROWTH DILUTION TITRES OF THE T_1 VACCINE STRAIN OF *M. mycoides* AFTER DILUTION IN BLOOD OF CBPP-SUSCEPTIBLE CATTLE OR BROTH

Time lapse (h)	Diluent	Dilution factor											
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	10^{-11}	10^{-12}
1	Broth	$10^{9\cdot3}$	$10^{9\cdot3}$	$10^{7\cdot7}$	$10^{7\cdot0}$	$10^{5\cdot3}$	$10^{4\cdot3}$	$10^{4\cdot0}$	$10^{3\cdot7}$	$10^{2\cdot0}$	$10^{1\cdot0}$	—	—
"	Blood	$10^{6\cdot0}$	$10^{6\cdot0}$	$10^{3\cdot3}$	$10^{3\cdot3}$	$10^{3\cdot0}$	—	—	—	—	—	—	—
24	Broth	$10^{9\cdot3}$	$10^{8\cdot3}$	$10^{7\cdot3}$	$10^{6\cdot3}$	$10^{5\cdot7}$	$10^{4\cdot7}$	$10^{3\cdot7}$	$10^{2\cdot0}$	$10^{2\cdot0}$	—	—	—
"	Blood	$10^{5\cdot3}$	$10^{3\cdot7}$	—	—	—	—	—	—	—	—	—	—
48	Broth	$10^{10\cdot0}$	$10^{9\cdot0}$	$10^{8\cdot7}$	$10^{8\cdot3}$	$10^{8\cdot0}$	$10^{5\cdot0}$	$10^{4\cdot7}$	$10^{3\cdot3}$	$10^{4\cdot0}$	$10^{3\cdot0}$	—	—
"	Blood	$10^{9\cdot7}$	$10^{4\cdot5}$	—	—	—	—	—	—	—	—	—	—
72	Broth	$10^{9\cdot7}$	$10^{9\cdot4}$	$10^{9\cdot0}$	$10^{9\cdot0}$	$10^{9\cdot0}$	$10^{8\cdot3}$	$10^{9\cdot7}$	$10^{9\cdot0}$	$10^{10\cdot5}$	$10^{9\cdot3}$	—	—
"	Blood	$10^{5\cdot3}$	$10^{5\cdot6}$	—	—	—	—	—	—	—	—	—	—
96	Broth	$10^{8\cdot7}$	$10^{9\cdot5}$	$10^{8\cdot3}$	$10^{9\cdot0}$	$10^{9\cdot0}$	$10^{9\cdot7}$	$10^{9\cdot6}$	$10^{10\cdot5}$	$10^{9\cdot3}$	$10^{9\cdot3}$	—	—
"	Blood	$10^{9\cdot0}$	$10^{4\cdot0}$	—	—	—	—	—	—	—	—	—	—

Mean dilution titre recorded by 50% endpoint of Reed & Muench (1938).
— = No detection of *M. mycoides*.

Neutrophils showed autoradiographic blackening which was caused by phagocytosed *M. mycoides* labelled with tritiated thymidine. The neutrophil in Fig. 2 was found in a smear prepared after 7 h contact of the blood with the organisms. Phagocytosis of the T_1 strain by leucocytes was observed from approximately 30 min to 24 h (final sample). In many cases part of the clump of *M. mycoides* could be seen adjacent to the leucocyte cell membrane and the remainder appeared to be within the cell. Presumably the clump of *M. mycoides*, in Fig. 1, was in the process of being phagocytosed. Neutrophils showed evidence of degranulation. Lymphocytes (Fig. 3), with ingested mycoplasma, were not observed as frequently as were neutrophils. Leucocytes were seen on the periphery of clumps of *M. mycoides* within 30 min.

However, this could have been an artifact produced in preparing the blood smears. Five to 7 h later, however, clumps of *M. mycoides* were seen where the white blood cells had migrated into the central areas of the clumps of organisms and it is unlikely that this could have been the result of making the smears. Examination of the paraffin sections prepared from the gelatin embedded blood/organism mixture, showed a similar picture to the smears. Numbers of leucocytes were visible on the edge and within clumps of organisms. No evidence of autoradiographic blackening was observed in control smears of blood containing unlabelled organisms, which were prepared simultaneously.

DISCUSSION

A marked decrease in number of viable organisms was clearly demonstrated *in vitro* when the T_1 broth culture vaccine was mixed with normal bovine blood, plasma and serum. There was no decrease in viable counts of the organism when it was diluted in broth, and only a small loss of viability, after 48 h, when diluted in inactivated serum or inactivated plasma. Thus the decrease in viable counts in plasma and serum was due to a heat labile inhibitory factor present in plasma and serum, probably complement. This finding is in agreement with that of Priestley (1952) who found that plasma and serum were bactericidal to mycoplasma.

tail, other lymph nodes separately identified in Table 1, lung lobes, trachea, turbinate, liver, spleen, kidney and the tail-tip (site of inoculation). One g. of each tissue was weighed, inoculated into 9 ml. of the isolation medium of Davies and Read (1968) and titrated. The last tube showing growth was noted. Subcultures of the tissues were

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TABLE IV

CULTURE PLATE TITRES OF THE T_1 VACCINE STRAIN OF *M. mycoides* AFTER DILUTION IN SERUM OR PLASMA FROM CBPP-SUSCEPTIBLE CATTLE OR BROTH

Time lapse (h)	Diluent	Dilution factor 10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
1	Broth	$2{\cdot}8\times10^{8}$	$2{\cdot}7\times10^{7}$	$3{\cdot}4\times10^{6}$	$5{\cdot}8\times10^{5}$	$3{\cdot}3\times10^{4}$	$3{\cdot}9\times10^{3}$	$2{\cdot}3\times10^{2}$	—
„	Plasma non-inact.	$2{\cdot}7\times10^{4}$	—	—	—	—	—	—	—
„	Plasma 56°C	$2{\cdot}2\times10^{8}$	$1{\cdot}6\times10^{7}$	$2{\cdot}6\times10^{6}$	$7{\cdot}8\times10^{4}$	$2{\cdot}6\times10^{3}$	$1{\cdot}1\times10^{3}$	$1{\cdot}5\times10^{2}$	—
„	Serum non-inact.	$3{\cdot}5\times10^{3}$	—	—	—	—	—	—	—
„	Serum 56°C	$1{\cdot}6\times10^{8}$	$2{\cdot}8\times10^{7}$	$3{\cdot}9\times10^{6}$	$2{\cdot}3\times10^{5}$	$3{\cdot}0\times10^{4}$	$2{\cdot}7\times10^{3}$	$2{\cdot}0\times10^{2}$	—
24	Broth	$5{\cdot}6\times10^{7}$	$5{\cdot}1\times10^{7}$	$5{\cdot}0\times10^{7}$	$5{\cdot}1\times10^{6}$	$1{\cdot}4\times10^{5}$	$4{\cdot}7\times10^{3}$	$1{\cdot}0\times10^{3}$	$5{\cdot}0\times10^{1}$
„	Plasma non-inact.	$3{\cdot}5\times10^{4}$	—	—	—	—	—	—	—
„	Plasma 56°C	$3{\cdot}1\times10^{8}$	$2{\cdot}8\times10^{7}$	$2{\cdot}5\times10^{6}$	$4{\cdot}2\times10^{4}$	$1{\cdot}3\times10^{4}$	$2{\cdot}0\times10^{3}$	$3{\cdot}2\times10^{2}$	—
„	Serum non-inact.	$3{\cdot}3\times10^{4}$	—	—	—	—	—	—	—
„	Serum 56°C	$3{\cdot}2\times10^{8}$	$3{\cdot}9\times10^{7}$	$2{\cdot}1\times10^{6}$	$3{\cdot}0\times10^{5}$	$2{\cdot}4\times10^{4}$	$3{\cdot}1\times10^{3}$	$4{\cdot}0\times10^{2}$	—
48	Broth	$4{\cdot}2\times10^{8}$	$2{\cdot}9\times10^{8}$	$3{\cdot}0\times10^{8}$	$2{\cdot}3\times10^{8}$	$2{\cdot}5\times10^{6}$	$2{\cdot}8\times10^{4}$	$5{\cdot}5\times10^{3}$	$1{\cdot}0\times10^{2}$
„	Plasma non-inact.	—	$2{\cdot}9\times10^{4}$	—	—	—	—	—	—
„	Plasma 56°C	$1{\cdot}9\times10^{8}$	$1{\cdot}4\times10^{7}$	$1{\cdot}4\times10^{6}$	$1{\cdot}5\times10^{5}$	$1{\cdot}4\times10^{4}$	$2{\cdot}7\times10^{3}$	—	—
„	Serum non-inact.	$5{\cdot}0\times10^{5}$	—	—	—	—	—	—	—
„	Serum 56°C	$3{\cdot}8\times10^{8}$	$3{\cdot}0\times10^{7}$	$2{\cdot}2\times10^{6}$	$3{\cdot}3\times10^{5}$	$2{\cdot}9\times10^{4}$	$2{\cdot}3\times10^{3}$	$4{\cdot}0\times10^{2}$	—
72	Broth	$2{\cdot}4\times10^{8}$	$2{\cdot}0\times10^{8}$	$3{\cdot}3\times10^{8}$	$3{\cdot}4\times10^{8}$	Too many to count		—	—
„	Plasma non-inact.	$1{\cdot}5\times10^{8}$	—	—	—	—	—	—	—
„	Plasma	$4{\cdot}9\times10^{8}$	$7{\cdot}4\times10^{7}$	$1{\cdot}2\times10^{7}$	$3{\cdot}7\times10^{6}$	$8{\cdot}6\times10^{3}$	$8{\cdot}0\times10^{3}$	—	—
„	Serum non-inact.	—	—	—	—	—	—	—	—
„	Serum 56°C	$5{\cdot}8\times10^{8}$	$4{\cdot}7\times10^{7}$	$3{\cdot}0\times10^{6}$	$2{\cdot}1\times10^{5}$	$2{\cdot}2\times10^{4}$	$2{\cdot}3\times10^{3}$	—	—

Mean titre recorded as CFU/ml.
— = No detection of *M. mycoides*

The examination of stained blood films and autoradiographic techniques suggested that part of the decrease of titre in whole blood was due to the phagocytosis of organisms by leucocytes, apart from the complement effect. There was also a marked increase in the number of organisms in the mixed broth culture and blood after 48 h incubation. This may have been the multiplication of organisms which had not been phagocytosed or killed by the complement. However, Davies (1969b) and Windsor (personal communication) have shown that in broth culture, the doubling time of the T_1 strain of *M. mycoides* is between 2 and 5 h depending on the size of the initial inoculum of the organisms, and that the logarithmic phase of growth is complete in 48 h. If the rate of increase of the number of organisms in blood/broth mixture was similar to that in broth alone, it might be expected that the rise in titre, if due only to this process, would have been observed much earlier. The length of time before such increase occurred, and in particular the rapidity of its appearance strongly suggest

tail, other lymph nodes separately identified in Table 1, lung lobes, trachea, turbinate, liver, spleen, kidney and the tail-tip (site of inoculation). One g. of each tissue was weighed, inoculated into 9 ml. of the isolation medium of Davies and Read (1968) and titrated. The last tube showing growth was noted. Subcultures of the tissues were

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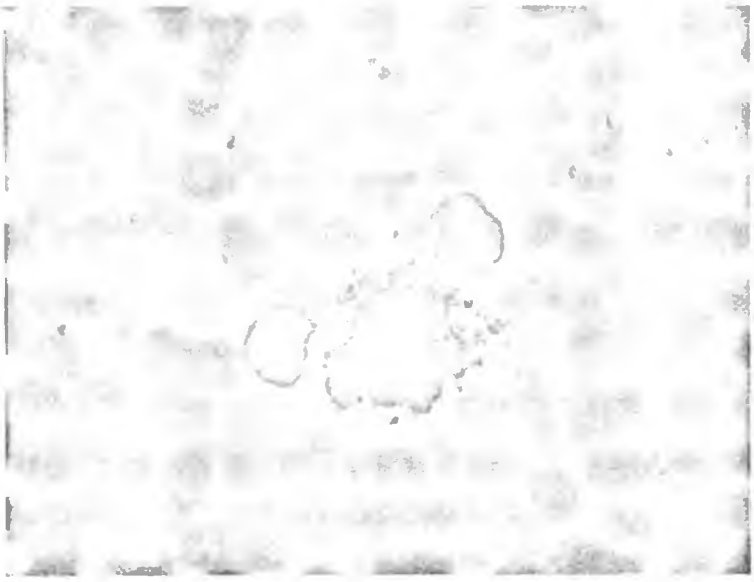

FIG. 1. An autoradiograph of *M. mycoides* labelled with tritiated thymidine and stained with Giemsa.

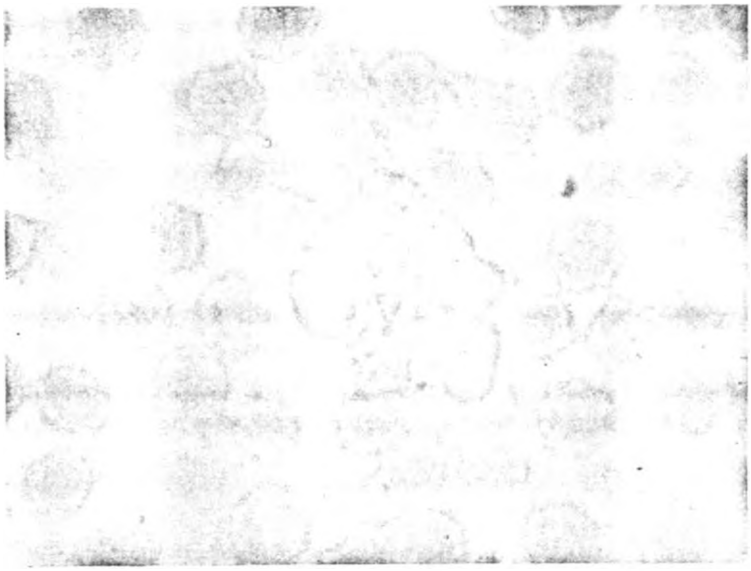

FIG. 2. A neutrophil, with phagocytosed *M. mycoides* labelled with tritiated thymidine.

tail, other lymph nodes separately identified in Table 1, lung lobes, trachea, turbinate, liver, spleen, kidney and the tail-tip (site of inoculation). One g. of each tissue was weighed, inoculated into 9 ml. of the isolation medium of Davies and Read (1968) and titrated. The last tube showing growth was noted. Subcultures of the tissues were

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FIG. 3. A lymphocyte with phagocytosed *M. mycoides* labelled with tritiated thymidine.

tail, other lymph nodes separately identified in Table 1, lung lobes, trachea, turbinate, liver, spleen, kidney and the tail-tip (site of inoculation). One g. of each tissue was weighed, inoculated into 9 ml. of the isolation medium of Davies and Read (1968) and titrated. The last tube showing growth was noted. Subcultures of the tissues were

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that large numbers of viable organisms were at that time released from the leucocytes, presumably following their disruption. This increase in titre was not observed during the course of the experiment when *M. mycoides* was mixed with either non-inactivated plasma or serum which suggests that the increase in titre in the blood/T_1 mixture after 48 h of incubation was due to the release of the organisms from the phagocytes. Presumably phagocytosis protects the organisms from the effects of the complement and indeed by 48 h the level of the complement in serum left at room temperature was found to be very low (Kakoma & Masiga, unpublished data) and this may be the reason why the titre remained high after it increased at 48 h.

The reduction in numbers of *M. mycoides* when they were mixed with either blood cells — tryptose broth or blood cells — inactivated plasma was not as marked as that observed when *M. mycoides* was mixed with whole blood. This difference may have been due to insufficient complement being available, in the former two mixtures, for the enhancement of phagocytosis. An alternative explanation could be that many leucocytes may have been killed during the process of separating the cells from the plasma.

Wilson (1953) has reported that the release of viable bacteria from phagocytes may occur in certain circumstances. Lloyd (1966) has also reported that *M. mycoides* contains a toxin that causes necrosis of tissues. This necrosis has been observed when cultures of pathogenic strains of *M. mycoides* come in contact with bovine leucocytes (Provost, 1964). It is possible that some of the ingested *M. mycoides* produce a toxin which subsequently kills the phagocytes and this leads to the release of viable organisms.

The isolation of *M. mycoides* from the lymph nodes of cattle for periods ranging from 14 days to 3 months after infection has been recorded by Hudson (1965), Davies (1969a), Lindley & Pedersen (1968) and Masiga (unpublished data). However, the recovery of organisms from the blood appears to be possible for a relatively short period after inoculation; for example, Turner *et al.* (1961) found a bacteraemia which appeared 5 min after vaccination of cattle with the V_5 strain of *M. mycoides* and which persisted for only 2 h. Similarly, in the present *in vitro* experiment, no bacteraemia was detected later than 50 h after vaccination. A notable difference between the findings of Turner *et al.* (1961) and the results of the present experiment was the intermittent nature of the bacteraemia demonstrated by the latter in 4 of 6 animals in which bacteraemia was demonstrated. No explanation can at present be offered for this difference, but it should be noted that Turner *et al.* (1961) used the V_5 strain of *M. mycoides* and not the T_1 strain and that they used the tail tip route of vaccination.

The marked rise in the titre of the mixture of blood and T_1 broth cultures occurred after 48 h incubation, whereas an increase in the number of organisms circulating in the blood of cattle was first indicated by the bacteraemia detected 8 h after vaccination. Despite the considerable difference between these intervals, the observable fate of the organisms *in vitro* offers a possible explanation for the intermittent bacteraemia which was shown by 4 of the 6 animals in which bacteraemia was detected.

Many factors play a part in governing the number of mycoplasma organisms in the blood at any one time after vaccination; among these will be the rate of entry of injected mycoplasma into blood or lymph channels; the extent to which they may be phagocytosed by leucocytes, both at the site of vaccination and in the blood by the circulating macrophages; the bactericidal activity of the complement; and the rate of multiplication of organisms in blood and other tissues. With all these factors involved it is probable that the numbers of circulating mycoplasma will vary considerably in the period following their injection; and this may account for the fact that they were detected only at irregular intervals. The ingestion and subsequent release of mycoplasma by leucocytes *in vitro* suggests that the intermittent bacteraemia which occurred *in vivo* may have been due to the release of large numbers of organisms into the blood by the disruption of leucocytes due to the toxic activity of *M. mycoides*.

tail, other lymph nodes separately identified in Table 1, lung lobes, trachea, turbinate, liver, spleen, kidney and the tail-tip (site of inoculation). One g. of each tissue was weighed, inoculated into 9 ml. of the isolation medium of Davies and Read (1968) and titrated. The last tube showing growth was noted. Subcultures of the tissues were

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FATE OF THE T_1 STRAIN OF *MYCOPLASMA MYCOIDES* IN CATTLE FOLLOWING VACCINATION

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INTRODUCTION

Cattle vaccinated with the T_1 broth culture vaccine of *Mycoplasma mycoides* var. *mycoides* (*M. mycoides*) have been found, on challenge, to be solidly immune to contagious bovine pleuropneumonia (CBPP) (Davies, Masiga, Shifrine and Read, 1968; Gilbert, Davies, Read and Turner, 1969; Windsor, Masiga and Read, 1972; and Masiga and Read, 1972). However, the immune mechanisms involved remain unknown (Gilbert and Windsor, 1971). Lloyd (1967) failed to transfer immunity by injecting susceptible cattle with bovine immune sera prior to subcutaneous challenge, and therefore suggested that the immunity involved in CBPP was probably of a cellular type. Lindley and Pedersen (1967), using the KH_3J strain of *M. mycoides*, isolated the organism and detected its antigens in local lymph nodes 2 months after primary vaccination. Davies (1969) recovered *M. mycoides* from normal cattle 14 days after vaccination with the T_1 broth culture and from stressed cattle 42 days after vaccination.

This paper reports a further investigation of the persistence of *M. mycoides* organisms and associated antigens, and the development of antibodies, in cattle vaccinated with the T_1 broth culture vaccine.

MATERIALS AND METHODS

Vaccine. The T_1 broth culture vaccine (Brown, Gourlay and MacLeod, 1965) was used to vaccinate animals by the tail-tip route. The conventional dose employed in East Africa (0·5 ml. of a culture containing approximately 2×10^9 organisms per ml.) was used.

Cattle. Ten cattle were obtained from an area of Kenya known to be free from CBPP. They were 1 to 2 years of age and of 150 to 200 kg. body weight.

Twenty ml. of blood were obtained by venepuncture twice daily and transferred immediately into 80 ml. of the "isolation medium" of Davies and Read (1969). Blood collected at the same time was allowed to clot and serum was separated by centrifugation. Six animals were killed 1, 2, 3, 71, 116 and 204 days, respectively, after primary vaccination. The remaining 4 animals were killed at various stages during the course of complement-fixing (CF) antibody development, namely, at the onset, peak and decline of CF activity, and after 2 weeks when the CF antibody could no longer be detected.

The tissues recovered from each animal were regional lymph nodes draining the tail, other lymph nodes separately identified in Table 1, lung lobes, trachea, turbinate, liver, spleen, kidney and the tail-tip (site of inoculation). One g. of each tissue was weighed, inoculated into 9 ml. of the isolation medium of Davies and Read (1968) and titrated. The last tube showing growth was noted. Subcultures of the tissues were

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carried out in Newing's tryptose broth (Gourlay, 1964). Mycoplasma isolated were identified and confirmed as *M. mycoides* by the growth inhibition test (Davies and Read, 1968). All tissues were subjected to the agar gel diffusion test (AGT) described by Masiga and Stone (1969). The tests allow for the specific detection of galactan as well as other *M. mycoides* antigens.

Serology. The complement fixation test (CFT) described by Campbell and Turner (1936) and the slide agglutination serum test (SAST) of Gourlay (1964) were carried out on all sera.

RESULTS

Tissues

Table 1 shows the tissues from which *M. mycoides* was isolated. Organisms were recovered from the tail-tip of all the animals killed during the 24 days following primary vaccination, but could not be detected at the site of inoculation in those killed from the 36th day onward. The only other tissues from which *M. mycoides* was isolated were lymph nodes of the iliac, inguinal and sacral regions, which drain the site of vaccination. These yielded organisms up to 71 days after vaccination. *M. mycoides* was not recovered from the blood of animals vaccinated by the tail-tip route.

The tissues in which precipitating antigens were detected by the AGT are also shown in Table 1. Antigens were first detected in the liver and the right iliac lymph node, 72 h. after vaccination. *M. mycoides* or related antigens had a wider distribution in the body tissues of animals killed from the 9th day onward. Antigens were detected periodically in the kidney, spleen and lung, as well as in lymph nodes draining regions other than the tail. In none of the animals were antigens detected in the upper respiratory tract. Galactan was detected in kidney and liver (4), spleen kidney and liver (5), and liver, left cardiac lung lobe and regional lymph nodes (7); these 3 animals were killed 9, 11, and 36 days after vaccination, respectively. Galactan was more widely distributed in the two animals (Nos. 9 and 10) killed 116 and 204 days after primary vaccination: the sites which proved positive in either one or both were the left cardiac and left diaphragmatic lobes of the lungs, the kidney, liver and spleen, the anterior mediastinal, right bronchial, left popliteal, left prefemoral, right medial sacral, right and left submandibular and left parotid lymph nodes. Antibody to *M. mycoides* was detected, in the left diaphragmatic lung lobe and the right prescapular lymph node, only in No. 10 killed 204 days after primary inoculation.

Serology

The results (Table 2) show the serological response of cattle vaccinated with T_1 broth culture vaccine. The earliest CF antibody was recorded after 5 days in No. 9. Animals 1, 2 and 3 were killed before the onset of CF antibody. Three other animals (4, 5 and 6) were killed during the onset, height and decline of the CFT response, and hence the duration of CF antibody could not be determined. Of the remaining 4 animals, 2 had CFT titres for 23 days, 1 for 12 days and the remaining animal for 10 days (see Table 2). All the animals that lived beyond 72 h. showed a CFT titre of more than 160, the highest titre

TABLE I

ISOLATION OF *M. MYCOIDES* AND DETECTION OF MYCOPLASMA ANTIGENS IN TISSUES OF CATTLE VACCINATED WITH T_1 STRAIN OF *M. MYCOIDES* BY THE TAIL TIP ROUTE

No. of animal	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*
Days killed after vaccination	*1*	*2*	*3*	*9*	*11*	*24*	*36*	*71*	*116*	*204*
Tissue										
L.C.	—	—	—	—	—	—	Ag	—	Ag	Ag
L.D.	—	—	—	—	—	—	—	—	Ag	Ab^{Ag}
Kidney	—	—	—	Ag	Ag	—	—	—	Ag	Ag
Liver	—	—	Ag	Ag	Ag	Ag	Ag	—	Ag	—
Spleen	—	—	—	—	Ag	—	—	—	Ag	Ag
Post. med. L.N.	—	—	—	—	—	Ag	Ag	—	—	—
Ant. med. L.N.	—	—	—	—	WAg	Ag	Ag	WAg	—	Ag
L. medial sac. L.N.	$+(10^3)$	—	—	$+(10^6)$	$+(10^2)$Ag	Ag	$+(10^7)$	—	—	—
R. medial sac. L.N.	+	—	—	—	$+(10^2)$	—	$+(10^7)$Ag	+Ag	Ag	—
L. submand. L.N.	—	—	—	WAg	—	—	—	WAg	Ag	—
R. submand. L.N.	—	—	—	—	WAg	—	Ag	—	Ag	Ag
L. Parotid L.N.	—	—	—	—	—	—	WAg	—	Ag	Ag
L. prescap. L.N.	—	—	—	—	—	—	—	—	Ag	Ag
R. prescap. L.N.	—	—	—	—	—	—	—	Ag	Ag	Ab^{Ag}
L. prefem. L.N.	—	—	—	—	WAg	—	—	—	Ag	Ag
R. prefem. L.N.	—	—	—	—	Ag	—	—	—	—	—
L. lateral sac. L.N.	—	—	—	+Ag	—	Ag	—	—	—	—
R. lateral sac. L.N.	+	—	—	(10^4)	—	—	—	—	—	—
L. sup. ing. L.N.	—	—	—	—	Ag	Ag	$+(10^2)$	WAg	—	—
R. sup. ing. L.N.	—	—	—	—	Ag	—	$+(10^6)$	—	—	—
L. deep. ing. L.N.	—	—	—	—	—	WAg	+	—	WAg	Ag
R. deep. ing. L.N.	—	—	—	—	WAg	$+10^0$	Ag	—	—	—
L. bronchial L.N.	—	—	—	—	WAg	—	—	—	—	—
R. bronchial L.N.	—	—	—	—	WAg	—	—	Ag	WAg	Ag
L. popliteal L.N.	—	—	—	—	—	—	—	—	WAg	Ag
R. popliteal L.N.	—	—	—	—	Ag	—	—	—	—	—
L. int. iliac. L.N.	—	—	$+(10^2)$	—	—	Ag	—	—	—	—
R. int. iliac. L.N.	$+(10^2)$	—	+Ag	Ag	+Ag	$+(10^5)$Ag	$+(10^5)$Ag	—	—	—
Tail	$+(10^5)$	$+(10^1)$	$+(10^6)$	$+(10^2)$	$+(10^7)$	+	—	—	—	—

Key: Ag = Antigen detected in AGT
Ab = Antibody detected in AGT
WAg = Weak antigen detected in AGT
+ = *M. mycoides* isolated from tissues+approximate titres
(10^2) etc. = approximate titres
L.C. = Left cardiac lung lobe
L.D. = Left diaphragmatic lung lobe
L.N. = Lymph node
SUP. ING. = Superficial inguinal

Seven other tissues (turbinate, trachae, left and right apical, right cardiac, right diaphragmatic lung lobes and right parotid lymph node) were examined and found negative.

being 2560 (9). After the onset of CF antibodies the development of the antibody titres was rapid: No. 5 had a negative titre in the morning, a titre of 20 by the evening and 80 the following day; and No. 9 had a titre of 40 in the morning which rose to 160 in the evening. The evening CF titres were doubled in the majority of the animals.

TABLE 2

THE SEROLOGICAL RESPONSE OF CATTLE VACCINATED WITH THE T_1 STRAIN

	Complement fixation test				*Slide agglutination serum test*		
Animal number	*First appearance of antibody (days)*	*Peak titre (reciprocal)*	*Day on which peak titre attained*	*Disappearance of antibody (days)*	*First appearance of antibody (days)*	*Peak (days)*	*Disappearance of antibody (days)*
1	K	K	K	K	K	K	K
2	K	K	K	K	K	K	K
3	K	K	K	K	K	K	K
4	7	160	8	K	7	9	K
5	7	320	9	K	6	7	K
6	7	1280*	16	K	7	8	K
7	6	1280	14	23	7	8	18
8	8	160	14	10	7	9	21
9	5	2560	9	23	5	7	24
10	13	320	16	12	11	19	16

* Reciprocal of CF titre. K = Killed before parameter could be measured.

The development of the agglutinating antibody is also recorded in Table 2. All the animals had antibody which was detected by the 5th day after primary vaccination and persisted up to 24 days in No. 9.

DISCUSSION

M. mycoides was recovered from the regional lymph nodes of all except 3 of the animals vaccinated with T_1 broth culture vaccine.

Whereas *M. mycoides* was recovered from the tails in each of 6 animals and the regional lymph nodes of 7 after primary vaccination, it was not recovered from any other tissue examined. This finding is similar to that of Lindley and Pedersen (1968) who, following the s.c. inoculation at the side of the neck, were unable to recover the KH_3J strain from sites other than the regional lymph nodes. Davies (1969), however, recovered *M. mycoides* from the trachea of one of 24 animals vaccinated at the tail tip with the T_1 strain of *M. mycoides*.

Whereas Lindley and Pedersen (1968) suggested that *M. mycoides* antigens persisted in the tissues of vaccinated animals for as long as the organism, the results of the present experiment indicate that the antigens can persist in animal tissues over periods when the organism is no longer detectable.

Davies (1969) recovered the T_1 strain from regional lymph nodes draining

the tail of non-stressed and stressed animals 14 and 42 days respectively after primary vaccination. The present findings showed that this strain persisted in animal tissues for longer periods. Davies (1969) concluded that because *M. mycoides* was not recovered from any tissues other than the regional lymph nodes draining the tail, the immunological message was transported from the local lymph nodes to other parts of the body. It is interesting to note, however, that in the present trials *M. mycoides* antigens or related antigens became widespread throughout the body within 10 days of vaccination. It is known that common antigens exist between *M. mycoides* and bovine tissues (Shifrine and Gourlay, 1965). The antigens detected in various tissues of the vaccinated animals might have been "altered bovine tissue antigens". It has been suggested by Weissman, Keiser and Bernheimer (1963) that in auto-immune diseases, such as rheumatoid arthritis, degrading enzymes released from the subcellular particles may denature native body tissues which then become auto-antigens. This hypothesis was further supported by Hirshorn, Schreibman, Verbo and Grunskin (1964), who advanced the theory that the release of lytic enzymes alters native proteins which are no longer recognized as "self". Kakoma, Masiga and Windsor (1972) have suggested that CBPP is an autoimmune disease. If this is the case, then some of the antigens observed in these experiments have been "altered host tissue antigens".

At 204 days both *M. mycoides* galactan or related host antigens, and antibody were detected by the AGT in the diaphragmatic lobe and the right prescapular lymph node of animal 10. The detection of galactan or related antigens 204 days after primary vaccination seems to be significant. Gourlay (1965) demonstrated that galactan could be excreted in urine. It would be expected that most, if not all, the galactan from the original organism in the vaccine dose, approximately 10^8 organisms, would be excreted by 204 days. It is, therefore, reasonable to suggest that the galactan which was detected at this time had probably been either tissue bound, or that its nature had been changed to resemble galactan of the host, or vice versa.

Lindley and Pedersen (1968) found no evidence of multiplication of the KH_3J strain in animal tissues following vaccination. In the present experiments the T_1 strain persisted in one tissue up to 71 days and at 36 days the organism was isolated from 5 tissues. Although the findings in these experiments did not suggest that the organism increased, it would be difficult to explain how it could persist in tissues for 71 days without multiplication. It is reasonable to suggest that to stimulate immunity against CBPP, the organism must lodge in the animals tissues for a period and also that it must multiply in the tissues. This may be the reason why dead vaccines against CBPP are thought by many to be useless. Gilbert and Windsor (1971) suggested that animals which had been vaccinated with 10^5 organisms were more susceptible to CBPP than the control animals. It appears, therefore, that to elicit immunity against CBPP a minimum number of organisms is required. If less than this number of organisms is inoculated into animals then they will be sensitized rather than immunized. Masiga and Boarer (1973) demonstrated in vitro that phagocytosis occurs with the T_1 strain and that complement has a bactericidal action on the organism. It seems probable that when animals are vaccinated a large number of the

organisms are destroyed by the body defence mechanisms. If the vaccine dose is below a certain minimum number, suggested by Gilbert and Windsor (1971) as 10^7, then insufficient numbers survive to stimulate immunity and instead hypersensitivity results.

Provost, Perreau and Queval (1963) found that lysed *M. mycoides* when used as a vaccine were not efficacious against CBPP, and Shifrine and Beach (1968) described experiments in which dead vaccines did not protect cattle against CBPP. It is, therefore, difficult to explain what part in immunity against CBPP the persisting antigens play. It is reasonable, however, to suggest that *M. mycoides* vaccine stimulates the immune mechanism, but that the persisting antigens maintain the immunity at a local level. The antigens become wide spread within 10 days of vaccination. As Gilbert (personal communication) also found galactan in the skin of vaccinated and infected animals, it could play a role of maintaining immunity at a local level since it is wide spread in animals that have had experience of *M. mycoides*.

Lindley and Pedersen (1968) and Davies (1969) suggested that the duration of CF antibody coincided with the persistence of *M. mycoides* in vaccinated animals, but the results of our experiments show that this may not be necessarily true for the T_1 strain. The longest period over which the CF antibodies persisted was 23 days, but the organism was still recoverable after 71 days. The experience gained in this laboratory shows that CF antibody does not persist in the animal after primary vaccination with the T_1 strain for periods longer than 8 weeks. Hudson (1965) recovered the KH_3J strain 3 months after primary vaccination, and Lindley and Pedersen (1968) after 2 months. Thus *M. mycoides* seems to persist for much longer periods than the CF antibody, and the antigens persist for even longer periods. The results reported in this paper show that the appearance and duration of the agglutinating and CF antibodies coincided. All animals had agglutinating and CF antibodies within 5 days and both types of antibody persisted 24 and 23 days respectively. On the other hand the detection of antibody activity in the tissues of one animal 204 days after vaccination may have some significance. Lloyd (1967) who failed to immunize passively cattle susceptible to CBPP, using immune sera suggested that humoral antibodies do not play a part in the immunity of CBPP, but that it may be due to some local factor. Davies (1969) also suggested that the immunity in CBPP could be associated with cell bound antibodies.

Although bacteraemia has been demonstrated (Masiga and Boarer, 1972 ~~in press~~) after subcutaneous vaccination behind the shoulder of cattle with the T_1 strain no bacteraemia has been observed when the tail-tip route was used. Although the role played by bacteraemia in immunity against CBPP following vaccination with the T_1 strain is difficult to explain, animals which had been vaccinated either by the subcutaneous route behind the shoulder (Gilbert and Windsor, 1971) or by the tail-tip route (Davies *et al.*, 1968) were solidly immune on challenge. It appears, therefore, that bacteraemia is not essential for the initiation and the development of immunity against CBPP. Viable organisms are thought to be essential for the stimulation of immunity (Provost *et al.*, 1963, Shifrine and Beach, 1968), but the minimum period for which they must persist in animal tissues to elicit the immunity is a subject of another investigation.

SUMMARY

Mycoplasma mycoides var. mycoides (*M. mycoides*) was recovered from lymph nodes draining the tail region of cattle vaccinated with the T_1 strain of *M. mycoides* for up to 71 days. *M. mycoides* antigens were not detected until 3 days after vaccination using the agar gel diffusion test. They were wide spread in the animal tissues by the 11th day and were detected up to 204 days. The earliest time at which complement fixing (CF) and agglutinating antibodies were detected was 5 days. Both CF and agglutinating antibodies persisted up to 30 days following vaccination. It is suggested that viable organisms stimulate immunity against contagious bovine pleuropneumonia and the associated antigens maintain the immunity.

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