A COMPARISON OF ANTIGENIC PROTEINS IN DIFFERENT *ANAPLASMA* ISOLATES

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A Thesis submitted to the Faculty of Science,
University of the Witwatersrand, Johannesburg,
in fulfilment of the requirements for a
Masters degree.

Onderstepoort, 1994

ABSTRACT

Anaplasmosis is a tick transmitted, haemoparasitic disease of bovines, caused by *A. marginale* and *A. centrale*, which occurs worldwide and results in losses of significant economic importance. Bovines that survive infection are immune to reinfection with a homologous isolate and partially protected against heterologous isolates. South African isolates of *A. marginale* and *A. centrale* are compared.

The *A. marginale* Underberg isolate and *A. centrale* vary when compared by immunoprecipitation, but have some proteins in common. Rabbit antisera, made against *A. marginale* Underberg initial bodies and against separated *A. marginale* Underberg proteins, show distinct immunogenic differences among the isolates on immunoblots. The antisera produced show that the isolates contain proteins having varying molecular weights but which share the same epitopes.

Antisera collected from animals infected with *Anaplasma* showed that the Underberg and Barkly West *A. marginale* isolates cross-react more with each other on immunoblots than with *A. centrale*. The 105, 57 and 40kDa proteins are common between the three isolates. Several proteins differ between the isolates and it is therefore important to take into account these factors when developing improved vaccines.

DECLARATION

I declare that this dissertation is my own, unaided work. It is being submitted for the degree of Masters of Science in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.

Jeanni Fehrsen

11th day of January, 1994

Use of experimental animals in this thesis was approved by the University of Witwatersrand Animal Ethics Screening Committee

Clearance certificate no: 92/23/3

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ACKNOWLEDGEMENTS

I wish to thank my supervisors Dr B A Allsopp and Prof J Alexander for their guidance and valuable criticism throughout this project. Also to Dr E S Visser for her help with the final manuscript. Sincere thanks to Ollie Matthee from the Protozoology Section, Onderstepoort for the handling of the animals. I would also like to thank my colleagues at the Molecular Biology and Protozoology Sections for their advice and assistance and my friends and family for their support.

LIST OF ABBREVIATIONS

A	ampere
APS	ammonium peroxydisulphate
BSA	bovine serum albumin
°C	degrees Celsius
CF	complement fixation
DNA	deoxyribonucleic acid
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
IEF	isoelectric focusing
IFA	immunofluorescent antibody
Ig	immunoglobulin
kDa	kilodalton
M	molar
MHC	major histocompatibility complex
ml	millilitre
mM	millimolar
mm	millimetre
mg	milligram
nm	nanometre
nM	nanomolar
NP-40	Nonidet-P40
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCV	packed cell volume
pI	isoelectric point
PMFS	phenylmethylsulfonyl fluoride
RBC	erythrocyte
SAC	*Staphylococcus aureus* Cowan I
SDS	sodium dodecyl sulphate
T cell	T lymphocyte
Th cell	helper T lymphocyte
Tc cell	cytotoxic T lymphocyte

[1] kDa is used in this thesis to quote the molecular mass ratio of proteins to prevent the use of many zeros.

TCA	trichloroacetic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
TLCK	N-α-p-tosyl-L-lysyl-chloromethylketone
Tris	tris(hydroxymethyl)-aminomethane
v/v	volume to volume
w/v	weight to volume
g	force of gravity
µCi	micro Curie
µg	microgram
µl	microlitre
µm	micrometre

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CHAPTER ONE

LITERATURE REVIEW

1.1. Anaplasmosis - History and classification

Anaplasmosis is a haemoparasitic disease of cattle commonly known as gallsickness in South Africa. The infectious organism was originally discovered by Sir Arnold Theiler who, in the early years of this century, proved that the marginal points that he observed in the erythrocytes of bovines were the causative agents of gallsickness. He called the organism *Anaplasma marginale* (Theiler, 1910) and during his attempts to isolate pure samples of the infectious agent he isolated another organism that caused milder infections. The parasites were more centrally placed in the erythrocytes than those of *A. marginale* and he called this organism *Anaplasma centrale* (Theiler, 1911).

A. marginale is the representative species of the genus *Anaplasma*, family Anaplasmataceae, order Rickettsiales (Ristic, 1981). Recently the sequence of the 16S ribosomal RNA gene has been determined and has confirmed the relatedness of *A. marginale* to the *Rickettsia* and *Ehrlichia* genera (Weisburg *et al*, 1991).

1.2. Host range and spread

All organisms of the genus *Anaplasma* are obligate intracellular parasites, they infect a range of wild and domestic Bovidae in tropical and subtropical regions and result in losses of significant economic importance (Palmer & McGuire, 1984). In addition to causing infections in domestic stock the organism also infects water buffalo, bison, African antelopes, American deer and camels. Common laboratory animals (rabbits, guinea pigs, rats, mice) are not susceptible to *Anaplasma* (Ristic, 1981). In South Africa anaplasmosis is enzootic in most of the Transvaal and Natal and in the North-eastern Orange Free State and South-eastern Cape Province (Potgieter, 1979).

1.3. Transmission of the disease

Twenty tick species have been implicated in the transmission of *Anaplasma* worldwide (Stich *et al.*, 1989). *Boophilus decoloratus, B. microplus, Rhipicephalus simus, R. evertsi evertsi* and *Hyalomma marginatum rufipes* have been shown to transmit anaplasmosis in Southern Africa. In addition *A. marginale* colonies have been demonstrated in midgut material from *R. simus* (Potgieter *et al.*, 1983). All these tick species are widely distributed, facilitating the extensive dissemination of anaplasmosis in Southern Africa (Potgieter, 1979; Potgieter & Van Rensburg, 1987). In the USA, *Dermacentor* ticks are the main vectors and *Anaplasma* has been demonstrated in midgut material (Kocan *et al.*, 1982) and in the salivary glands where it is thought to replicate before transmission to the bovine host (Stiller *et al.*, 1989). *A. marginale* overwinters in *D. andersoni* (Logan *et al.*, 1987) and trans-stadial transmission occurs (Stich *et al.*, 1989).

Mechanical transmission may also result from the direct transfer of blood by biting flies or by blood-contaminated instruments (Ristic, 1981; Potgieter *et al.*, 1981). Trans-placental transmission is thought to be possible but a very high parasitaemia is needed, such as may be found in the course of an acute infection during gestation (Zaugg & Kuttler, 1984).

1.4. Life cycle

In the bovine host, *Anaplasma* invades the erythrocytes by invagination as a single entity, probably a single *Anaplasma* cell, termed the initial body, which is 0.2-0.5µm in diameter. The initial bodies then multiply by binary fission which results in an inclusion body, 0.6-0.9µm in diameter, incorporating up to eight initial bodies. The initial body is surrounded by a double membrane whereas the subunits of the inclusion body are contained in a single membrane (Simpson *et al.*, 1967). During an infection the lifetime of large inclusion bodies is short and it seems that they cause the destruction of erythrocytes containing them (Lotze & Yiengst, 1942). The organism exits the erythrocyte either by the reverse of invagination or after erythrocyte lysis (Simpson *et al.*, 1967).

Anaplasma has a complex life cycle in the tick vector to accomplish its transfer from a blood meal to the midgut epithelial cells (Friedhoff, 1990). Five stages in tick midgut epithelial cells have been identified from small (5.5µm), electron dense colonies to larger (10.5 µm) reticulated forms (Kocan *et al.*, 1982). The morphology of the colonies in the tick cells change as moulting progresses and the infectivity also differs between the stages, some stages are apparently non-infective (Kocan *et al.*, 1990).

Phase contrast and fluorescence microscopy have shown that the Oregon isolate contains an appendage which is lacking in other USA isolates. Adsorption studies with polyclonal antisera to the Oregon isolate indicates that the appendage bears antigens not present on the initial body itself (Palmer, 1989). It is proposed by Kocan (1986) that the isolates with appendages belong to another species, *Anaplasma caudatum*.

1.5. The disease

Infection with *Anaplasma* is followed by a prepatent period of 20 to 40 days during which no symptoms are evident. The length of this period is directly related to the amount of parasite material with which the animal was infected (Ristic, 1981). The subsequent patent period is characterised by erythrocyte invasion and haemolytic anaemia, during this phase erythrocytes are removed from the circulation by phagocytosis. Fever, weakness, constipation, icterus, loss of appetite, lowered milk production and even abortion or death can prevail during the acute phase of the infection (Palmer & McGuire, 1984; Palmer *et al.*, 1985).

Animals which survive the primary infection become carriers of a subclinical infection and they are then immune to reinfection with the homologous parasite. Heterologous challenges, however, may result in further infection (McGuire *et al.*, 1984). Parasitaemia and anaemia are generally mild in young calves but the reason for this is not known; promptness of the immune response and the presence of fetal antibodies have been suggested as possible explanations (Ristic, 1981).

Morris *et al.* (1971) found that the degree of anaemia is not proportional to the severity of the parasitaemia since uninfected erythrocytes are apparently sensitised to phagocytosis by macrophages. There is some indication that opsonins could be responsible for this phenomenon. Opsonins are factors that bind to cells and increase their susceptibility to phagocytosis (Herbert *et al.*, 1985). Opsonins eluted from the erythrocytes of bovines infected with *Anaplasma* were found to attach to uninfected bovine erythrocytes whereas this was not observed in eluates from uninfected bovines.

Erythrocyte membrane proteins may be altered during the course of *Anaplasma* infection. The extent to which bovine erythrocyte membrane carbohydrates are altered during infection was determined by comparing lectin binding to *Anaplasma*-infected erythrocytes and uninfected erythrocytes (Goff *et al.*, 1986). Increased binding was found with infected erythrocytes suggesting that the erythrocytes are altered to have more binding sites exposed. Nordelo and Ysern-Caldentey (1982) found that additional proteins were glycosylated and two new proteins appeared in the membrane fraction of infected erythrocytes. The two new proteins could be parasite proteins that are presented on the surface of the erythrocyte membrane

1.6. Treatment of anaplasmosis

Tetracyclines are the only drugs which are effective against anaplasmosis. These compounds retard and inhibit the cycle of development of the parasite and complete elimination of *Anaplasma* is possible if the drugs are administered early in infection. In latent infections the parasite can be eliminated by multiple treatments with the drugs (Ristic, 1981). Some *Anaplasma* isolates show drug tolerance and this makes it difficult to cure carrier animals of anaplasmosis completely, especially in the field (Kuttler, 1983). The only way to conclusively prove carrier status is to subinoculate blood into a susceptible animal, but this is time consuming and expensive and thus adds to the problems of eliminating *Anaplasma*. The development of DNA probes and PCR techniques could aid in the diagnosis of carriers (Trueblood *et al.*, 1991; Visser *et al.*, 1991; Eriks *et al.*, 1989; Goff *et al.*, 1988).

1.7. Immune response to *Anaplasma*

1.7.1. Humoral immune response

The transfer of serum from an animal immune to *Anaplasma* to a susceptible bovine does not confer protection against infection (Ristic, 1981). Erythrocytes are removed indiscriminately during anaplasmosis regardless of whether they are parasitised. It is thought that the fixed and free-serum erythrocyte autohaemagglutinins which appear in *Anaplasma*-infected bovines could be the effectors of the anaemia which occurs during anaplasmosis.

Allen *et al.* (1981) compared the serum protein response (serum albumin levels, α, β and γ globulin levels and white blood cell count) of cattle infected with a virulent *A. marginale* isolate and an attenuated *A. marginale* strain. The protein responses in the cattle infected with the virulent strain rose to higher levels than those in the cattle infected with the attenuated strain.

Murphy *et al.*, (1966) showed by chromatographic analysis and the complement fixation (CF) test that 30 days after peak parasitaemia the CF reactive immunoglobulins (Ig) comprised 25% IgG and 75% IgM. At peak parasitaemia the distribution was 92% IgM and 8% IgG. IgM is the first antibody produced after an immunogenic stimulus and plays a role in the activation of the complement system (Herbert *et al.*, 1985). Cyclic recurrences of erythrocyte invasion occur in *Anaplasma* carriers which are similar to plasmodial infections (Murphy *et al.*, 1966). The high level of IgM could be due to the persistence of low level parasitaemia which results in continual antigenic stimulus similar to the initial stimulus (Murphy *et al.*, 1966).

Corrier *et al.* (1981) suppressed the humoral immune response of *Anaplasma* carriers by treating with cyclophosphamide. The bovines developed clinical anaplasmosis and decreased levels of IgM, IgG and leucocytes and different immunofluorescent antibody (IFA) titres were detected compared to the carriers not treated with cyclophosphamide. According to Corrier *et al.* (1981) protective immunity to anaplasmosis is thought to be primarily cell-mediated, if humoral immunity does not have a protective role these results may indicate that the humoral response helps to maintain a state of equilibrium in the *Anaplasma*-host relationship. The low level of parasites in the carrier results in continual antigenic stimulus which protects the animal from clinical anaplasmosis.

Recently, Tebele *et al.* (1991) immunized bovines with the membrane fraction of the *A. marginale* Norton Zimbabwe strain and found protection against the homologous strain. There was a significant correlation between antibody titre and level of protection against anaemia. These findings could either indicate that the humoral immune response does have a protective role against clinical anaplasmosis or that the antibodies had a neutralizing effect by blocking infection.

Calves are more resistant to anaplasmosis than adult bovines and this could be attributed to the fact that IgG is transferred to calves via the colostrum (Murphy *et al.*, 1966). Zaugg and Kuttler (1984) claim that the presence of colostral antibodies against *Anaplasma* only delays the manifestation of infection, it does not provide long lasting protection.

1.7.2. Cell mediated immune response

Immunity to intracellular parasites is usually due to the cellular immune response (Carson *et al.*, 1976) and in *Anaplasma* immunity is associated with the continuous low-level presence of the parasite. Carson *et al.* (1976) propose that the antibodies detected by serological tests play a role in causing anaemia rather than in protection. Animals showing an early cell mediated response prior to the patent period of the disease have a greater chance of survival than animals with little or no evidence of a cell mediated response before the patent period (Ristic, 1981).

Table 1-1 compares aspects of the immune response of *Anaplasma* with that to *Theileria* and *Cowdria*, which are also blood parasites that cause disease in bovines. There seem to be close similarities which are probably the result of the protective mechanism being the same (Emery, 1981; Morrison *et al.*, 1987; Goddeeris *et al.*, 1986; Emery *et al.*, 1988; Stewart, 1987; Du Plessis *et al.*, 1991).

Table 1-1. Comparison of aspects of the bovine host immune response to *Anaplasma* with that to *Theileria* and *Cowdria*.

Characteristic	*A. marginale*	*T. parva*	*C. ruminantium*
Passive immunity transferable by antibody?	No	No	No
Immunity conferred by antibody against parasite proteins?	No	No	No
Immunity against homologous parasite produced by infection and treatment?	Yes	Yes	Yes
Parasite seen transiently during immunization?	Yes	Yes	Yes
Any immunity without infection?	Yes (killed vaccines)	No	No
Parasitized cells seen transiently when immune animals re-challenged?	Yes	Yes	Yes
Any immunity to heterologous parasites?	Variable	Very variable	Very variable
Immunity transferred with syngeneic immune T cells?	Not done	Yes, Tc	Yes, probably Tc
Immunity transferred with syngeneic immune Th cells?	Not done	Not done	No
Specific Tc cell killing of parasitized cells *in vitro*?	Not done	Yes	Not done
Monoclonal antibody against MHC blocks killing?	Not done	Yes	Not done
Specificity of immunity varies according to immunizing parasite?	Yes	Yes	Yes
Specificity of immunity varies according to MHC phenotype of host?	Not done	Yes	Yes

It appears that the anti-*cowdria* effector cells in mice are almost certainly Tc cells expressing Lyt2$^+$ and that the similarities with the *T. parva* immunity situation do not arise because of any similarity between the parasites but because of the similarity between the immune effector mechanisms. Comparable cellular immunity studies have not been performed on *Anaplasma*, but the fact that passive immunity is not transferred by antibodies and that infection is necessary for effective, long lasting immunity implies an important role for a cellular protective mechanism.

The role of the cellular immune response in cattle vaccinated with various *Anaplasma* vaccines was determined by Carson and Buening (1979) using the leucocyte migration inhibition test (LMIT). Animals inoculated with either virulent or attenuated strains showed a greater increase in LMIT than did animals which were given the inactivated vaccine, even though there was a degree of protection in the latter animals. The higher level of cell mediated immune response in the first two cases persisted but the lower level response resulting from the inactivated vaccine did not persist (Buening, 1973). The persistence of the cellular immunity could be due to the carrier status of the animals which is similar to findings with *Cowdria* vaccinated animals.

1.8. *Anaplasma ovis*

Anaplasma ovis occurs in sheep but bovines are not susceptible to this parasite. Sheep are slightly susceptible to *A. marginale* and could therefore be carriers of anaplasmosis (Ryff *et al.*, 1964). Recently Barry and Van Niekerk (1990) found that *A. ovis* causes a mild febrile disease in Boer goats and that infection with *A. ovis* can result in abortions if the goats are subjected to physical stress. The disease usually passes unnoticed in the field, however, and *A. ovis* is not of any great economic importance.

1.9. Anaplasmosis in the wild

The infection of bovines with *Anaplasma* isolated from wild animals usually results in mild reactions. This could pose a threat to cattle that are in close contact with wild animals (as is often the case in Africa) as some isolates may become virulent (Kuttler & Zaugg, 1988). It is generally accepted that antelopes are the natural reservoirs of *Anaplasma* in South Africa, infections having been found in blesbok, duiker and black wildebeest (Potgieter, 1979).

1.10. Control of the disease

Current anaplasmosis control measures include control of arthropod transmission, the use of antibiotic prophylaxis and the vaccination of susceptible cattle (Palmer, 1989). Control of arthropod transmission using acaricides is labour intensive, expensive and creates a highly susceptible cattle population. Perhaps the most important problem, however, is the appearance of acaricide-resistant ticks (Norval, 1983). Antibiotic prophylactic control leaves cattle vulnerable if the program is disrupted, it too is expensive and it is used only in the USA and even there only on a limited scale (Palmer, 1989).

Vaccination is the most efficient and economical way to protect cattle against anaplasmosis. The presence of post-infection immunity provides a strong basis for vaccine development. Theiler recognised the potential of *A. centrale*, a less virulent form of *A. marginale*, for vaccination against anaplasmosis (Theiler, 1911). He showed that while *A. centrale* does not result in complete immunity it gives sufficient protection against *A. marginale* to prevent severe disease symptoms. The strain of *A. centrale* isolated by Theiler is still being used today as a live blood vaccine against anaplasmosis in South Africa and certain other countries (Palmer, 1989; Potgieter, 1979; Wilson *et al.*, 1980). This vaccine, however, suffers from various disadvantages. It has a limited shelf life (7 days), its infectivity is variable and the *A. centrale* vaccine does not always induce adequate immunity against *A. marginale*. Despite all this the only improvements made to the vaccine during the last 80 years have been in the area of production quality control (Potgieter, 1979).

Current immunoprophylactic methods include infection with a live blood vaccine (*Anaplasma* parasitized erythrocytes) or whole killed initial bodies (Norman, 1973; Potgieter, 1979). Several different inocula are used in different parts of the world and each has its advantages and disadvantages.

The *A. centrale* live blood vaccine causes mild infections and animals normally recover spontaneously, but it is not as avirulent as was originally assumed. It is recommended that animals are vaccinated under the age of 6 months as severe reactions due to vaccination occur in older animals (Potgieter, 1979; Potgieter & Van Rensburg, 1983). The effect of the *A. centrale* vaccine on Friesian cows and calves was studied by Pipano *et al.* (1985) by comparisons of the packed cell volume (PCV), parasitaemia and clinical symptoms. As compared to the calves the adult cows showed a higher parasitaemia, a lower PCV, often needed chemotherapy and were more susceptible to subsequent *A. marginale* challenge.

A virulent *A. marginale* strain can be used as a vaccine but antibiotic treatment is needed after infection. The degree of protection usually depends on the virulence of the strain used, however, degree of virulence and seriousness of side effects are related (Palmer, 1989). A minimal infective dose of a virulent strain can be used but the response varies with the isolate used, route of inoculation, age and breed of cattle (Palmer, 1989).

An attenuated *A. marginale* strain which is not tick transmissible and which therefore allows greater control over the spread of the disease is used as a vaccine in the USA and some South American countries. The immunized animals are protected against severe clinical disease when challenged with several heterologous isolates and this is the most effective form of immunization available in the USA (Palmer, 1989).

Isolates of *A. marginale* made from deer have also been tested for use as vaccines. An *A. marginale* stock isolated from deer and attenuated in sheep showed similar virulence in cattle to *A. centrale*. Bovines vaccinated with this attenuated stock showed a high level of immunity against virulent *A. marginale* challenges under experimental conditions and in field trials in Colombia (Kuttler *et al.*, 1984).

There are advantages to immunizing with a vaccine consisting of killed whole *A. marginale* organisms. There is no requirement for cold storage facilities and the morbidity which may occur with infection with live organisms is avoided. A further significant benefit is the avoidance of the risk of accidentally transmitting other infectious agents. This is particularly important if vaccines are to be exported from one country to another. Unfortunately killed vaccines do not result in the same degree of protection as live vaccines and the presence of erythrocyte stroma induces anti-erythrocyte isoantibodies which cause isoerythrolysis in calves ingesting these colostral antibodies (Palmer, 1989). In order to overcome some of these difficulties while improving the economics of vaccine storage, Potgieter and Bester (1981) tested lyophilised *A. marginale* Barkly West for use as a vaccine. The material was viable for 6 months, that was a great improvement on the life-span of the live blood vaccine (1 week) but there were some disadvantages. Vaccinations had to be intravenous or intramuscular which are not as convenient as the subcutaneous *A. centrale* vaccination. The vaccine had to be stored at -20°C, and if left at 4°C for seven days it lost its infectivity. These problems have prevented the widespread use of the lyophilised *A. marginale* vaccine.

1.11. *In vitro* culturing of *Anaplasma*

There is at present no satisfactory *in vitro* culture system for *Anaplasma* and parasite material has therefore to be obtained from infected animals. *In vitro* culturing of *Anaplasma* would aid investigations of the biological properties of the organism by providing a defined source of material. Davis *et al.* (1978) showed that *Anaplasma* can be propagated in short-term bovine erythrocyte cultures. No increase in the number of infected cells could be observed but metabolic activity was shown by the uptake of ^{14}C-methionine and ^{3}H-thymidine. Cultures were only maintained for 5 days and uninfected erythrocyte cultures showed no incorporation of label. Mazzola and Kuttler (1980) reported an increase of the number of *Anaplasma* bodies in erythrocyte cultures, maintaining viable organisms for 16 days

Anaplasma has been observed in bovine erythrocytes and in cells of adult ticks (Kocan *et al.*, 1982). Hidalgo *et al.* (1989a) investigated the potential of a cell line derived from embryonic cells of *Dermacentor variables* for cultivation of *A. marginale*. They managed to maintain the culture through passages over an eleven month period. The material was not infective for cattle and probably represented a non-infective tick stage in the life cycle of *A. marginale*. Tick gut material containing different stages of the parasite are also not infective when inoculated into bovines (Hidalgo *et al.* 1989b).

A. marginale was established in a mosquito cell line by phagocytosis of infected erythrocytes by *Aedes albopictus* cells which were maintained for 52 days, but after 21 days the cultures were no longer infective (Mazzola *et al.*, 1976).

1.12. Diagnosis

The acute phase of *Anaplasma* infection is easily diagnosed on the basis of clinical symptoms and microscopic examination of Giemsa stained blood smears (Ristic, 1981). The identification of carrier animals is much more difficult as the low levels of *Anaplasma* present can usually not be detected in blood smears. Serological tests are used to confirm clinical cases and to detect carriers. The serological tests only indicate that the animal has been exposed to the disease and do not directly demonstrate carrier status.

The serological tests which have been adapted for the diagnosis of anaplasmosis include the IFA test, the CF test, the card agglutination test and the enzyme-linked immunosorbent assay (ELISA) (Trueblood *et al.*, 1991; Nakamura *et al.*, 1988; Shkap *et al.*, 1990). The specificity of the various serological tests may be reduced when other blood parasites are present, for example *Babesia*. Anti-erythrocyte antibodies are present in the serum of *Babesia*-infected bovines which react with the erythrocyte contamination in the antigen preparation used in the serological assays. Cross-reactions have been shown between *A. marginale* and three *Plasmodium* species and between *Plasmodium* and *Babesia* by the IFA test. *Plasmodium* invades erythrocytes by invagination in a similar manner as *Anaplasma* and this common mechanism of entry might be the reason for the cross-reaction (Dimopoullos & Fmerty, 1976). The serological assays also cannot distinguish between natural infections and infections due to vaccination (Luther *et al.*, 1980) and have high rates of false positive and false negative results (Barbet & Allred, 1991). ELISA assays using monoclonal antibodies and cloned genes are being developed that could overcome these problems (Trueblood *et al.*, 1991).

A sensitive and specific method for the detection of *Anaplasma* organisms is to use a DNA probe with the nucleic acid of the organism as the specific target. DNA probes have been isolated and used to study the carrier status of animals and the epidemiology of anaplasmosis (Visser *et al.*, 1991; Eriks *et al.*, 1989; Goff *et al.*, 1988).

1.13. Identification of specific proteins and comparison of isolates

A great deal of work has been done to compare the USA isolates of *A. marginale* and *A. centrale* with each other and much evidence of differences has accumulated. Kuttler (1967) found a reduction of virulence when *A. centrale*-immunised bovines were challenged with *A. marginale* although the opposite was not true. This lack of cross-protection has also been observed between different *A. marginale* isolates and in fact such experiments have provided little evidence that the differences between *A. centrale* and *A. marginale* are greater than those between *A. marginale* isolates (Palmer, 1989). Isolates which probably consist of mixed populations and not cloned lines of *A. marginale* and *A. centrale* were used in the cross-immunity experiments. One cannot predict which sub-population will predominate in an infection in a specific animal and thus the cross-immunity experiments do not yield precise data.

Studies have been carried out to compare *Anaplasma* isolates at the protein level. Radiolabelled proteins from the Washington and Florida isolates of *A. marginale* were separated by two-dimensional gel electrophoresis and the majority of proteins were found to be common between the two isolates. A few major proteins, however, were found to be variable in molecular weight and primary protein structure (Barbet *et al.*, 1987). In another study the Illinois and Florida isolates of *A. marginale* were compared on immunoblots using sera from infected bovines. Proteins in the 108-91kDa and the 47-27kDa regions were detected and both isolate-specific and genus-specific antigens of *Anaplasma* were identified (Adams *et al.*, 1986). Adams *et al.* (1989) also showed that antisera from an *A. centrale*-infected animal reacted to two proteins of 41kDA and 38kDa from *A. centrale* but only to the 41kDa homolog from *A. marginale* (Waeol isolate). The antisera from the animal infected with the Waeol isolate only reacted with the 41kDa proteins in both *A. centrale* and *A. marginale*. The 41kDa protein seems to be genus specific.

Nakamura *et al.* (1991) also compared *Anaplasma* isolates by using bovine antisera on immunoblots. The Kochinda and California isolates of *A. marginale* and the Akashima and Aomori isolates of *A. centrale* were compared and the major protein band detected was 38-40kDa in size. This band it reacted strongly with homologous antisera but weakly with heterologous antisera. Shkap *et al.* (1990) compared the *A. marginale* Israel-NT isolate and *A. centrale* on immunoblots and found that antisera from bovines infected with these isolates predominantly reacted with proteins in the 33-36kDa region.

Nakamura *et al.* (1991) suggest that the proteins detected by them in the 38-40kDa region, the 41 and 38kDa proteins of Adams *et al.* (1989) and the 33-36kDa proteins identified by Shkap *et al.* (1991) are the same molecules and also the same as the 36kDa protein identified by Palmer and McGuire (1984) - see following paragraph. This region consists of more than one protein (Nakamura *et al.*, 1991) and therefore could contain genus-common, isolate-common and isolate-specific proteins.

Palmer and McGuire (1984) followed a different approach to study *Anaplasma* proteins. Rabbit antiserum against *A. marginale* was produced that neutralised the infectivity of initial bodies for bovines. Five proteins were immunoprecipitated with this antiserum, they had molecular weights of 105, 86, 61, 36 and 31kDa. Surface radiolabelling of purified initial bodies showed that these were all membrane surface proteins. To identify the proteins that induced the neutralizing antibodies, monoclonal antibodies were produced against the surface proteins. A panel of monoclonal antibodies was produced against whole organisms of the Virginia and Washington isolates of *A. marginale*. Some of these monoclonal antibodies could differentiate between isolates while others recognised common determinants between the different USA isolates (McGuire *et al.*, 1984). The isolate-common monoclonal antibodies were used to identify the common epitopes among isolates (McGuire *et al.*, 1984). The epitopes recognized by these antibodies were limited to two surface proteins of *Anaplasma*, the molecular weights in the *A. marginale* Florida isolate were 105kDa and 36kDa (Palmer *et al.*, 1988a). These proteins were designated Am105 and Am36 respectively.

The monoclonal antibodies that recognized epitopes on Am105 were able to neutralise the infectivity of initial bodies of the Florida isolate (Palmer *et al.*, 1986a). This neutralising-sensitive epitope is conserved among 16 *A. marginale* isolates and *A. centrale* (Palmer *et al.*, 1988a). The monoclonal antibodies were used to immunoaffinity purify the 105kDa protein which was in fact a complex of two proteins, MSP-1a and MSP-1b, 105 and 100kDa respectively in size. When used to immunise cattle, the MSP-1 complex induced protection against homologous challenge and heterologous challenges (Palmer *et al.*, 1989). The gene coding MSP-1a, *msp*1α, has been cloned and expressed in *E. coli* and is being developed and tested for use as a subunit vaccine (Barbet *et al.*, 1987). The coding region for the MSP-1b, *msp*1β, has also been cloned, sequenced and expressed in *E. coli* (Barbet & Allred, 1991).

The MSP-1a and MSP-1b proteins which were expressed by the different USA *A. marginale* isolates were found to vary in size. Antibodies reactive to the polypeptides showed a variation from 105 to 86 kDa and 100 to 97 kDa respectively (Oberle *et al.*, 1988). The *msp*1α genes from four different isolates were cloned and sequenced and the size variation could be explained by the number of times a tandemly repeated sequence occurred within the gene (Allred *et al.*, 1990). The *msp*1β gene also contains tandemly repeated sequences but the number of repeats is lower than that in *msp*1α (Barbet & Allred, 1991).

The isolate-common monoclonal antibodies also recognised Am36 (McGuire *et al.*, 1984). Am36 showed small molecular weight variations and appeared to be conserved between the different isolates. Am36 was also immunoaffinity purified and used in protection studies and partial but significant protection to anaplasmosis was achieved in cattle which were immunised with Am36 (Palmer *et al.*, 1988b).

Immunoprecipitations with antisera from chronic and acute *Anaplasma* infected bovines showed that the 86kDa protein (designated MSP-3) was the most common protein in all stages of infection (Palmer *et al.*, 1986b). This protein has been immunoaffinity purified after which it was found to react with serum from carrier animals it is now being tested for use in a serological assay (McGuire *et al.*, 1991).

1.14. Aims of this project

Because the current Anaplasmosis blood vaccine is not entirely satisfactory (see section 1.10) it is important to investigate ways to improve it. An obvious possibility is to develop a recombinant vaccine expressing one or more *Anaplasma* proteins in an appropriate delivery vector but in order to do this the proteins bearing protective epitopes have to be identified. Protective epitopes have been recognized in the American isolates (see section 1.2.13.), but these may not necessarily be relevant to protection in animals in Africa or elsewhere. Cross-protection trials, morphological and serological studies and analysis of individual proteins all show that various *A. marginale* isolates and *A. centrale* are significantly different. Cross-protection trials in Zimbabwe showed that cattle immune to the Florida isolate of *A. marginale* were only partially protected against the Norton isolate from Zimbabwe (Tebele *et al.*, 1991; Tebele & Palmer, 1991). This indicates the need to examine the South African isolates of *A. marginale* and *A. centrale* for their own specific protective antigens and epitopes.

The primary aim of this project, then, was to identify and compare antigenic proteins from a range of South African isolates of *Anaplasma*. The approach we have followed is to produce anti-*Anaplasma* sera in rabbits, as described by Palmer and McGuire (1984), and then to use these antibodies for the immunogenicity studies.

Several uses could be made of the data generated by this study. Some of the proteins identified should be relevant to the protection of cattle against anaplasmosis and cloned *Anaplasma* genes from Southern Africa, together with those from other geographical areas, could eventually be used to generate a universal anaplasmosis vaccine. To date three sequences are available, those of the two MSP-1 proteins and that of MSP-5 (Visser *et al.*, 1992).

Improvement of serological assays is another benefit which may arise from this study. The currently available tests have serious weaknesses, such as cross-reactions with other blood parasites and false positives and negatives. Identification of immunogenic *Anaplasma* proteins (immunodominant, isolate common or isolate specific) followed by cloning and expression in a suitable system, would provide a source of molecularly pure antigen. At present the occurrence of false positives is mainly a result of contamination of the *Anaplasma* antigen used with bovine erythrocyte material. The development of improved tests will entail a knowledge of the immune response in infected animals to identify proteins which give rise to circulating antibody. In chapter 4 the kinetics of the production of antibodies in bovines are monitored and three isolates are compared for cross-reactivity. This study should provide useful information on the level of antibodies against specific proteins during the course of infection.

CHAPTER TWO

IMMUNOPRECIPITATIONS WITH RABBIT AND BOVINE ANTISERA

2.1. INTRODUCTION

This chapter describes the work carried out to identify *Anaplasma* proteins by immunoprecipitation using antisera raised both in rabbits and in cattle. The two sources of antiserum were employed in order to identify which proteins, out of all the proteins expressed by *Anaplasma*, would be recognized by a bovine during the course of infection. We planned to raise the bovine sera by infecting animals with live *Anaplasma* and to raise the rabbit sera by inoculation of purified *Anaplasma* organisms. Since rabbits are not susceptible to anaplasmosis no infection would develop but the resultant antisera would be expected to contain antibodies which would recognize all *Anaplasma* proteins.

The isolates chosen for this study were *Anaplasma centrale* and *Anaplasma marginale* Underberg. These are isolates, not cloned cultures, so there is the possibility that each may contain mixed populations of immunotypes. *A. centrale* was chosen because it has been used as a vaccine since 1911 and is therefore widely disseminated throughout South Africa. *A. marginale* Underberg was chosen because it is a virulent isolate thought to be typical of the type of organism which causes outbreaks of anaplasmosis.

It is difficult to purify *Anaplasma* organisms so as to completely eliminate contamination by erythrocyte proteins and various methods have been tried by other workers in order to get over this obstacle. Budden and Dimopoullos (1977) used erythrocyte affinity columns but found that the *Anaplasma* initial bodies which they obtained were not serologically active. Hart *et al.* (1981) utilized haemolysin to release *A. marginale* from the erythrocytes and followed this by treating with phospholipase C, trypsin, neuraminidase and differential centrifugation to remove erythrocyte material. These purified initial bodies were also not infective.

Because of the difficulties involved in purification of the parasite it has been usual to employ crude preparations for the study of *Anaplasma*. Dzuris *et al* (1987) isolated viable *A. marginale* by first lysing the erythrocytes with 0.83% NH_4Cl after which they were passed through a French press and sedimented over Renografin. An alternative method described by Potgieter and van Rensburg (1983) is a modification of the method described by Shkap *et al.* (1990). Briefly, water and sonication are used to lyse the erythrocytes and centrifugation further purifies the parasites. Because of its simplicity and effectiveness we decided to use this method and a complete description of the procedure is given in section 2.2.2.

The methods we used to produce antibodies in rabbits were adapted from the work of Palmer & McGuire (1984) and Harlow & Lane (1988). The general principles to which we gave attention are described here and complete experimental details are given in section 2.2.3. In order to generate a good immune response adjuvants must be used to prevent rapid dispersal of the antigen and to stimulate the rate of phagocytosis. The primary reaction produces mainly IgM, which is not ideal for subsequent serology because of its relatively low affinity for antigen. A second (booster) exposure results in a faster and more potent response, resulting in the production of IgG which has a higher affinity for the antigen. The level of stimulus achieved is related to the dose and to the schedule of inoculations and may vary between individual antigens. For this reason each new experiment needs optimization of immunization procedures.

Rabbit antisera raised against partially purified initial bodies would be expected to contain antibodies to bovine erythrocyte material which would confuse the identification of *Anaplasma* proteins. Two possibilities exist for overcoming this difficulty, absorption of rabbit antisera with bovine erythrocyte material and exclusive labelling of *Anaplasma* proteins in short term *in vitro* cultures. Both of these methods were employed in this study.

The method for the specific labelling of *Anaplasma* proteins was adapted from the work of Palmer *et al* (1988) and involved incubation of *Anaplasma*-infected erythrocytes with ^{35}S methionine. The erythrocytes do not synthesize proteins whereas the infecting organisms remain metabolically active for a short period, thereby incorporating the label. The labelled proteins may then be conjugated with specific antiserum and the antigen-antibody complexes immunoprecipitated with *Staphylococcus aureus* Cowan I (SAC). This bacterium expresses protein A, which binds specifically and strongly to the Fc regions of many IgG subclasses of most animals. The resulting complexes are recovered by centrifugation (Kessler, 1975; Palmer *et al.*, 1985) and then analyzed by polyacrylamide gel electrophoresis (PAGE) followed by autoradiography. All these procedures are described in detail in sections 2.2.5-2.2.10.

Figure 2-1 summarizes the experimental design followed in this study. *Anaplasma* proteins from two different isolates of the parasite were labelled with ^{35}S and immunoprecipitated with specific antisera from two sources. The sources were bovines infected with anaplasmosis and rabbits immunized with partially purified *Anaplasma* organisms. The resulting labelled proteins were separated and compared by PAGE.

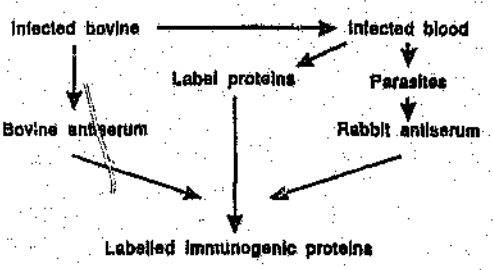

Figure 2-1. Scheme showing antiserum production for immunoprecipitations.

2.2. MATERIALS AND METHODS

2.2.1. Source of parasites and bovine antisera

Infected and uninfected bovine blood and bovine sera were donated by the Protozoology section, Veterinary Research Institute, Onderstepoort. Bovine sera were either freshly collected or obtained from the frozen stock collection. Blood from 5 uninfected bovines was pooled to obtain a mixture of blood types for the uninfected erythrocyte controls. Blood infected with *A. centrale* (40% parasitaemia) was obtained from the vaccine donor bovines. *A. marginale* Underberg-infected blood (31% parasitaemia) was obtained from a bovine infected with the original *A. marginale* Underberg stabilate. Before infection of the bovines they were shown to be serologically negative on the card agglutination test and no *Anaplasma* DNA could be detected by a DNA probe (Visser *et al.* 1991).

2.2.2. Isolation of parasites (initial bodies)

The method of Potgieter and van Rensburg (1983) was followed with some alterations. Infected and uninfected bovine blood was collected in heparin, centrifuged at 2 000xg for 10min and the plasma and buffy coat removed. The erythrocytes were washed 3 times in phosphate buffered saline (PBS) and stored at -70°C. The cells were thawed, lysed with 3.5 volumes sterile water and centrifuged at 1000xg to remove any remaining white blood cells. The supernatant was centrifuged at 17 000xg for 30min and the resulting supernatant aspirated off, the sediment was resuspended in water and centrifuged again. The sediment was washed twice with PBS containing 0.2% EDTA (PBS-EDTA) and finally resuspended in PBS-EDTA. The suspension was sonicated twice for 15 seconds at an amplitude of 12 microns in an MSE sonicator, centrifuged at 17 000xg and the supernatant plus jelly-like layer carefully aspirated off. The pellet was washed twice in PBS-EDTA, resuspended in PBS-

EDTA containing 1mM phenylmethylsulfonyl fluoride (PMFS) and 0.1mM N-α-p-tosyl-L-lysyl-chloromethylketone (TLCK) as protease inhibitors, and the final preparation was stored at -70°C. Uninfected blood was treated in the same way as the infected blood, but the last two washing steps were omitted.

2.2.3. Production of antisera

2.2.3.1. Rabbit antiserum against uninfected bovine erythrocytes

Sera were prepared as described by Palmer and McGuire (1984). Each of two rabbits was inoculated (subcutaneously over a large area) five times, each time with 0.5ml washed, uninfected bovine erythrocytes (≈1 x 10^{10} cells). On day 0 the cells were mixed 1:1 with complete Freund's adjuvant, on days 7 and 14 the cells were mixed 1:1 with incomplete Freund's adjuvant and on days 28 and 35 the cells were administered without adjuvant. The rabbits were bled before inoculation and on days 42 and 178. The antiserum was designated CTRL.

2.2.3.2. Rabbit antiserum against *A. centrale*

These sera were prepared by inoculating rabbits as described in section 2.2.3.1. Three rabbits were used to produce antiserum against bovine erythrocytes infected with *A. centrale* (antiserum designated anti-ACE) and three rabbits were inoculated with *A. centrale* initial bodies (antiserum designated anti-AMI). ≈4 x 10^9 parasites per inoculum. The inocula were prepared and administered using the same schedule as in section 2.2.3.1.

2.2.3.3. Rabbit antiserum against *A. marginale* (Underberg)

These sera were prepared as described by Harlow and Lane (1988). Two rabbits were used to produce antiserum against bovine erythrocytes infected with *A. marginale* by inoculating 0.5ml washed erythrocytes into each rabbit on days 0, 28 and 56, mixed 1:1 with complete Freund's adjuvant on day 0, 1:1 with incomplete Freund's adjuvant on day 28 and without adjuvant on day 56. The antiserum was designated anti-AMI. Three rabbits were used to produce antiserum against *A. marginale* (Underberg) initial bodies by inoculating ≈2 x 10^{10} parasites into each rabbit on days 0, 28 and 56, the inocula being prepared as described above. This antiserum was designated anti-AMI. The rabbits were bled before inoculation and on days 28 and 70.

2.2.3.4. Preparation of antiserum

Blood was collected and left to clot at room temperature for one hour. The clot was allowed to contract at 4°C for at least an hour and then centrifuged at 3 000xg for 10min. The antisera were stored at -20°C and thawed aliquots were kept at 4°C.

2.2.3.5. Absorption of antiserum

Antisera were absorbed with uninfected bovine erythrocyte powder prepared by acetone precipitation (see sections 2.2.1 and 2.2.8.). Acetone powder (1% w/v) was added to serum and incubated for at least 30min at room temperature with agitation. The powder was pelleted by centrifugation at 10 000xg for 10min and discarded.

2.2.4. Preparation of samples for polyacrylamide gel electrophoresis

The initial bodies were thawed on ice, lysed with 0.1 volumes of lysis buffer (10% NP-40, 1% SDS) and incubated at room temperature for 5min (Nakamura *et al.* 1988). An equal volume of PAGE sample buffer (0.5 M Tris, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.001% bromophenol blue) was added, samples were boiled for 3min and microfuged for 10min before loading on to the gels. Molecular weight markers, MW-SDS-6H (Sigma) or ^{14}C-labelled molecular weight markers (NEN, Du Pont) were used to estimate molecular weights on gels and blots. Molecular weight markers were boiled for one minute before loading on to gels.

2.2.5. Radiolabelling of *Anaplasma* proteins

Anaplasma proteins were labelled with ^{35}S-methionine as described by Palmer *et al.* (1985). Fresh blood from an infected bovine was centrifuged at 2 000xg for 10min, the plasma and buffy coat removed and the red cells washed twice with PBS and once with Eagle's minimal medium. The washed packed erythrocytes (1.5ml) were diluted into 12ml Eagle's minimal essential medium without methionine. 125μCi (0.126nM) ^{35}S-methionine (NEN products, Du Pont) per ml erythrocytes was added and incubated at 37°C with 5% CO_2 for 48 hours. After the incubation step, the erythrocytes were washed twice in PBS, disrupted in an equal volume of disruption buffer (50mM Tris pH 8, 5mM EDTA, 1mM PMFS, 0.1M TLCK, 1% NP-40 and 0.1% SDS) and stored at -70°C. Uninfected bovine blood was treated in the same way as a control culture.

2.2.6. Trichloroacetic acid precipitation

The amount of radiolabel incorporated into the *Anaplasma* proteins was determined as described by Harlow and Lane (1988). Disrupted erythrocytes (5µl) were mixed with 5µl bovine serum albumin (BSA, 10mg/ml) and spotted on glass fibre filters (GF/C, Whatman). The filters were incubated in 10% trichloroacetic acid (TCA) on ice for 30min, the TCA was replaced and left at room temperature for 5min with agitation. TCA replacement and incubation steps were repeated twice and finally the filters were incubated in 95% ethanol for 5min and left to dry. Scintillant (Ready-solv EP, Beckman) was added to the samples which were counted in a liquid scintillation analyzer (1600 CA Tri-Carb, Packard).

2.2.7. Preparation of *Staphylococcus aureus*

Protein A-bearing *Staphylococcus aureus* Cowan I (SAC) was donated by the Bacteriology Section, VRI, Onderstepoort and was fixed to produce solid phase protein-A for collecting immune complexes as described by Harlow and Lane (1988). *S. aureus* was grown to saturation in 500ml Penassay medium (0.15% beef extract, 0.15% yeast extract, 0.5% peptone, 0.1% glucose, 0.35% NaCl, 0.37% K_2HPO_4 and 0.13% KH_2PO_4), harvested by centrifugation at 7 000xg for 10min, the pellet resuspended in PBS containing 0.02% sodium azide and washed twice. The final pellet was resuspended at 10% w/v in PBS with 0.02% sodium azide. The suspension was stirred, formaldehyde added slowly to a final concentration of 1.5% and the mixture left stirring for 90min at room temperature. The cells were washed, resuspended at 10% w/v in PBS with 0.02% sodium azide, heat killed by incubation at 80°C for 5min and left to cool in an ice bath. The cells were washed twice, resuspended at 10% v/v and stored at -70°C.

2.2.8. Preparation of bovine erythrocyte powder

Bovine erythrocyte powder was prepared by acetone precipitation as described by Harlow and Lane (1988). Uninfected bovine erythrocytes were washed three times as described in section 2.2.2, four volumes of acetone were added and mixed vigorously. After 30min on ice the precipitate was pelleted at 10 000xg for 10min, the pellet was resuspended in cold acetone, left on ice for 10min and pelleted again. The final pellet was dispersed on filter paper to dry.

2.2.9. Immunoprecipitation

The method of Palmer *et al.* (1985) was followed. Radiolabelled, disrupted erythrocytes were thawed on ice, sonicated (see section 2.2.2.) and centrifuged for 10min at 10 000xg. Siliconized tubes were used for the immunoprecipitation reactions: 100µl of either rabbit (see Table 2-1) or bovine antiserum (see section 2.2.1) were added to the labelled proteins (2×10^5 to 10^6 TCA-precipitable cpm, see section 2.2.6.) and incubated for 30min on ice. Rabbit anti-bovine whole serum (50µl, SIGMA) was added to the immunoprecipitation reactions containing bovine antiserum and incubated on ice for a further 30min. SAC (100µl) was added to the reactions, incubated for 30min on ice, centrifuged at 2 000xg for 30 seconds and the supernatant aspirated off. The precipitates were washed three times with washing buffer (0.1M NaCl, 1% NP-40, 0.05M Tris pH 8), the final pellet resuspended in PAGE sample buffer and the proteins separated by PAGE.

2.2.10. Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis (PAGE) was performed as described by Laemmli (1970). The resolving gel contained 0.375M Tris pH 8.8, 0.1% w/v SDS, 0.05% v/v TEMED (NNN'N'-tetramethyl-ethylenediamine), 0.075% ammonium peroxydisulphate (APS) and either 10% w/v or 7% and 17.5% (for the gradient gels) acrylamide, with N,N'-methylene bisacrylamide (bis) to achieve 2% crosslinking. The stacking gel contained 4% acrylamide (2% crosslinking), 0.125M Tris pH 6.8, 0.1% SDS, 5.3% glycerol, 0.25% v/v TEMED and 0.075% w/v APS. Electrophoresis buffer contained 25mM Tris, 192mM glycine and 0.1% SDS. Electrophoresis was carried out in a vertical electrophoresis apparatus at 10V/cm for 8h (10% gels) and 11h (gradient gels). All gels were 10% unless stated otherwise in the legends of the figures. Molecular weight standards were run on each gel to determine the molecular weights of the unknown proteins. A calibration curve was constructed for each gel, relating the molecular weights of the standards to their mobilities, by fitting the data to a fourth order polynomial equation. We performed the regression using a program called Grafit (Erithacus Software) and the molecular weights of the unknown proteins were predicted using the resulting formula. Gels were either stained with Coomassie blue to visualise all the proteins, or transferred from the acrylamide gels to nitrocellulose membranes. Proteins immobilized on nitrocellulose were detected with by means of a protein stain, india ink, or with antibodies (Towbin *et al.*, 1979; Gershoni and Palade, 1983; Harlow and Lane, 1988).

2.2.11. Fluorography

After electrophoresis, the radiolabelled proteins were fixed in the gels with a 25% methanol and 7% acetic acid solution for one hour. To enhance the signal of ^{35}S about ten fold (Harlow and Lane, 1988) the gels were incubated in Enlightning (Biotechnology Systems, NEN, Du Pont) for 30min before they were dried on a slab gel drier (Hoefer Scientific Instruments) and exposed to X-ray film (Cronex 4) at -70°C.

2.2.12. Transfer of proteins from gels to nitrocellulose membranes

Proteins were transferred to nitrocellulose membranes (Hybond-C, Amersham) with a semi-dry transfer unit (TE 70 Semiphor™, Hoefer Scientific Instruments). After electrophoresis, the gels were soaked in transfer buffer (40mM Tris, 40mM glycine, 0.4% SDS and 20% methanol) for at least 10min. The nitrocellulose membranes were treated according to the manufacturer's instructions. The gels and membranes were sandwiched between buffer-soaked blotter paper and transferred at 100mA for 30min. After transfer the membranes were rinsed in PBS to remove remaining acrylamide, the marker lanes were cut off and stained with India ink (see section 2.2.14) and the remainder of the membrane was used for immunoblotting.

2.2.13. Coomassie blue staining

Polyacrylamide gels were stained with 0.2% Coomassie blue in 50% methanol and 10% acetic acid for at least one hour at room temperature with agitation and destained in 20% methanol and 10% acetic acid (Laemmli, 1970; Harlow and Lane, 1988).

2.2.14. India ink staining

Proteins were detected on nitrocellulose by staining with India ink (Hancock and Tsang, 1985). The membranes were blocked with PBS Tween 20 (0.4%, Merck) twice for 5min and incubated in PBS Tween 20 (0.3%) plus 0.1% India ink until bands were visualised. The membranes were destained with PBS to remove background staining when necessary.

2.2.15. Immunoblotting

The following steps were all carried out with agitation. The membranes were blocked for one hour in PBS containing 5% milk powder (ELITE fat free instant milk powder). Antisera were diluted in PBS containing 1% w/v milk powder. Rabbit antiserum was diluted 1/200 and bovine antiserum

diluted 1/100. Membranes were incubated in primary antibody for 2-16h at room temperature. The membranes were rinsed three times in PBS and then washed once for 10min and twice for 5min in PBS. The secondary antibodies were peroxidase-conjugated anti-rabbit, anti-goat and anti-bovine IgG (The Binding Site, England). These were diluted 1/1000 and incubated at room temperature for 1-2h. The washing steps were repeated and substrate added. The substrate solution was freshly made up as follows: 60mg 4-chloro-1-naphthol (Sigma) was dissolved in 10ml ice cold methanol; 60µl hydrogen peroxide was added to 100ml cold PBS and the two solutions were mixed. The membranes were incubated in the substrate for 10-60min for the colour to develop, rinsed in water to enhance the colour, dried and stored in the dark.

2.3. RESULTS

2.3.1. Isolation of parasites

Anaplasma parasites (initial bodies) had to be partially purified from bovine erythrocytes before further studies could be carried out on the parasites. The presence of initial bodies in the final preparation was confirmed microscopically and the presence of parasite proteins in the preparation is shown by electrophoresis in Fig. 2-2a, a Coomassie blue-stained PAGE 7-17.5% gradient gel of *A. centrale* proteins and uninfected erythrocyte control proteins (RBC). The *A. centrale* preparation contains more protein bands than the erythrocyte preparation, thus these must be *A. centrale* proteins. The *A. centrale* preparation contains some erythrocyte proteins, these bands are especially prominent in the 80kDa region, at 45kDa and there is a common doublet at 200kDa.

The molecular weight standards run on the gel of Fig. 2-2a were fitted to a polynomial equation to obtain a graph (Fig. 2-2b). The molecular weights of the unknown proteins were deduced from the graph. A similar curve was constructed for each gel shown in this study.

Figure 2-3 compares the initial body preparations of *A. centrale*, *A. marginale* Underberg and *A. marginale* Barkly West. The same erythrocyte proteins were observed in the initial body preparations as were observed as in Fig. 2-2. *Anaplasma* proteins were present in the preparations and differences and similarities in the band patterns were evident in the three isolates. Common proteins were seen in the 30kDa and 50 to 66kDa regions. Proteins were present in the 40kDa region and in the region above the 66kDa molecular weight marker in all the isolates but the banding patterns were not identical.

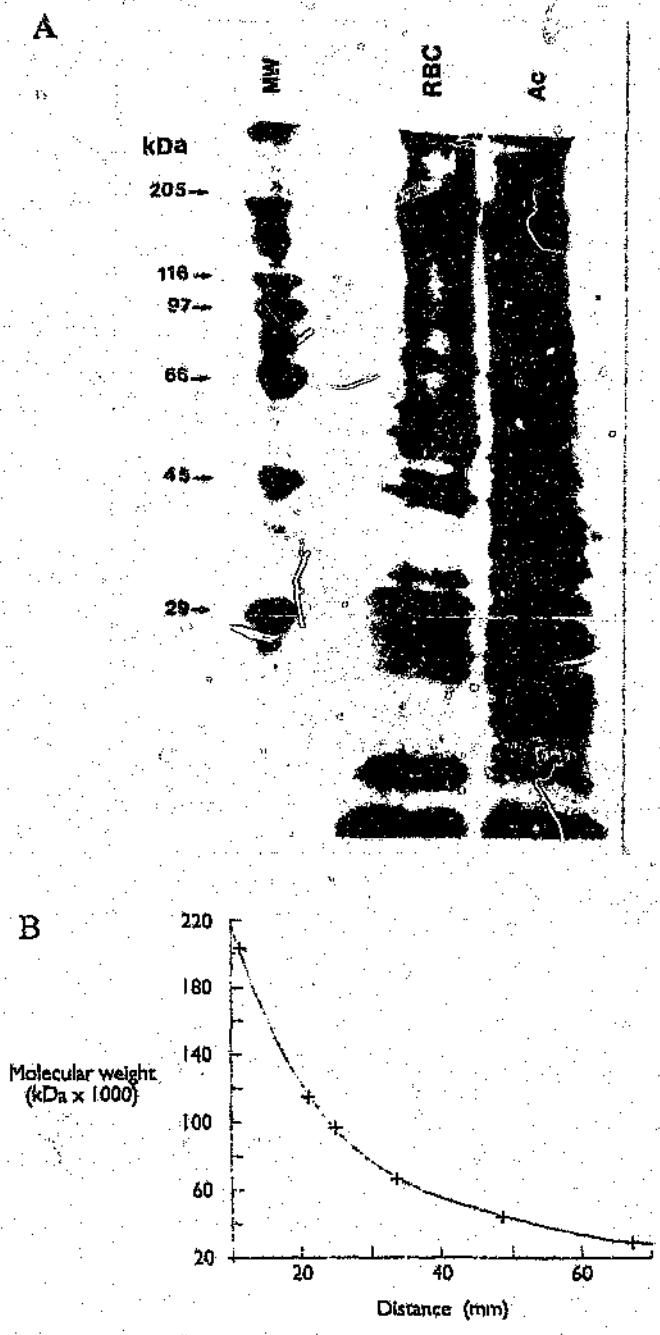

Fig. 2-2. (A) Coomassie blue stained 7%-17.5% gradient polyacrylamide gel of *A. centrale* (AC) and uninfected erythrocyte (RBC) proteins. The molecular weight marker (mw) sizes are indicated by the arrows. (B) Graph showing molecular weight standards of gel (A) plotted against mobility, fitted to a polynomial equation ($y = a + bx + cx^2 + dx^3 + ex^4$) from which molecular weights of the unknown proteins were determined.

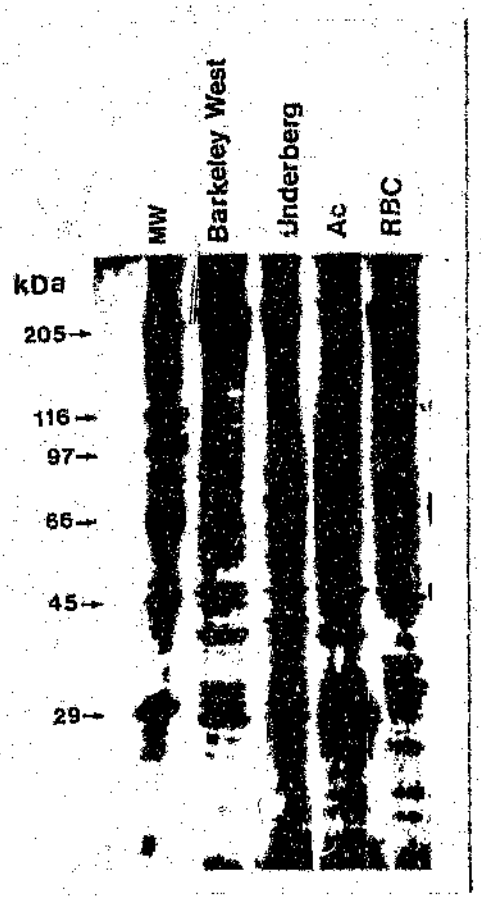

Fig. 2-3. Coomassie blue stained 7%-17.5% gradient polyacrylamide gel of *A. marginale* Barkly West, *A. marginale* Underberg, *A. centrale* (AC) and uninfected erythrocyte (RBC) proteins. The molecular weight marker (mw) sizes are indicated by the arrows.

2.3.2. Production of antisera

As shown by the scheme in Fig. 2-1 rabbit antiserum was made to identify immunogenic *Anaplasma* proteins by immunoprecipitation. Table 2-1 lists the antisera made.

TABLE 2-1. Table identifying rabbit anti-*Anaplasma* sera and controls

Serum identity	Number of Rabbits	Inoculum
CTRL	2	Uninfected erythrocytes
anti-ACE	3	*A. centrale* infected erythrocytes
anti-ACI	3	*A. centrale* initial bodies
anti-AME	3	*A. marginale* infected erythrocytes
anti-AMI	3	*A. marginale* initial bodies

Figures 2-4 and 2-5 show that the antibodies recognised *Anaplasma* proteins on immunoblots. Figure 2-4 shows rabbit antisera against *A. centrale* reacted with *A. centrale* proteins on an immunoblot. Antisera were absorbed with erythrocyte acetone powder to remove antibodies against erythrocyte proteins before use in the immunoblots. Serum collected prior to inoculations are shown in lanes 1, 3 and 5. No reaction of the preinoculation antiserum with *A. centrale* proteins was observed showing that the animals had no cross-reactive antibodies to *Anaplasma*. The reaction of the antiserum collected on day 178 with *A. centrale* proteins is shown in lanes 2, anti-ACE, 4, CTRL and 6, anti-ACI. Negligible reaction was seen in lane 4 with the control antiserum, most of the antibodies were absorbed out with the acetone powder, except in the high molecular weight region. The major bands detected by both anti-ACE and anti-ACI are the doublet in the 40kDa region and a lighter band in the 22kDa region.

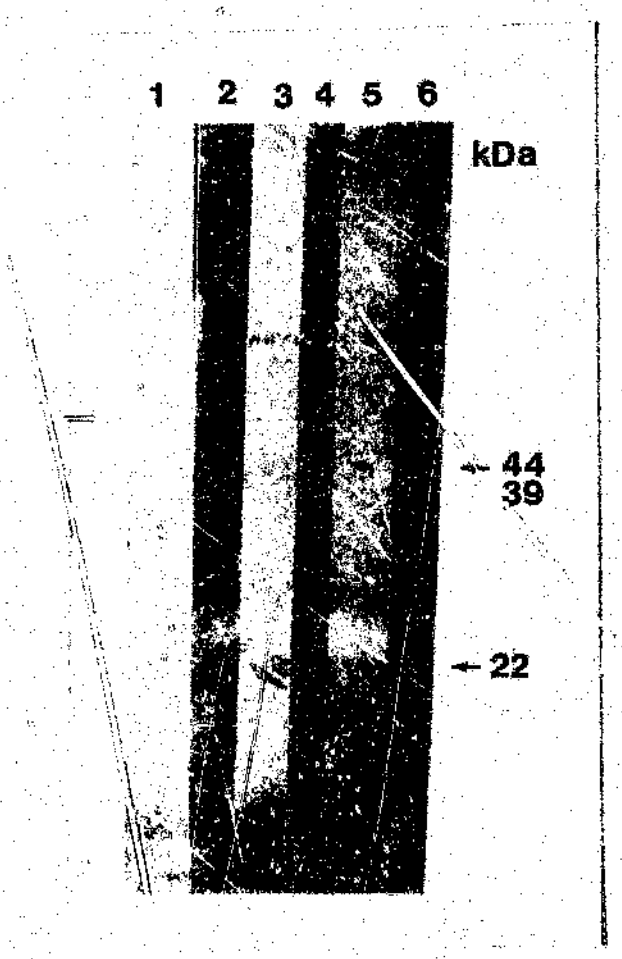

Fig. 2-4. Immunoblot of *A. centrale* proteins, separated by polyacrylamide gel electrophoresis and transferred to nitrocellulose, were reacted with antisera (at 1/200 dilution) as follows: in lane 2, anti-ACR, lane 4, CTRL and lane 6, anti-ACI and the respective pre-inoculation antisera in lanes 1, 3 and 5. Molecular weight sizes (in kDa) are indicated by the arrows.

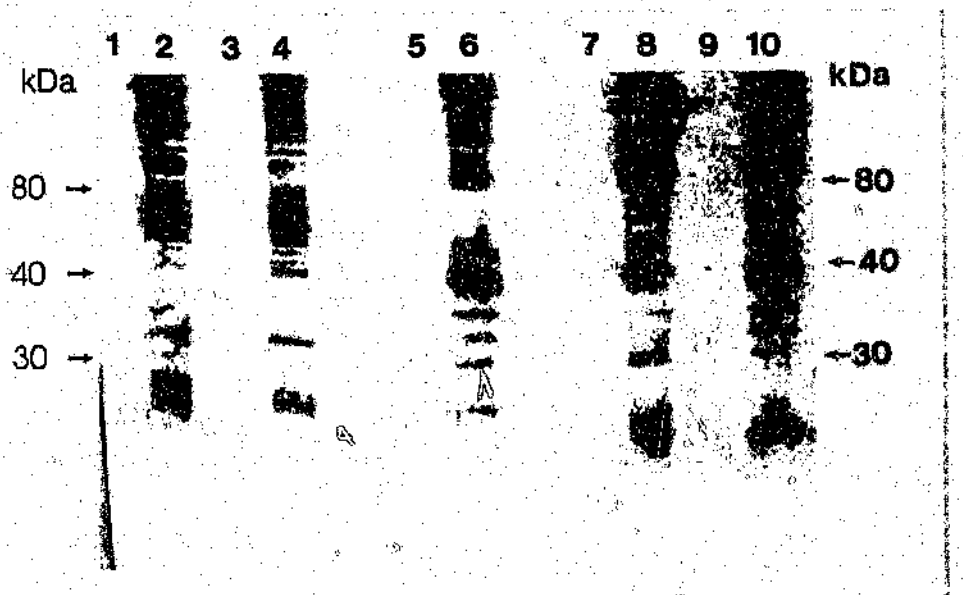

Fig. 2-5. Immunoblot of *A. marginale* Underberg proteins, separated by polyacrylamide gel electrophoresis and transferred to nitrocellulose, were reacted with antisera (at 1/200 dilution) as follows: lanes 2 and 4, anti-AME; lanes 6, 8 and 10, anti-AM1, and the respective pre-inoculation antisera in lanes 1, 3, 5, 7 and 9. Molecular weight sizes (in kDa) are indicated by the arrows.

Antisera made against *A. marginale* Underberg were shown to react with *A. marginale* Underberg proteins on an immunoblot (Fig. 2-5). The preinoculation antisera of individual rabbits are shown in lanes 1, 3, 5, 7 and 9. No antibodies to *A. marginale* were present in the rabbit sera prior to inoculation. The antiserum collected on day 70 are shown in lanes 2 and 4, anti-AME and lanes 6, 8 and 10, anti-AMI. The intensities of the bands detected in lanes 6, 8 and 10 are not identical, which shows the importance of using more than one animal for the production of a fully representative antiserum. The profiles of anti-AME differed to anti-AMI. The most prominent bands recognised by anti-AMI were in the 40kDa region, anti-AME on the other hand recognised bands in the 60-70kDa region. Both anti-AME and anti-AMI prominently recognised a ≈20kDa protein. Rabbit antisera was successfully produced against both *A. centrale* and *A. marginale* and could be used for the identification of *Anaplasma* proteins.

2.3.3. Radiolabelling of *Anaplasma* proteins

Complete elimination of erythrocyte proteins from initial body preparations is difficult and a way to circumvent this problem is to exclusively radiolabel the *Anaplasma* proteins. *A. centrale* and *A. marginale* Underberg infected blood samples, together with uninfected blood controls, were grown in short-term *in vitro* cultures to label the parasite proteins with ^{35}S-methionine. Figure 2-6 shows an autoradiogram of the control erythrocyte culture proteins (RBC) separated by electrophoresis and visualised by fluorography. No erythrocyte proteins were labelled. Thus the labelled proteins observed in the infected cultures would be expected to be *Anaplasma* proteins, as shown by Palmer *et al.* (1985). Figure 2-7 shows the labelled proteins obtained from the *A. centrale* and *A. marginale* Underberg infected samples. Common bands and isolate specific bands were seen; the major bands at 148 and 134kDa of *A. centrale* were absent in *A. marginale* Underberg, but the bands in the 30 to 40kDa region of *A. marginale* Underberg were much more prominent than in *A. centrale*.

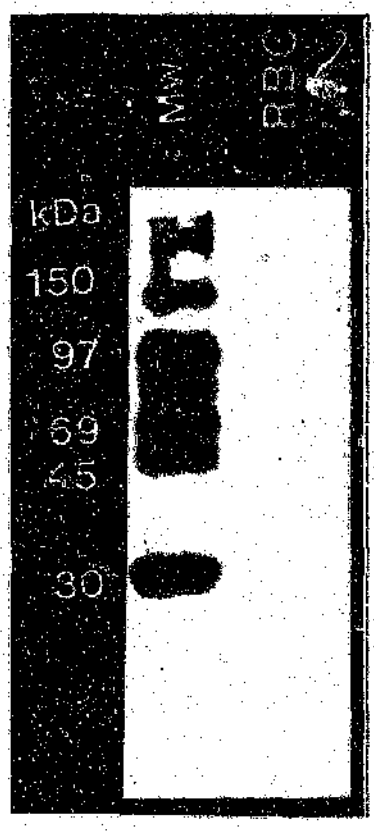

Fig. 2-6. Autoradiograph of control, uninfected erythrocyte (RBC) ^{35}S-methionine labelled proteins, separated by electrophoresis. Molecular weight marker (MW) sizes are indicated on the side.

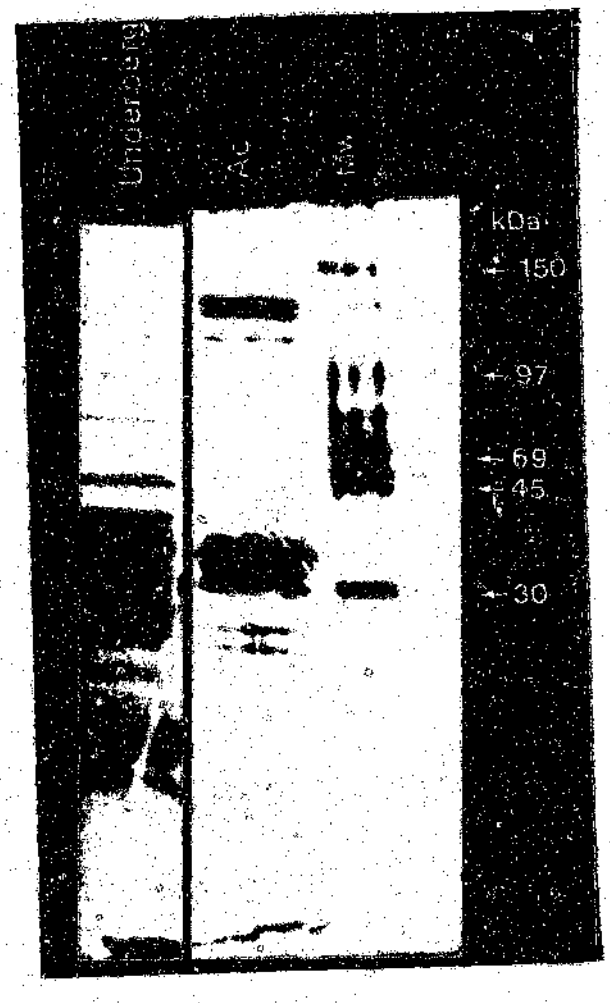

Fig. 2-7. Autoradiograph *A. marginale* Underberg and *A. centrale* ^{35}S-methionine labelled proteins, separated by electrophoresis. Molecular weight marker (MW) sizes are indicated by the arrows.

2.3.4. Immunoprecipitations with rabbit antisera

To identify radiolabelled *Anaplasma* proteins which are immunoreactive, rabbit antisera (section 2.3.2) were used to immunoprecipitate the ^{35}S-methionine-labelled *Anaplasma* proteins. These proteins were then separated by electrophoresis and visualised by fluorography. Figure 2-8 shows *A. marginale* Underberg proteins precipitated with anti-AMI as well as a number of control reactions. Lanes 2 and 3 show *A. marginale* Underberg proteins precipitated with anti-AMI however the antiserum in lane 2 was absorbed with bovine erythrocyte powder. The *Anaplasma* proteins precipitated with anti-AMI (lanes 2 and 3) had molecular weights of 75, 63, 42 and 29kDa, the 42kDa band being the most prominent. In lane 3, two additional proteins of 154 and 105kDa are detected. Antibodies to these two *Anaplasma* proteins were removed by the bovine erythrocyte powder (lane 2). Cross-reactive epitopes between erythrocyte and *Anaplasma* proteins could explain this result. In lane 4 the proteins were omitted and in lane 5 the antiserum was omitted, the absence of bands indicating that SAC alone will not precipitate any proteins. The bands observed were therefore formed by a specific reaction between the *Anaplasma* proteins and the antibodies. In lane 6 the CTRL antiserum was used and no bands were detected. Table 2-2 lists the molecular weights of the proteins identified.

Figure 2-9 shows the results of immunoprecipitation reactions of *A. centrale* and *A. marginale* with rabbit sera. *A. marginale* Underberg was used in lanes 2, 4 and 6 and *A. centrale* in lanes 3, 5, 7 and 8 (see figure legend for antisera used in each lane). *A. marginale* proteins, precipitated by anti-AME (lane 2) had molecular weights which corresponded to the proteins precipitated by anti-AMI (Fig. 2-8, Table 2-2) with the exception of the 148 and 39kDa proteins, which were recognised only by anti-AMI, and the 29kDa protein, recognised only by anti-AMI. *A. centrale* proteins were precipitated by anti-ACI (lane 3) and anti-AMI (lane 8), see Table 2-2 for the molecular weights of the proteins. The 105, 75 and 42kDa proteins were present at very low levels in all the lanes, including the CTRL antiserum lane (Fig. 2-9, lane 5 and 6). These therefore appear to be non-specific bands.

Lanes 9 and 10 show the labelled proteins of *A. centrale* and *A. marginale* Underberg (not immunoprecipitated); the bands that gave the strongest signal were different to the bands that were precipitated by the antisera. This indicates that the most abundant proteins were not the most immunogenic. Although proteins could be identified by immunoprecipitation with the rabbit antisera (Figs. 2-8 and 2-9), the intensities of these bands was very low. The bands in Fig. 2-8 were stronger than those in Fig. 2-9, the intensities of the proteins on the gels differ and some reaction with CTRL serum was also observed. We are therefore forced to conclude that immunoprecipitation of labelled *Anaplasma* proteins with the rabbit antisera was not very reproducible.

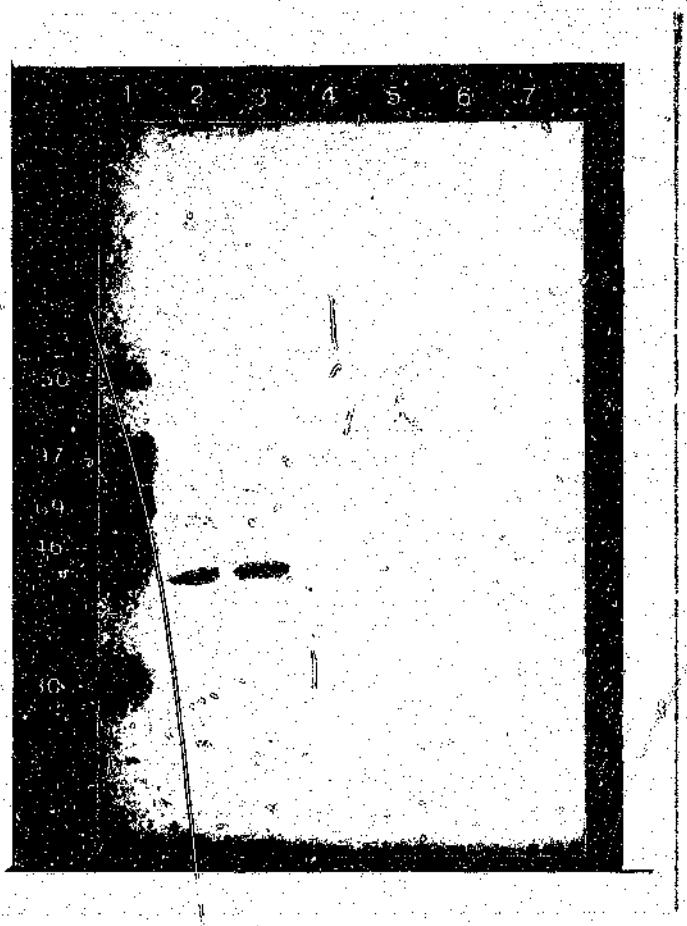

Fig. 2-8. Autoradiograph of *A. marginale* Underberg ^{35}S-methionine labelled proteins immunoprecipitated with rabbit antisera and separated by electrophoresis. Lane 1 shows ^{14}C labelled molecular weight markers and the sizes are indicated by the arrows. In lane 4 the proteins were omitted from the reaction and lane 7 shows the uninfected erythrocyte control culture, not immunoprecipitated. Antiserum used in precipitation reactions were as follows:

Antiserum

lane 2. anti-AMI, absorbed with bovine erythrocyte powder.

lane 3. anti- AMI

lane 4. anti- AMI

lane 5. no antiserum

lane 6. CTRL

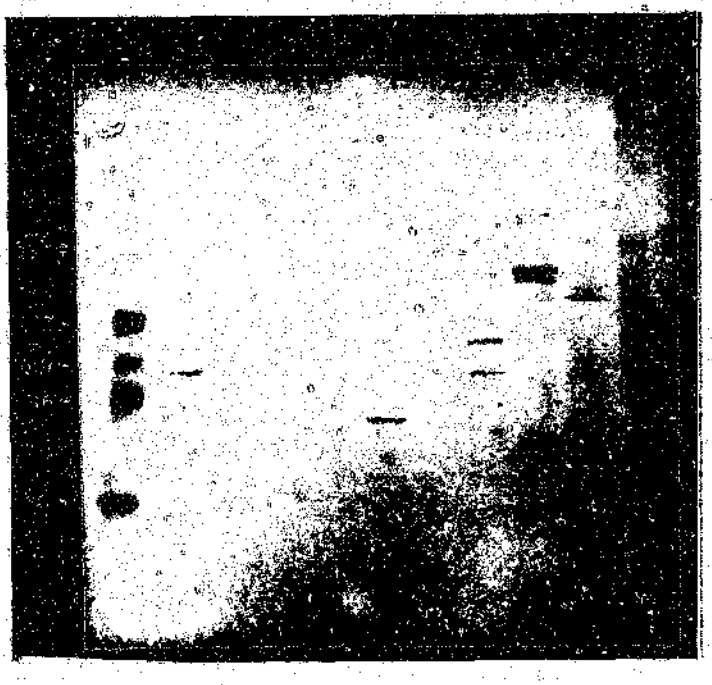

Fig. 2-9. Autoradiograph of *A. marginale* Underberg and *A. centrale* ^{35}S-methionine labelled proteins immunoprecipitated with rabbit antisera and separated by electrophoresis. Lanes 1 and 11 shows ^{14}C labelled molecular weight markers and the sizes are indicated by the arrows. The proteins and antisera used were as follows:

Proteins	Antiserum
lane 2: Underberg	anti-AME
lane 3: *A. centrale*	anti-ACE
lane 4: Underberg	CTRL
lane 5: *A. centrale*	CTRL
lane 6: Underberg	anti-ACE
lane 7: *A. centrale*	anti-AME
lane 8: *A. centrale*	anti-AM1
lane 9: *A. centrale*	not immunoprecipitated
lane 10: Underberg	not immunoprecipitated

Table 2-2 List of the molecular weights of *A. centrale* and *A. marginale* Underberg proteins immunoprecipitated by rabbit antisera.

Molecular weight in kDa	
A. centrale	*A. marginale*
154	154
148	148
127	
105*	105*
75*	75*
63	63
42*	42*
	39
36	
	29

* Also precipitated by the CTRL antiserum.

2.3.5. Immunoprecipitations with bovine antisera

To identify radiolabelled *Anaplasma* proteins that reacted with bovine antisera, immunoprecipitations were attempted using the bovine antisera described in section 2.2.1. The results are shown in Fig. 2-10. Antisera from three different *Anaplasma*-infected bovines were used for each isolate. The *A. marginale* Underberg proteins precipitated with homologous antisera (lanes 2 to 5) gave stronger signals than the *A. centrale* proteins precipitated with homologous antisera (lanes 6 to 9). The *A. marginale* Underberg proteins recognised by the bovine sera had molecular weights of 154, 148, 134, 105, 92, 82, 63, 57, 42, 39, 32 and 29kDa. The dark spot in lane 4 was caused by contamination in the cassette. No such spot was observed on a shorter exposure in a different cassette (results not shown). The *A. centrale* protein bands were much less intense and had molecular weights of 148, 82, 63, 42, 36 and 32kDa. The antiserum used in lanes 2 and 6 was the pre-infection antiserum from the *A. marginale* Underberg-infected animal (section 2.2.1). This pre-infection control serum also resulted in precipitation of proteins, the molecular weights being 134, 82, 63, 57, 42, 39 and 29kDa (Fig. 2-10, lanes 2 and 6). The reaction of *Anaplasma* proteins with control antiserum will be considered in the next section. The bands which were not recognized by control antiserum (the 154, 148, 134, 105 and 92 kDa proteins) may be *Anaplasma* specific but this conclusion needs to be treated with caution.

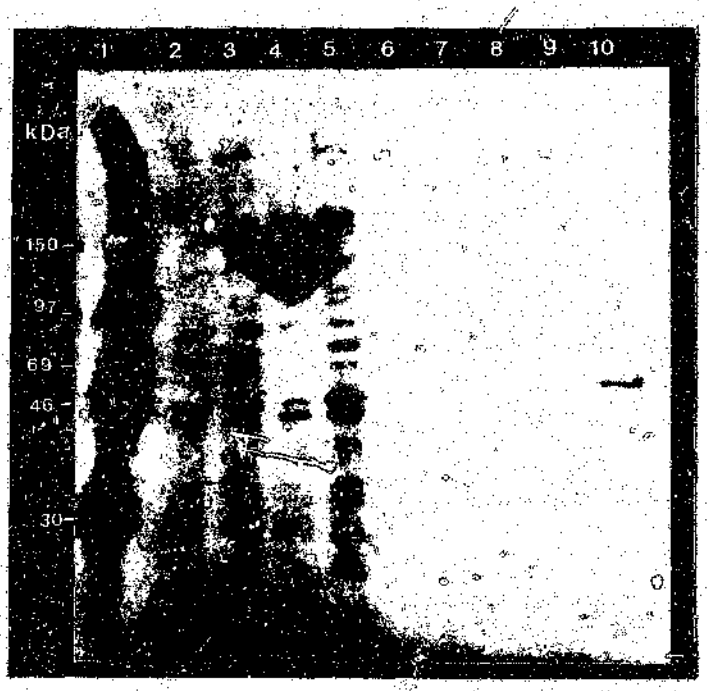

Fig. 2-10. Autoradiograph of *A. marginale* Underberg and *A. centrale* ^{35}S-methionine labelled proteins immunoprecipitated with bovine antisera and separated by electrophoresis. Lane 1 shows ^{14}C labelled molecular weight markers and the sizes are indicated by the arrows. The proteins and antisera used were as follows: lanes 2 to 5, *A. marginale* Underberg proteins, preinfection bovine antiserum in lane 2 and antisera from 3 different *A. marginale* Underberg infected bovines in lanes 3, 4 and 5, lanes 6 to 9 *A. centrale* proteins, lane 6, preinfection bovine antiserum and lanes 7, 8 and 9 antisera from 3 different *A. centrale* infected bovines. In lane 10 *A. marginale* Underberg proteins were immunoprecipitated with the same antiserum as in lane 7.

4. DISCUSSION

Rabbit antisera was produced even though bovine antisera from the *Anaplasma* infected animals were available. Bovine antisera only comprise antibodies against proteins that are recognised by the animal in the immune response due to the *Anaplasma* infection. Rabbit antisera would hopefully recognise a wider range of *Anaplasma* proteins. Palmer and McGuire (1984) made rabbit antiserum against *A. marginale* which was capable of neutralising the infectivity of initial bodies for bovines. Antisera against the South African isolates were made in a similar way, these were anti-ACE, anti-ACI, anti-AME and anti-AMI. It was hoped that these rabbit antisera would recognise different proteins, as compared to those which reacted with the bovine antisera, and perhaps also different sets of proteins in the different *Anaplasma* isolates. It was realised that the rabbit antiserum was produced against *Anaplasma* initial bodies which contained some bovine erythrocyte proteins. However this was accepted as inevitable because of the loss of infectivity and serological activity have been shown to occur in very pure preparations (Budden and Dimopoullos, 1977; Hart *et al.*, 1981).

While radiolabelled *Anaplasma* proteins were precipitated by some of the rabbit antisera the negative control serum (CTRL) also precipitated two proteins (75 and 42kDa) which appeared to be *A. marginale* and *A. centrale* proteins (Fig. 2-9, lanes 4 and 5). These bands are not visible in the *A. marginale* lane in Fig. 2-8 (lane 6). The bands in Fig. 2-9 (lane 4 and 5) are very faint and might also be visible in Fig. 2-8 if it were exposed for longer. This appears to be a non-specific reaction; these proteins are abundant on a Coomassie blue stained gel in *A. marginale* Underberg and *A. centrale* (Fig. 2-3) and could associate with the antibodies non-specifically. Alternatively they could be associated with erythrocyte membrane fractions which are pelleted together with SAC during the washing steps.

The observation that pre-infection bovine antiserum also precipitated labelled proteins (Fig. 2-10, lanes 2 and 6) was unexpected. The animal was seronegative for *Anaplasma* (card agglutination test) and no *Anaplasma* DNA could be detected using an *Anaplasma* specific DNA probe. The history of the animal may offer an explanation. It was vaccinated with *Corynebacterium pyrogenes* vaccine, which is a general immunostimulant, and it had an *Eperythrozoon* infection. *Eperythrozoon* also belong to the family Anaplasmataceae. Goff *et al.* (1986) shows by agglutination experiments that erythrocytes from *Eperythrozoon wenyoni*-infected bovines had altered agglutination properties and therefore altered erythrocyte membranes. They obtained similar results with *Anaplasma* infected erythrocytes. Thus the pre- tion sera might have contained antibodies that recognised altered membranes and the labelled *Anaplasma* proteins were associated with erythrocyte membrane fractions which were pelleted together with SAC during the washing steps. Other possibilities are that the animal had been exposed to *Anaplasma* or that the pre-infection antiserum contained antibodies that cross-reacted with

Anaplasma proteins at a low level not detectable by the card agglutination test.

Table 2-2 compares the *A. marginale* Underberg and *A. centrale* proteins precipitated by rabbit antisera. Several common proteins were precipitated but some proteins were different between the two isolates. Palmer and McGuire (1984) identified *Anaplasma* proteins with molecular weights of 105, 86, 61, 36 and 31kDa by immunoprecipitation with rabbit antisera prepared in a manner similar to the rabbit antisera used in this study. Similar sized proteins were identified in the isolates in this study: Palmer and McGuire's 86kDa protein could be equivalent to our 82kDa protein, their 61kDa equivalent to our 63kDa and their 36kDa equivalent to our 42kDa.

The approach followed in this chapter had some drawbacks. It was necessary to use fresh blood from infected bovines in order to establish the *in vitro* cultures for labelling. The rabbit antiserum, prepared against the parasites from the same culture, took more than 80 days to produce (with altered schedule 2.2.3.3). Blood from the same animal was used to establish the cultures for labelling and as source of antigen for production, so the labelled cultures had to be stored untill the antisera were available, hence the signals produced by the labelled proteins were weak. Because of the unexplained reactions with control rabbit and bovine antisera it is not possible to state conclusively that our experiments identified *Anaplasma* proteins. We decided to adopt alternative approaches, free from the difficulties experienced here, and these are discussed in chapters 3 and 4.

CHAPTER THREE

IMMUNOBLOTTING WITH MONOSPECIFIC ANTISERA

3.1. INTRODUCTION

In this chapter we describe a different approach which was followed to identify *Anaplasma* specific proteins because of the problems encountered with the immunoprecipitations as discussed in chapter two. The rabbit antiserum against *A. marginale* Underberg initial bodies (anti-AMI, section 2.2.3.3.) was used on immunoblots to identify *Anaplasma* specific proteins and to compare the proteins of several isolates. The resolution of the immunoblots was improved (compared to the immunoblots in chapter 2) by using bigger, gradient gels, enabling clear identification of proteins. If the identified *Anaplasma* specific proteins are common to all the isolates that might be encountered in the field, they could be useful in the development of improved vaccines and serological tests. So after the proteins were identified, monospecific antisera against the specific *A. marginale* Underberg proteins were made. These monospecific sera were again used on immunoblots to look for homologous proteins in the other isolates. In addition to *A. centrale* and *A. marginale* Underberg, all the other *A. marginale* isolates that were available were compared. These included *A. marginale* Stuart, 7812-1, Barkly West and Escourt isolates which were isolated in 1976, 1990, 1981 and 1984 respectively (De Waal, D.T., personal communication).

Immunoblots were mainly used in this study and a new substrate for detection was used that increased the sensitivity of the blots. Immunoblots are protein blots that are treated with specific antibodies; the primary antibody which reacts with the protein on the membrane is visualised by a secondary anti-antibody that is conjugated to an enzyme which reacts with a specific substrate. A frequently used conjugate enzyme is horseradish-peroxidase which may be detected by reaction with 4-chloro-1-napthhol to form a purple precipitate (the use of this reagent is described in Chapter 2). An alternative and newly developed detection method, enhanced chemiluminescence (ECL, Amersham), uses a luminol-containing substrate instead of the 4-chloro-1-naphthol. The conjugated horseradish-peroxidase catalyses the oxidation of luminol, a cyclic diacylhydrazide, in the presence of hydrogen peroxide. After oxidation, the luminol is in an excited state which decays to the ground state via a light emitting pathway. The light emitted can be captured on X-ray film. The process is represented schematically in Fig. 3-1. This method of detection is reputed to be ten times more sensitive than colorimetric methods and results can be obtained within minutes after addition of substrate (ECL, Western blotting detection system instruction manual, Amersham).

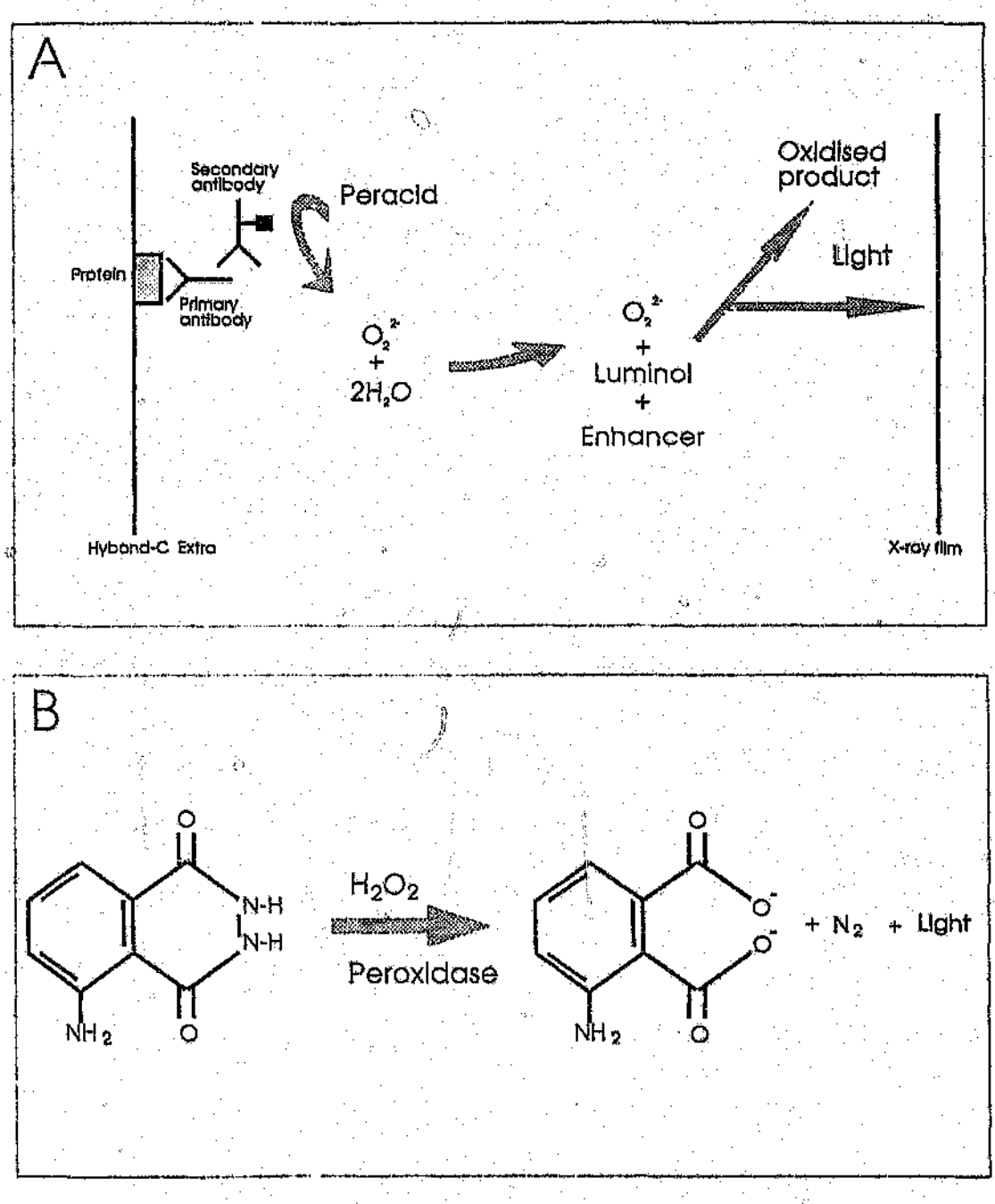

Fig. 3-1. Schematic representation of a) the principles of the ECL detection method and b) the oxidation of luminol. Diagrams copied from the ECL. Western blotting protocols booklet, Amersham

The system was used on some of the immunoblots in this study and it did prove to be a much more sensitive detection method than using 4-chloro-1-naphthol as the substrate for peroxidase. Hybond C-super. nitrocellulose cast on an inert support, was used instead of normal nitrocellulose because it was found to be more sensitive and much easier to handle.

In this study *A. marginale* Underberg proteins were separated by electrophoresis, the bands cut out and injected into rabbits or goats to produce monospecific antisera. To achieve better separation between some proteins isoelectric focusing was used, a technique which separates proteins according to their isoelectric points on a pH gradient. The monospecific antisera were used to probe immunoblots of different *Anaplasma* isolates in order to identify proteins that cross-reacted.

3.2. MATERIALS AND METHODS

3.2.1. Source of parasites and initial body isolation

Infected and uninfected bovine blood and bovine sera were donated by the Protozoology section, Veterinary Research Institute, Onderstepoort. *A. centrale* and *A. marginale* Underberg were obtained and treated as in section 2.2.1 and 2.2.2. For each of the *A. marginale* isolates used (Stuart, 7812-1, Barkly West and Escourt) 10 ml of frozen stabilate was obtained and initial bodies were isolated as described in section 2.2.2.

3.2.2. Polyacrylamide gel electrophoresis and transfer of proteins to nitrocellulose membranes

The initial bodies were prepared for electrophoresis as described in section 2.2.4. Protein concentrations of lysed initial bodies were determined by reading the absorbance at 260nm and 280nm and using the formula (Harlow and Lane, 1988):

$$(1.55 \times A_{280}) - (0.76 \times A_{260}) = x \text{ mg/ml}$$

PAGE was performed as described in section 2.2.10. Proteins were transferred to nitrocellulose (Hybond-C or Hybond-C Super, Amersham) as described in section 2.2.12. Protein molecular weight markers were detected on nitrocellulose membranes by staining with India ink (Hancock and Tsang, 1985) as described in section 2.2.14.

3.2.3. Coomassie blue staining

Polyacrylamide gels were stained with Coomassie blue as described in section 2.2.13 or by a quick method as follows: after electrophoresis gels were rinsed three times in distilled water and incubated in 0.05% Coomassie brilliant blue R (SIGMA) in water at room temperature for 10min with shaking. The gels were destained in water until bands were visualised (Harlow and Lane, 1988).

3.2.4. Electroelution of proteins

After preparative gel electrophoresis, gels were stained by the quick Coomassie blue method, proteins bands cut out (see section 3.3.3. for molecular weights) and the proteins electroeluted from the gel using a Biotrap (Scheicher & Schuell). This incorporates an inert membrane (BT2, Scheicher & Schuell) through which the proteins pass before being stopped by a second membrane (BT1, Scheicher & Schuell). The gel pieces were placed in the chamber in elution chamber buffer (25mM Tris, 192mM glycine, 0.1% SDS, pH 8.3) and the protein collected in the same buffer. The Biotrap was half submerged in the buffer and subjected to 80V for 16h or 200V for 4h. Before removal of the samples the current was reversed for a few seconds to remove proteins sticking to the outer membrane.

3.2.5. Isoelectric focusing

Isoelectric focusing (IEF) was performed as described by Guilian *et al.* (1984) in a vertical polyacrylamide slab gel system under denaturing conditions. The gel solution contained 10% glycerol, 2% Triton X-100 (Merck), 8M urea, 5.5% acrylamide (38:1, acrylamide:bis), 2.4% ampholytes pH 3.5 to 9.5 (Bio-lyte 3/10, Biorad), 188mM TEMED and 40mM APS. The solution was degassed before addition of TEMED and APS. The cathode buffer consisted of 0.02M sodium hydroxide, degassed before use; the anode buffer consisted of 0.02M acetic acid. Samples were mixed with an equal volume of freshly made sample buffer (15% glycerol, 2% Triton X-100, 8M urea, 15mM dithiothreitol, 2.4% ampholytes). The gels were pre-focused for 15min at 200V, 30min at 300V and 30min at 400V, the upper (cathode) buffer was then replaced, the samples loaded, and the gels focused for 15 hours at 400V and 1 hour at 800V. The gels were stained with Coomassie blue using freshly made solutions as follows: the gels were fixed with 20% trichloroacetic acid for 30min, transferred to solution A (33% ethanol, 10% acetic acid) containing 0.25% SDS for 10 to 30min, rinsed four times for 15min in solution A, stained for 30min in solution A containing 0.05% Coomassie blue and destained in 33% ethanol, 10% acetic acid and 10% glycerol.

3.2.6. Production of antiserum

3.2.6.1. Rabbit antiserum against *A. marginale* Underberg proteins

A. marginale Underberg proteins (1.5mg), separated by preparative PAGE, were stained by the quick Coomassie blue method (see section 3.2.3). Six protein bands were cut out (see section 3.3.2) and PBS added. The gel pieces were passed consecutively through 15, 21 and 25 gauge needles and the homogenate inoculated into rabbits. Six rabbits were used, each rabbit received a different protein and was inoculated on days 1, 28 and 56. The rabbits were bled as described in section 2.2.3.3.

3.2.6.2. Goat antiserum against *A. marginale* Underberg proteins.

Proteins in the 38 to 42kDa region, electroeluted from preparative gels (see section 3.2.4), were separated by isoelectric focusing (see section 3.2.5.), the two major bands cut out and treated as in section 3.2.6.1. Each of two goats was inoculated with one of the proteins on days 1 (0.25 mg mixed 1:1 with complete Freund's adjuvant) and 28 (0.25 mg mixed 1:1 with incomplete Freund's adjuvant). The goats were bled before inoculation and subsequently on days 21 and 40.

3.2.6.3. Preparation of antiserum

Blood was collected and left to clot at room temperature for one hour. The clot allowed to contract at 4°C for at least an hour and then centrifuged at 3000xg for 10min. Hipack protein-A and protein-G LTQ columns (Chromatochem) were used according to the manufacturers instructions to isolate IgG from both rabbit and goat sera

3.2.7. Immunoblotting

Immunoblots were treated as described in section 2.2.15. Two substrates were used for the peroxidase conjugated secondary antibodies 4-chloro-1-naphthol as described in section 2.2.15. and enhanced chemiluminescence (ECL, Amersham) which was used according to the manufacturers instructions and exposed to X-ray film (Cronex 4).

3.3. RESULTS

3.3.1. Comparison of isolates by immunoblotting

Six isolates of *Anaplasma* (see section 3.2.1) were compared on an immunoblot with anti-AMI (see Table 2-1). Protein concentrations of the lysed initial body preparations were approximated by UV absorbance and 20-30µg of each sample was loaded per lane. Figure 3-2 shows a 7-17.5% gradient gel, stained with Coomassie blue, on which samples of all six isolates, and an erythrocyte control sample (RBC) have been separated. Figure 3-3 shows the duplicate gel immunoblotted with anti-AMI at 1/200 dilution followed by detection with 4-chloro-1-naphthol. The most prominent bands are in the 40kDa region. Anti-AMI reacted strongly with 41, 39, 38 and 33kDa proteins in the homologous isolate (Underberg lane) but only with the 39kDa proteins of the heterologous *A. marginale* isolates. In *A. centrale* a 38kDa protein was recognised. The 61kDa protein seemed to be an *Anaplasma* common protein and the 70kDa protein seemed to occur in all *A. marginale* isolates (giving only a very faint signal in the Underberg isolate). The proteins between 70 and 100kDa varied significantly; 79, 94 and 101kDa in Barkly West, 92kDa in Am7812/1, 82kDa in Stuart, 82 and 85kDa in Underberg and 87kDa in *A. centrale*. The 87kDa band was also present in the RBC lane and appeared to be an erythrocyte protein.

3.3.2. Rabbit antiserum against *A. marginale* Underberg proteins

The major proteins in *A. marginale* Underberg identified by immunoblotting (Fig. 3-3) were of molecular weights 85, 82, 61, 41, 39 and 33kDa. each of these proteins was cut out of a gel (see section 3.2.6.1) and inoculated into a rabbit. Only the rabbit inoculated with the 82kDa protein produced antibodies. it seems likely that the other proteins were either insufficiently immunogenic or the amount of protein inoculated was too small. IgG was isolated from the serum of the rabbit that produced antibodies and designated anti-82U. this was used to probe an immunoblot (Fig. 3-4a) and signals were detected using the ECL substrate. Part of the immunoblot was used as a control (Fig. 3-4b), probed with the preinoculation antiserum.

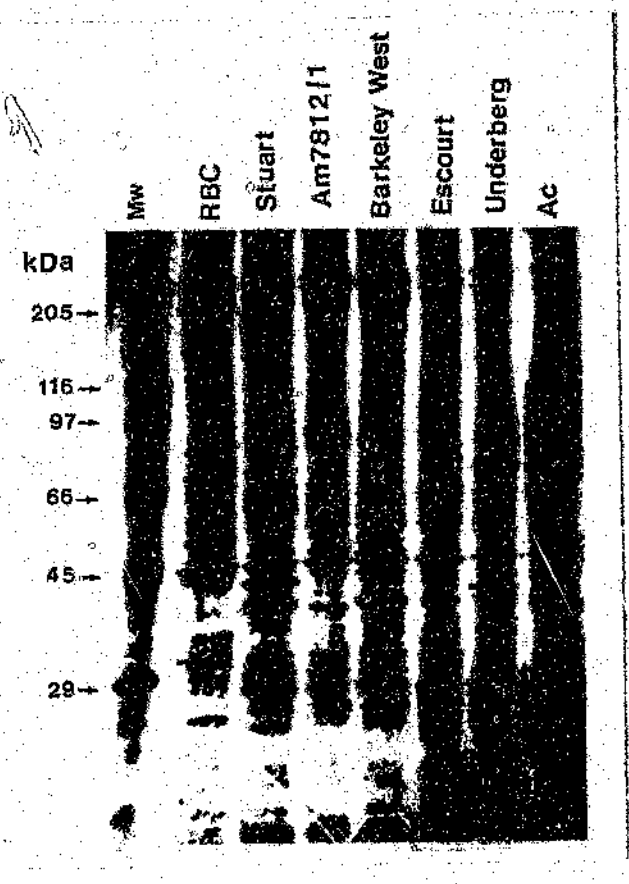

Fig. 3-2. Coomassie blue stained 7%-17.5% gradient polyacrylamide gel of proteins from uninfected erythrocytes (RBC). *A. marginale* Stuart, 7812/1, Barkly West, Escourt and Underberg isolates and *A. centrale* (AC). The molecular weight marker (mw) sizes are indicated by the arrows.

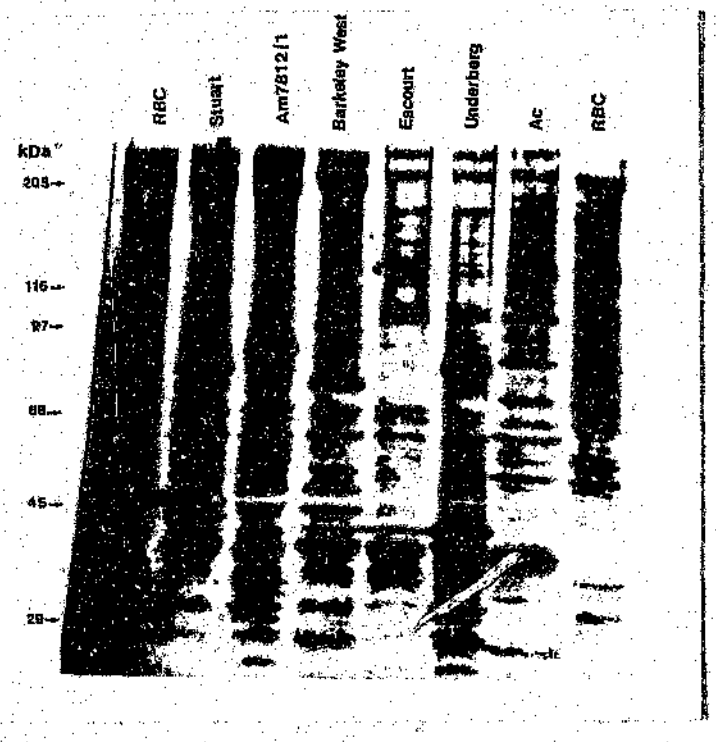

Fig. 3-3. Immunoblot of a 7%-17.5% gradient polyacrylamide gel of proteins from uninfected erythrocytes (RBC), *A. marginale* Stuart, 7812/1, Barkly West, Escourt and Underberg isolates and *A. centrale* (AC), blotted with anti-AM1 at 1/200 dilution. The position of the molecular weight markers and sizes are indicated by the arrows.

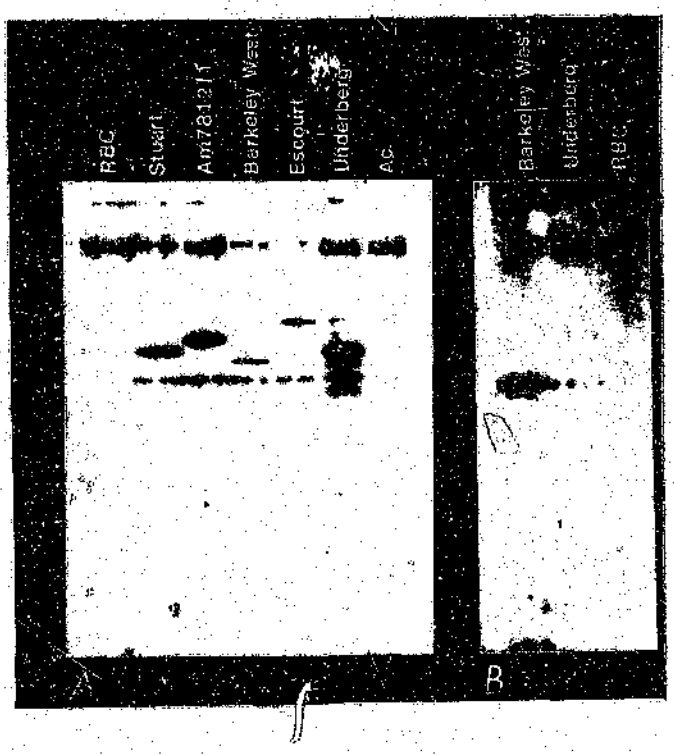

Fig. 3-4. *Immunoblot of a 7%-17.5% gradient polyacrylamide gel of proteins from uninfected erythrocytes (RBC), A. marginale* Stuart, 7812/1, Barkly West, Escourt and Underberg isolates and *A. centrale* (AC), reacted in panel A with anti-82U IgG and in panel B with the pre-inoculated antiserum. The antibodies were detected with ECL. The position of the molecular weight markers and sizes are indicated by the arrows.

It appears that the preinoculation serum contained antibodies reacting with the 69kDa *Anaplasma* protein and also with proteins in the 200kDa region, some of which were apparently bovine erythrocyte proteins (Fig. 3-4b). Figure 3-4a shows total proteins from six *Anaplasma* isolates immunoblotted with anti-82U. Bovine erythrocyte proteins were detected, these were in the same 200kDa region as those detected by the preinoculation serum. In the homologous reaction with Underberg proteins the anti-82U serum generated a strong signal at 82kDa as expected but there were also three closely associated lower bands. These lower bands could be breakdown products of the 82 kDa protein or the protein sample used for immunising the rabbit may have contained some of the lower proteins. *A. marginale* Stuart contains a protein of a similar molecular weight that cross-reacts with anti-82U, *A. marginale* 7812/1, Barkly West, Escourt and *A. centrale* have cross-reactive proteins of varied sizes: 92, 79, 108 and 112kDa respectively. The reaction with *A. centrale*, *A. marginale* Barkly West and Escourt proteins is weak compared to the other isolates, suggesting that the reactive epitopes of these isolates differ more from those of Underberg than do the Stuart and Am7812/1 epitopes. The size variation corresponds to the variation seen in Fig. 3-3 in the 70 to 100kDa region, so it is possible to relate the immunogens reacting with the monospecific antiserum (anti-82U) woth those reacting with the polyclonal antiserum (anti-AMI).

3.3.3. Goat antiserum against *A. marginale* Underberg proteins

The proteins in the 38 to 42kDa region were very prominent on Coomassie blue stained gels (Fig. 3-2) and on immunoblots (Fig. 3-3). The attempt to produce antiserum against the 41 and 39kDa proteins in rabbits failed, so a further attempt was made using goats. The proteins were difficult to separate by PAGE, so IEF was used in an attempt to achieve better discrimination. The *A. marginale* Underberg proteins in the 38 to 42kDa region were cut from preparative polyacrylamide gels, electroeluted from the gel and further separated by IEF. Figure 3-5 shows the eluted proteins separated on a 10% polyacrylamide gel, stained with Coomassie blue in lane 2 and probed with antiserum from the *A. marginale* Underberg infected bovine in lane 3. Figure 3-6 shows the separation achieved by IEF. The two most prominent bands (a and b in Fig. 3-6) were used to inoculate goats but only the animal inoculated with band 'a' demonstrated any reaction with *A. marginale* Underberg proteins. IgG was prepared from serum taken form this animal on day 70, this was designated anti-42U. Figure 3-7b shows that IgG isolated from the preinoculation serum of the goat did not react with proteins of *A. marginale* Barkly West, *A. marginale* Underberg or the erythrocyte control. Figure 3-7a shows the reaction of anti-42U with the different isolates on an immunoblot.

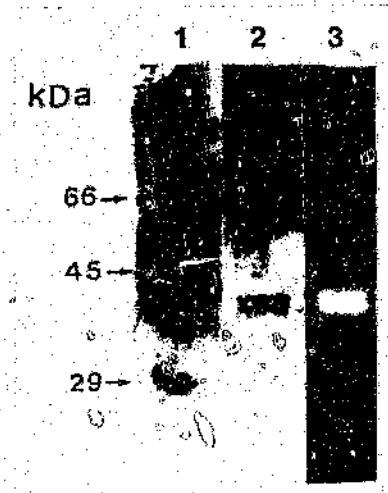

Fig. 3-5. Eluted proteins (38 to 42kDa) separated by electrophoresis, lane 2, the Coomassie stained gel and lane 3, immunoblotted with antiserum from an *A. marginale* Underberg infected bovine (section 2.2.1) and detected with ECL. Lane 1 shows the molecular weight markers and sizes are indicated by the arrows

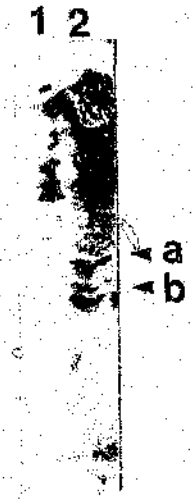

Fig. 3-6. Coomassie blue stained gel of eluted proteins (38 to 42kDa) separated by IEF, lane 1, pI markers (Pharmacia) and lane 2, the 38 to 42kDa region of proteins. Arrows indicate proteins used for antiserum production.

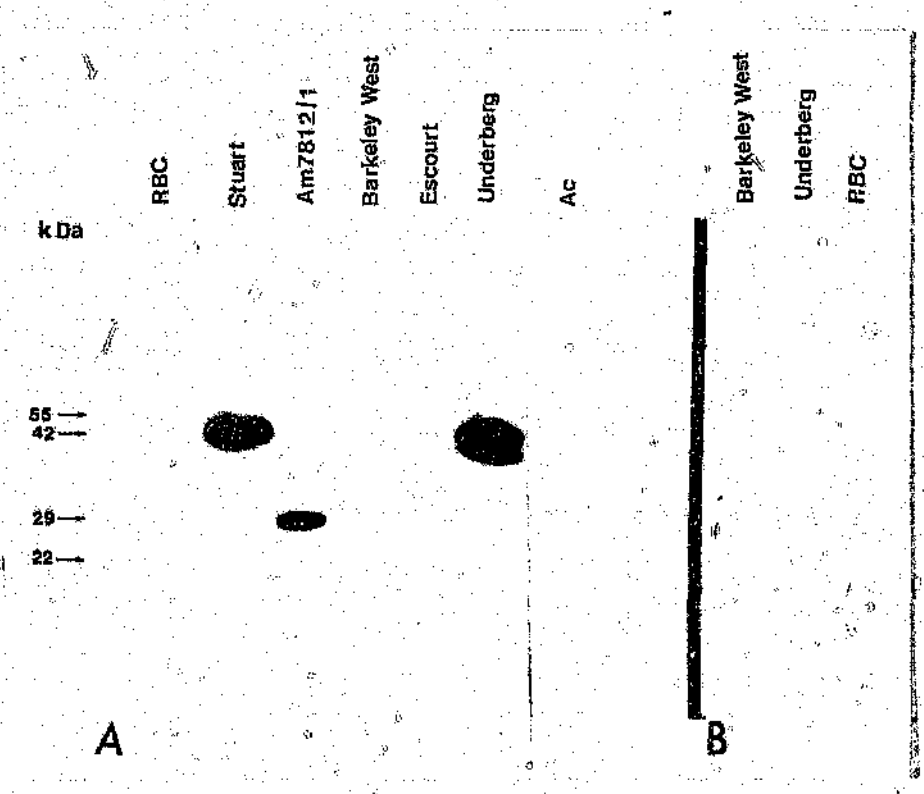

Fig. 3-7. Immunoblot of a 7%-17.5% gradient polyacrylamide gel of proteins from uninfected erythrocytes (RBC), *A. marginale* Stuart, 7812/1, Barkly West, Escourt and Underberg isolates and *A. centrale* (AC), blotted with the pre-inoculated antiserum in panel B and with anti-42U IgG in panel A. The antibodies were detected with ECL. The molecular weight marker (mw) sizes are indicated by the arrows.

Anti-42U reacted with a protein of 42kDa in both *A. marginale* Underberg and *A. marginale* Stuart and with proteins of molecular weights 29 and 22kDa in *A. marginale* 7812/1. No reaction was detected between anti-42U and erythrocyte control, *A. marginale* Barkly West, *A. marginale* Escourt and *A. centrale* proteins. *A. centrale* and the Barkly West and Escourt isolates cross-reacted very weakly with anti-82U (Fig. 3-4a). All the isolates produced prominent signals with anti-AMI in the 40kDa region (Fig. 3-3), *A. marginale* 7812/1 being the least reactive. These signals were not evident with anti-42U (Fig 3-7a). This indicates that anti-42U contains a different panel of antibodies to the proteins in the 40kDa region than anti-AMI. This is not unreasonable, since separated denatured proteins were used as inoculum for anti-42U whereas whole initial bodies were used for anti-AMI.

The lower protein bands of 29 and 22kDa of Am 7812/1 in Fig. 3-7a are not prominent in Fig. 3-3, possibly indicating that the antibodies were made against a minor protein component of the 40kDa region of *A. marginale* Underberg. The two bands seen in the 38-42kDa region on the 10% acrylamide gel shown in Fig. 3-5 were resolved into four bands by IEF (Fig. 3-6). It therefore appears likely that the 29 and 22kDa proteins of *A. marginale* 7812/1 have common epitopes with proteins of *A. marginale* Underberg in the 40kDa region.

3.4. DISCUSSION

Antisera, made against *A. marginale* Underberg initial bodies (anti-AMI) and against separated *A. marginale* Underberg proteins (anti-82U, anti-42U), were able to demonstrate distinct immunogenic differences among the isolates compared in this study. Anti-AMI showed extensive cross-reactivity between the isolates. The 39 and 61kDa proteins seem to be *Anaplasma*-common proteins and they are of similar molecular weights in all of the isolates. Proteins in the 70 to 100kDa region of all the isolates reacted with anti-AMI but the molecular weights varied considerably. This is similar to results obtained in the USA where the 105 and 100kDa proteins of the Florida isolate varied in size (see section 1.13; Oberle *et al.*, 1988; Allred *et al.*, 1990).

Antiserum was successfully produced against two *A. marginale* Underberg proteins of molecular weights 42 and 82kDa (anti-82U and anti-42U). The *A. marginale* Stuart isolate contained two proteins which cross-reacted with anti-82U and anti-42U and which had the same molecular weights as those in the Underberg isolate. This suggests that *A. marginale* Stuart and Underberg shared more epitopes with each other than with the other isolates. *A. marginale* Barkly West, *A. marginale* Escourt and *A. centrale* reacted weakly or not at all with anti-82U and anti-42U. This was unexpected as anti-AMI reacted equally well with all the isolates. Anti-42U might have been detecting a minor protein component of the 40kDa region. This is feasible since others have also found that this region consists of many proteins. Adams *et al.* (1986) found that bovine antisera reacted with proteins in the 40kDa region as either a diffuse band or a doublet. Shkap *et al.* (1991) also identified a group of proteins in this region and proposed that it consisted of more than one protein.

The antisera that we were able to produce showed that the isolates contained proteins having varying molecular weights but with shared epitopes. This is not an unexpected observation, size variations have also been observed among the USA isolates (Oberle *et al.*, 1988). Allred *et al.* (1990) sequenced the genes coding for the MSP-1a protein of four USA *Anaplasma* isolates and showed that the number of copies of tandem repeated sequences is responsible for size variations. Our results also indicate that *A. marginale* Barkly West, *A. marginale* Escourt and *A. centrale* are less closely related to *A. marginale* Underberg than are *A. marginale* Stuart and *A. marginale* 7812/1. Neither anti-42U nor anti-82U cross-react with all the isolates tested so they would not be useful in the development of an improved vaccine or serological test.

Unfortunately not a lot of information is generated by these results. In addition, our low success rate in the production of antibodies in both rabbits and goats suggested that a change of approach was necessary. When it was found that the increased sensitivity of ECL detection made it possible to use bovine antisera to probe immunoblots we decided to compare our *Anaplasma* isolates in a different fashion, as discussed in the next chapter.

CHAPTER FOUR

ANTIBODY DEVELOPMENT DURING COURSE OF INFECTION

4.1. INTRODUCTION

In this study we examined the development of antibodies in the bovine host during during the course of an *Anaplasma* infection. This approach was expected to be able to identify the immunogenic proteins responsible for stimulating the humoral immune response in the host. Those proteins that induce antibody production could be likely candidates for use as antigens in improved serological tests and could be useful in the development of improved vaccines.

Antiserum samples were collected weekly from *Anaplasma*-infected bovines and used to probe immunoblots to monitor the development of antibodies to *Anaplasma* as the infection progressed. During the initial part of our study the signals obtained with bovine sera on immunoblots, using the 4-chloro-1-naphthol detection system, had been too low to be useable. The increased sensitivity of immunoblotting using Hybond C-super and the ECL detection system (discussed in chapter 3) made it possible to use bovine antisera to detect proteins by immunoblotting.

Several researchers have looked at the antibody levels in *Anaplasma* infected bovine sera, at times ranging from day 23 to day 101 post infection and also in carrier animals (Nakamura *et al.*,1991, Adams *et al.*, 1986, McGuire *et al.*, 1991, Shkap *et al.*, 1991, Palmer *et al.*, 1985 and Adams *et al.*, 1989). None of the studies followed the course of infection and the detection methods used were not as sensitive as the ECL method used in this study. This is the first study which monitors the development of antibody production during the course of infection with the South African *Anaplasma* isolates.

Anaplasma centrale, *Anaplasma marginale* Underberg and *Anaplasma marginale* Barkly West were the three isolates compared in the experiments considered in this chapter. The origin of *A. centrale* and *A. marginale* Underberg are discussed in chapter one. *A. marginale* Barkly West was chosen because it is one of the less virulent isolates, even though it produces a much higher peak parasitaemia than does *A. marginale* Underberg. *Anaplasma marginale* Barkly West has not been passaged further through cattle

The serum samples were taken from day 1 (the day of infection) through the prepatent period as the organism multiplied and up to peak parasitaemia, at which time the animals were treated with tetracycline. After tetracycline treatment the bovines recovered but remained carriers of the disease. With this approach one can determine the time it takes for the animal to produce detectable antibodies against *Anaplasma* and which proteins appear to be the most immunogenic. This immunogenicity can also be compared between the isolates.

4.2. MATERIALS AND METHODS

4.2.1. Source and isolation of parasites

A. marginale Barkly West was isolated during an anaplasmosis outbreak in the Barkly West district (Cape Province) in 1981 (Potgieter *et al.*, 1981). Ticks were collected and fed on susceptible animals which resulted in *A. marginale* infections from which blood stabilates were made.

Infected and uninfected bovine blood and bovine sera were donated by the Protozoology section, Veterinary Research Institute, Onderstepoort. The *A. centrale* vaccine strain and *A. marginale* Onderberg isolates were obtained as described in section 2.2.1. *A. marginale* Barkly West blood (70% parasitaemia) was obtained from a bovine infected with the original *A. marginale* Barkly West stabilate from 1980. The method of Potgieter and van Rensburg (1983) was followed for isolation of initial bodies as described in section 2.2.2.

4.2.2. Determination of protein concentration

The protein concentration of the initial body preparations was determined by the filter paper dye-binding assay, described by Minamide and Bamburg (1990). The initial bodies were first lysed with one tenth volume of lysis buffer (see section 2.2.4.) before following the method. BSA was used to determine the standard curve. A dilution series for each sample was spotted in triplicate on to Whatman No. 1 filter paper which was then rinsed in methanol for 10 seconds and air dried. The filter paper was incubated in staining solution (0.5% Coomassie blue, 7% acetic acid) for 30 min at room temperature with agitation, destained with 7% acetic acid until most of the background was removed and air dried. Filter paper squares containing individual spots were placed in Eppendorf tubes and the Coomassie blue eluted by adding 1ml extraction buffer (66% methanol, 33% water, 1% ammonium hydroxide). The tubes were vortexed, left for 5 min at room temperature and vortexed again. Aliquots were placed in microtitre plates and the absorbance was read at 405-600nm in an automatic reader (EAR 400 AT colorimeter, SLT - Labinstruments, Austria).

4.2.3. Polyacrylamide gel electrophoresis and immunoblotting

Initial bodies and molecular weight markers were treated as described in section 2.2.4. Electrophoresis was carried out as described in section 2.2.10., with some alterations. The stacking gels contained 5% acrylamide, the separating gels 10% acrylamide and SDS was omitted from the gel solutions, making degnerating the gels solutions easier. The SDS in the electrophoresis buffer runs ahead of the proteins in the gel, thus the proteins stays in denaturing conditions. Acrylamide solutions were deionized with 2g Amberlite MB-1 (Sigma) per 100ml and degassed before pouring the gels. Acrylamide (30%) and bisacrylamide (1%) stock solutions were made up separately and mixed to achieve 2.5% crosslinking. The separating gels contained 0.375M Tris pH 8.8, 4.4mM TEMED, 4.4mM APS, 9.76ml acrylamide stock and 7.5ml bisacrylamide stock per 30ml solution. The stacking gels contained 0.187M Tris pH 6.8, 4.4mM TEMED, 4.4mM APS, 3.26ml acrylamide stock and 2.5ml bisacrylamide stock per 20ml solution. Electrophoresis buffer and running conditions were as described in section 2.2.10. Proteins were transferred to nitrocellulose (Hybond-C Super, Amersham) with a semi-dry transfer unit (TE 70 Semiphor™, Hoefer Scientific Instruments) as described in section 2.2.12. Protein molecular weight markers were detected on nitrocellulose by staining with India ink (Hancock and Tsang, 1985) as described in section 2.2.14. Immunoblotting was performed as described in section 2.2.15. and the antibodies were detected by ECL as described in section 3.2.7.

4.2.4. Collection of bovine antiserum

Sera from bovines infected with *A. centrale* and the *A. marginale* Barkly West and Underberg isolates were donated by the Protozoology Section, VRI, Onderstepoort. Sera samples were collected before infection and then once weekly for at least three months to give a time-course series of antiserum samples. Blood was collected (10ml) and left to clot at room temperature for one hour. The clot was allowed to contract at 4°C for at least an hour and then centrifuged at 3000xg for 10 min. Aliquots of the sera were stored at -20°C

4.3. RESULTS

The antibody response of the bovines to *Anaplasma* infection was detected by immunoblotting. Proteins of *A. marginale* Underberg, *A. marginale* Barkly West, *A. centrale* and uninfected erythrocytes were separated by electrophoresis on preparative gels. Gradient gels (made as described in section 2.2.10) were used in the experiments shown in Figs. 4-1, 4-4 and 4-10, but in the remainder of the experiments 10% acrylamide gels were used as described in section 4.2.3. The resolution of the gradient gels is better than that of linear gels but it is more difficult to ensure reproducibility when pouring the gradient gels. The variations subsequently made it difficult to compare results between different gels, for which reason 10% gels were more often used.

After electrophoresis the proteins were transferred to nitrocellulose, the membranes cut into 6mm strips and each strip incubated with a different antiserum (see section 4.2.4). Equal amounts of proteins were loaded onto each preparative gel (=1.5mg) but the peak percentage parasitaemia differed between the isolates (31% to 70%). Hence the ratio between parasite and erythrocyte proteins varied in each case and this had to be taken into account when comparing the different gels. Strips for each isolate were probed with the homologous antiserum as well as with the two heterologous antisera (Figs. 4-1 to 4-12). Tables 4-1a, 4-1b, 4-2 and 4-3 show the lane numbers corresponding to Figs. 4-1 to 4-12 together with the day post-infection upon which each antiserum sample was collected and the parasitaemia of the animal on that day.

TABLE 4-1a. Table showing the days on which antisera were collected from the *A. marginale* Underberg infected bovine and the parasitaemia of the animal on that day. Lane numbers correspond to those in Figs. 4-1, 4-4 and 4-10.

A. marginale Underberg.		
Lane number	**Days post-infection.** Figs. 4-1, 4-4, 4-10	**% Parasitaemia***
1	0	-
2	7	VR
3	14	NR
4	21	31%
5	28	
6	42	
7	47	
8	62	
9	76	NR
10	81	
11	90	NR
12	97	NR
13	104	
14**	111	NR
15***	118	
16	125	

* If accurate counting of parasite numbers not possible, parasitaemias were described as very rare (VR: 1 to 3 infected cells during a 3-5 minute examination) or not rare (NR: 1 or more infected cells per 15 fields).
** Fig. 4-1 only up to lane 14
*** Fig. 4-10 only up to lane 15

TABLE 4-1b. Table showing the days on which antisera were collected from the same *A. marginale* Underberg infected bovine as Table 4-1a and the parasitaemia of the animal on that day. Lane numbers corresponds to those in Fig. 4-7.

A. marginale Underberg		
Lane number	**Days post-infection. Fig. 4-7**	**% Parasitaemia***
1	0	
2	7	VR
3	21	31%
4	42	
5	47	
6	62	
7	76	NR
8	81	
9	90	NR
10	97	NR
11	104	
12	111	NR
13	118	
14	125	1%

* If accurate counting of parasite numbers not possible, parasitaemias were described as very rare (VR: 1 to 3 infected cells during a 3-5 minute examination) or not rare (NR: 1 or more infected cells per 15 fields).

TABLE 4-2. Table showing the days on which antisera were collected from the *A. marginale* Barkly West infected bovine and the parasitaemia of the animal on that day. Lane numbers corresponds to those of Figs. 4-2, 4-5, 4-8 and 4-11.

A. marginale Barkly West.		
Lane number	**Days post-infection. Figs. 4-2, 4-5, 4-8, 4-11**	**% Parasitaemia.***
1	0	
2	20	75%
3	35	VR
4	47	
5	49	4%
6	55	
7	62	
8	69	VR
9	76	
10	83	
11	90	
12	97	
13	104	VR
14	111	R
15	118	NR
16	125	1%
17	132	NR
18	146	5%
19**	153	
20	160	R

* If parasite numbers could not be counted accurately, parasitaemia was described as very rare (VR: 1 to 3 infected cells during a 3-5 minute examination), rare (R: less than 1 infected cell per 15 fields) or not rare (NR: 1 or more infected cells per 15 fields).

** Fig. 4-11 only up to lane 19.

TABLE 4-3. Table showing the days on which antisera were collected from the *A. centrale* infected bovine and the parasitaemia of the animal on that day. Lane numbers correspond to those in Figs. 4-3, 4-6, 4-9 and 4-12.

A. centrale		
Lane number	**Days post-infection** Figs. 4-3, 4-6, 4-9, 4-12	**% Parasitaemia**
1	0	
2	7	
3	14	
4	21	5%
5	28	35%
6	35	
7	42	
8	49	4%
9	56	16%
10	63	
11	70	
12	77	
13	84	4%
14	91	
15	98	

Figures 4-1, 4-2 and 4-3 show *A. marginale* Underberg proteins probed with antisera from *A. marginale* Underberg, *A. marginale* Barkly West and *A. centrale* infected bovines respectively. The homologous antiserum recognised more bands than the heterologous antisera and the *A. centrale* antiserum produced a fainter signal with *A. marginale* Underberg proteins (fig. 4-3) than the two *A. marginale* isolates (figs. 4-1 and 4-2). Table 4-4 lists the molecular weights of the proteins recognised by the different antisera. The most prominent bands recognised by the Underberg antiserum were the 22, 40-46, and 82kDa proteins. The 22kDa protein was not in the range of molecular weight markers used and was extrapolated to be between 20 and 22kDa in size. The 22 and 46kDa proteins were recognised by both Barkly West and *A. centrale* antisera, the 82kDa protein only by Barkly West antiserum and the 40kDa protein only by the *A. centrale* antiserum. No bands were detected by preinfection antisera with any of the isolates.

Signals were first detected on day 7 (Fig. 4-1, lane 2) with the Underberg antiserum, on day 20 (Fig. 4-2, lane 2) with the Barkly West antiserum and on day 35 (Fig. 4-3, lane 6) with the *A. centrale* antiserum. After day 21 (Fig. 4-1, lane 4) no additional proteins were detected by the homologous Underberg antiserum and the intensities stayed constant with two exceptions: the intensity of the 28kDa protein signal decreased after day 47 (lane 7) and for the 22kDa protein there was a sharp increase of intensity at day 62 (lane 8). The intensity of the 30kDa protein signal detected by the Barkly West antiserum in Fig. 4-2 decreased but the remaining signals appeared to be of relatively constant intensity. In the case of the *A. centrale* antiserum the intensity of the 57, 56 and 40kDa proteins decreased after day 63 (Fig. 4-3, lane 10). The bands recognised by the Barkly West antiserum in the 50 to 70kDa region were not very clear. This might be expected to be due to the difference in resolution between the gradient and 10% linear gels, but Fig. 4-3 is also a 10% linear gel and the 57 and 56kDa proteins are recognised as more distinct bands when compared to Fig. 4-2. It appears therefore that this is a real effect and the Barkly West antiserum did not strongly recognise proteins in the 50 to 70kDa region. This effect is also seen in Fig.4-5.

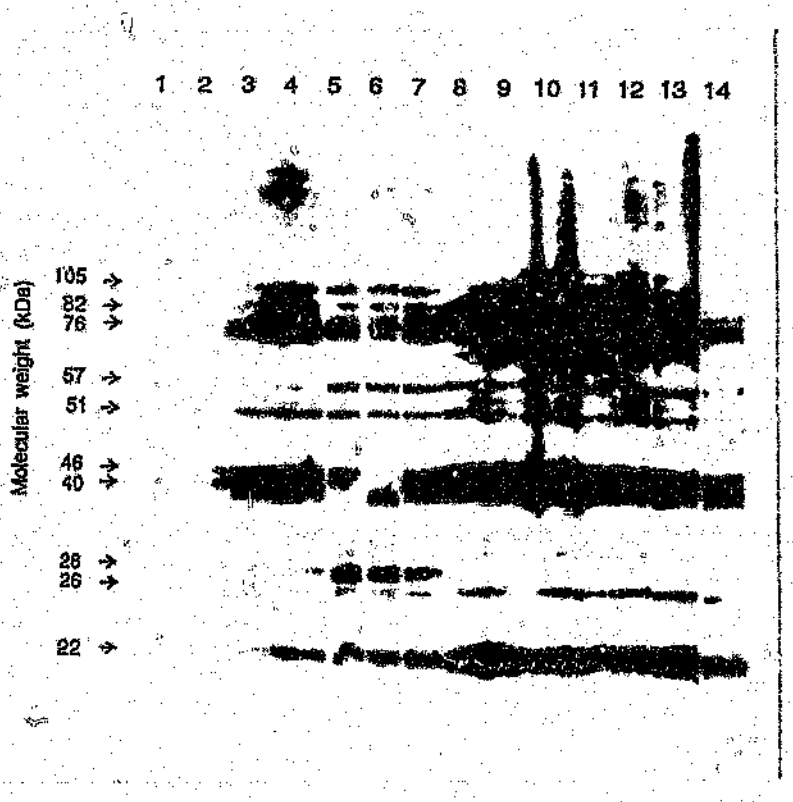

Fig. 4-1. *A. marginale* Underberg proteins immunoblotted with antisera from the *A. marginale* Underberg infected bovine. *A. marginale* Underberg proteins were separated by electrophoresis (on a 7%-17.5% gradient gel), transferred to Hybond C-Super (Amersham) and the membrane cut into strips. The strips were immunoblotted each with an antiserum collected during the course of infection from the *A. marginale* Underberg infected bovine. ECL was used to detect the bound antibodies. Table 4-1a shows which antiserum was used for each individual lane. The arrows indicate the molecular weight (in kDa) of the major protein bands.

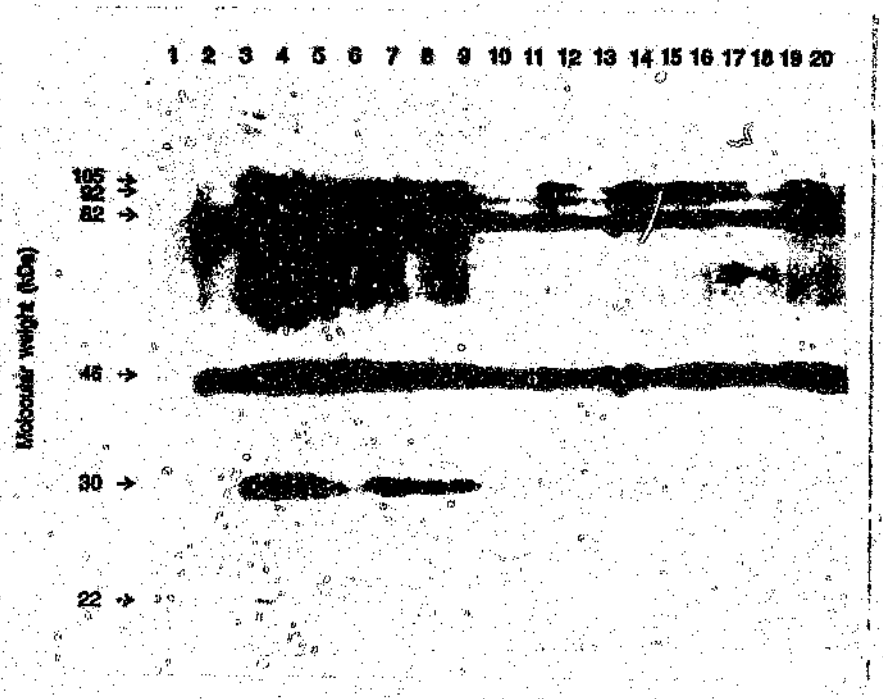

Fig. 4-2. *A. marginale* Underberg proteins immunoblotted with antisera from the *A. marginale* Barkly West infected bovine. *A. marginale* Underberg proteins separated by electrophoresis (on a 10% gel) and treated as described for Fig. 4-1 but the strips were immunoblotted with antisera collected during the course of infection of the *A. marginale* Barkly West infected bovine. Table 4-2 shows which antiserum was used for each individual lane. The arrows indicate the molecular weight (in kDa) of the major protein bands.

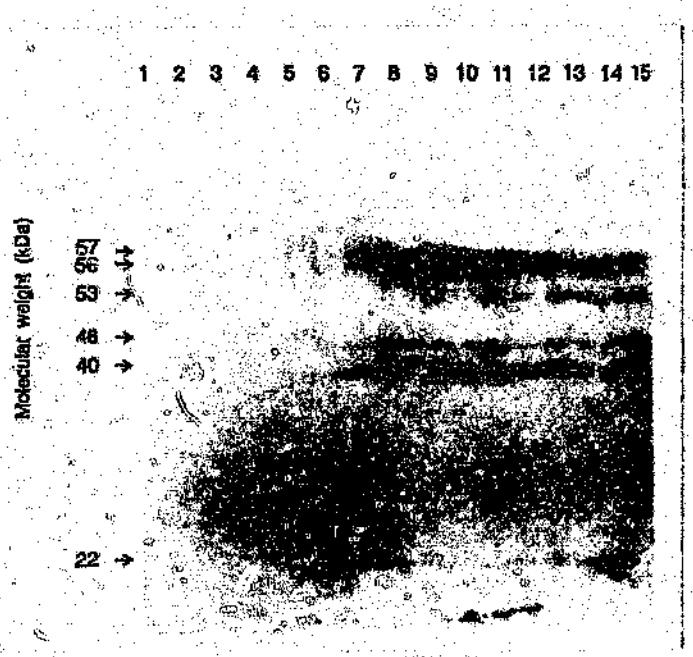

Fig. 4-3. *A. marginale* Underberg proteins immunoblotted with antisera from the *A. centrale* infected bovine. *A. marginale* Underberg proteins separated by electrophoresis (on a 10% gel) and treated as described for Fig. 4-1 but the strips were immunoblotted with antisera collected during the course of infection of the *A. centrale* infected bovine. Table 4-3 shows which antiserum was used for each individual lane. The arrows indicate the molecular weight (in kDa) of the major protein bands.

TABLE 4-4. Molecular weights (in kDa) of *A. marginale* Underberg proteins immunoblotted with serum from *A. marginale* Underberg, *A. marginale* Barkly West and *A. centrale* infected bovines.

A. marginale Underberg proteins		
Underberg serum Fig. 4-1	**Barkly West serum** Fig. 4-2	***A. centrale* serum** Fig. 4-3
105	105	
92	92	
86		
82	**82**	
76	76	
73		
69	69	
57	57	57
		56
53		53
51		
46	46	46
40#		40
32		
30	30	
28		
26		
22	22	22

#: Very broad band ranging from 38 to 41 kDa.
Most prominent bands are in bold letters.

Figures 4-4, 4-5 and 4-6 show *A. marginale* Barkly West proteins probed with antisera from the *A. marginale* Underberg, *A. marginale* Barkly West and *A. centrale* infected bovines respectively. Table 4-5 lists the molecular weights of the proteins recognised by the different antisera.

The Barkly West antiserum recognised fewer proteins in the homologous infection (Fig. 4-5) than were recognised by the Underberg antiserum in the homologous infection (Fig. 4-1). Proteins were detected from day 20 (Fig. 4-5, lane 2). No antisera were collected before day 20, which was when the parasitaemia peaked. Antibodies could probably have been detected earlier in the Barkly West infection as in the case of Underberg (Fig. 4-1). The major proteins recognised by the homologous antiserum were 114, 105, 89, 53, 46, 40 and 29kDa in size (Fig. 4-5) and the signals were of constant intensity. The intensity of the 92kDa protein increased sharply on day 118, this coincides with the third relapse of the animal when there were detectable levels of *Anaplasma* parasites in the blood smears. The intensity of the 29kDa protein decreased after day 47 but thereafter the signal remained constant.

The *A. marginale* Underberg antiserum recognised most of the *A. marginale* Barkly West proteins (Fig. 4-4) and the most prominently detected bands were 105, 75, 57, 53, 40 and 22kDa in size. The 22kDa protein was extrapolated to be between 20 and 22kDa in size. The signals initially appeared on day 21 with the Underberg antiserum (Fig. 4-4, lane 4) and there was a sharp increase in the intensity of the 22kDa band on day 81 (Fig. 4-4, lane 10). The proteins between 53 and 70kDa were more prominently detected with the Underberg antiserum than with the homologous Barkly West antiserum. This is similar to the finding in Fig. 4-2 where the Barkly West antisera only weakly recognised the *A. marginale* Underberg proteins in this region.

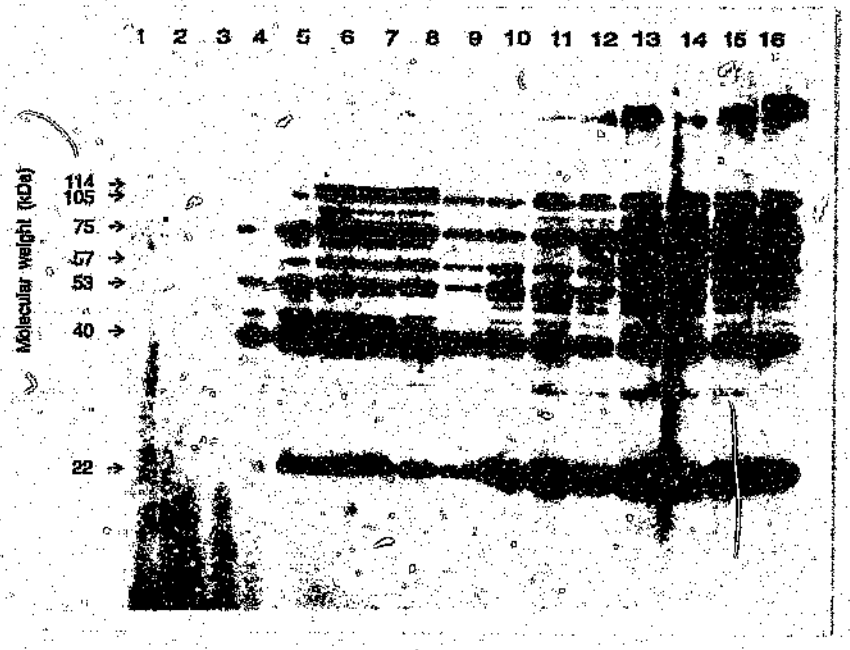

Fig. 4-4. *A. marginale* Barkly West proteins separated by electrophoresis (on a 7%-17.5% gradient gel) and treated as described for Fig. 4-1 but the strips were immunoblotted with antisera collected during the course of infection of the *A. marginale* Underberg infected bovine. Table 4-1a shows which antiserum was used for each individual lane. The arrows indicate the molecular weight (in kDa) of the major protein bands.

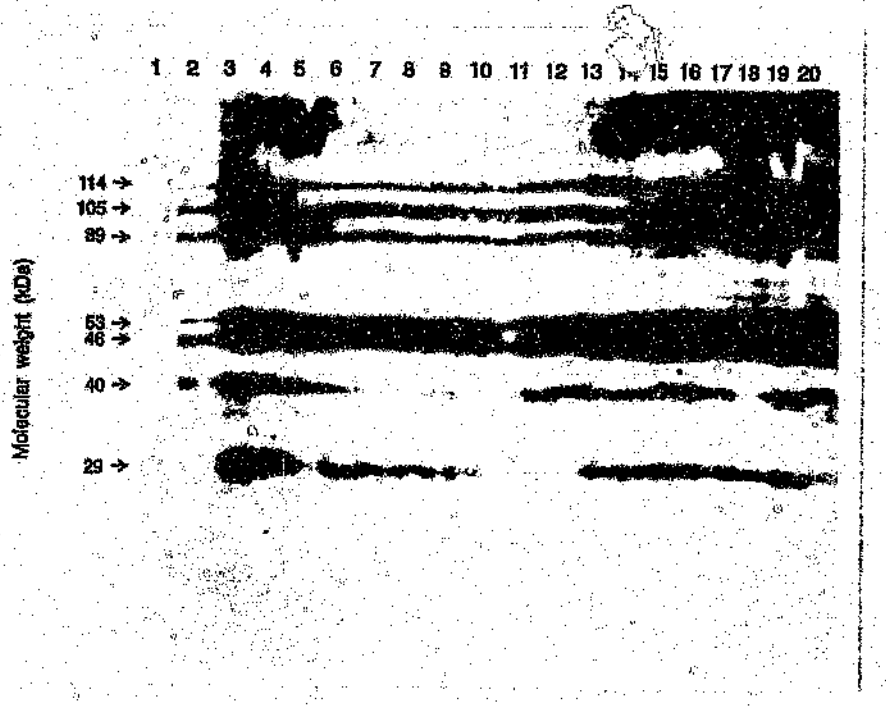

Fig. 4-5. *A. marginale* Barkly West proteins immunoblotted with antisera from the *A. marginale* Barkly West infected bovine. *A. marginale* Barkly West proteins separated by electrophoresis (on a 10% gel) and treated as described for Fig. 4-1 but the strips were immunoblotted with antisera collected during the course of infection of the *A. marginale* Barkly West infected bovine. Table 4-2 shows which antiserum was used for each individual lane. The arrows indicate the molecular weight (in kDa) of the major protein bands.

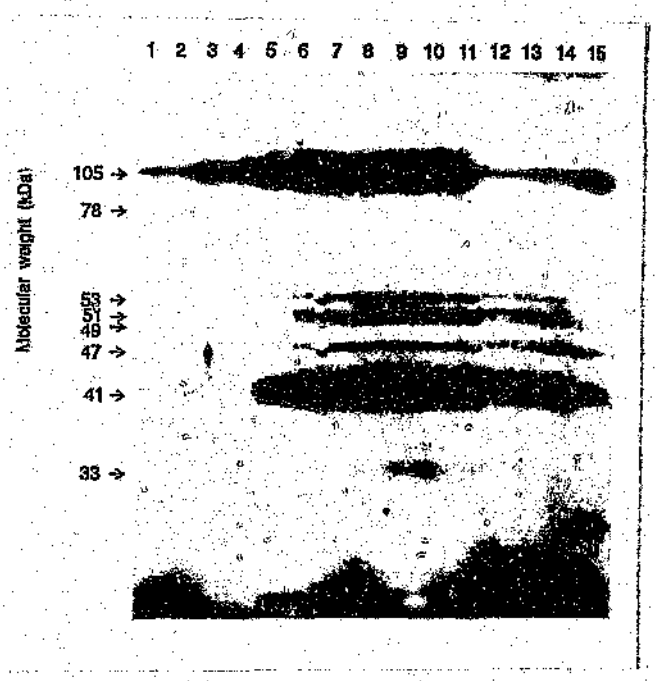

Fig. 4-6. *A. marginale* Barkly West proteins immunoblotted with antisera from the *A. centrale* infected bovine. *A. marginale* Barkly West proteins separated by electrophoresis (on a 10% gel) and treated as described for Fig. 4-1 but the strips were immunoblotted with antisera collected during the course of infection of the *A. centrale* infected bovine. Table 4-3 shows which antiserum was used for each individual lane. The arrows indicate the molecular weight (in kDa) of the major protein bands.

TABLE 4-5. Molecular weights (in kDa) of *A. marginale* Barkly West proteins immunoblotted with serum from *A. marginale* Underberg, *A. marginale* Barkly West and *A. centrale* infected bovines.

A. marginale Barkly West proteins		
Underberg serum Fig. 4-4	**Barkly West serum Fig. 4-5**	***A. centrale* serum Fig. 4-6**
114	**114**	
105	**105**	**105**
	92	
89	**89**	
		78
75	75	75
69	69	
57	57	57
53	53	53
51	51	51
49		49
46	46	46
40#	40#	40#
		36
33	33	33
	29	
22		22

#: Very broad band ranging from 38 to 41 kDa.
Most prominent bands are in bold letters.

A. centrale antiserum recognised fewer *A. marginale* Barkly West proteins than the antisera of the other two isolates. The most prominent bands recognised were 105 and 41kDa in size (Fig. 4-6). The intensity of all the bands decreased slightly from day 84 (Fig. 4-6, lane 13) and the 105kDa band decreased sharply on 77 (Fig. 4-6, lane 12). The 105kDa protein was recognised by the preinfection antiserum so there must have been an *Anaplasma* cross-reactive antibody present before infection with *A. centrale*.

Figures 4-7, 4-8 and 4-9 show *A. centrale* proteins probed with antisera from *A. marginale* Underberg, *A. marginale* Barkly West and *A. centrale* infected bovines respectively. Fewer *A. centrale* proteins were detected than in the two *A. marginale* isolates. Table 4-6 compares the *A. centrale* proteins recognised by the different antisera. The major proteins were 148, 134, 105, 63, 57 and 31kDa in size. The band most prominently recognised by the homologous antiserum was the very broad band in the 40kDa region (Fig. 4-9) which was also very prominent in the other two isolates. The signal decreased in intensity in *A. marginale* Underberg and *A. marginale* Barkly West but more so in *A. marginale* Barkly West (Figs. 4-7 and 4-8). The signal for the 31kDa protein detected only by *A. marginale* Underberg is detected for a short period, day 21 to day 62 (Fig. 4-7, lanes 3 and 6). The 148, 134 and 105kDa proteins were also quite prominent with the homologous antiserum (Fig. 4-9). Signals started to appear on day 28 (Fig 4-9, lane 5), which is when peak parasitaemia occurred, whereas with *A. marginale* Barkly West and *A. marginale* Underberg antisera signals started to appear on days 20 (Fig. 4-8, lane 2) and 21 (Fig. 4-7, lane 3). The signals for the 63 and 57kDa proteins were detected later on in the infection: on day 76 (Fig. 4-7, lane 8) by the *A. marginale* Underberg antiserum, on day 83 (Fig. 4-8, lane 10) by the *A. marginale* Barkly West antiserum and on day 42 by the *A. centrale* antiserum (Fig. 4-9, lane 7). This increase in intensity occurred after the second relapse of the animal when there were detectable levels of *Anaplasma* parasites in the blood smears.

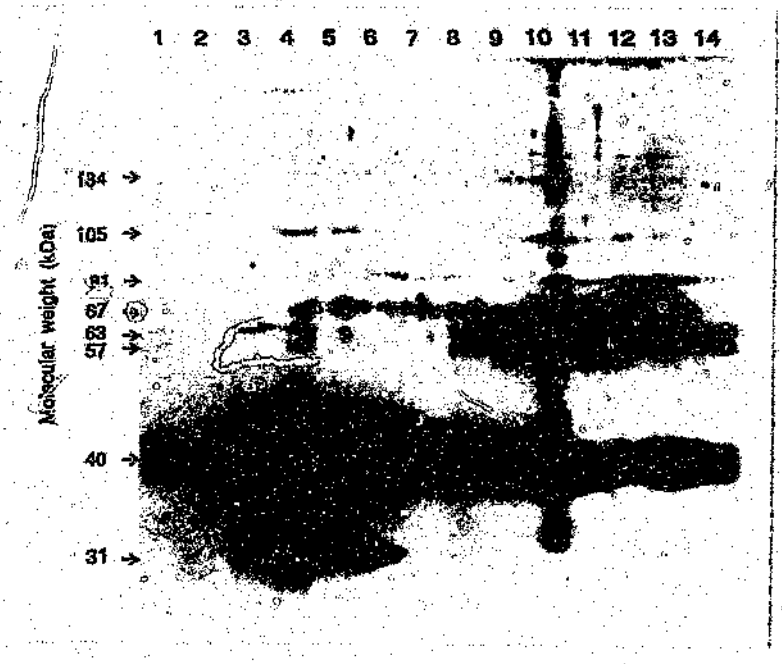

Fig. 4-7. *A. centrale* proteins immunoblotted with antisera from the *A. marginale* Underberg infected bovine. *A. centrale* proteins separated by electrophoresis (on a 10% gel) and treated as described for Fig. 4-1 but the strips were immunoblotted with antisera collected during the course of infection of the *A. marginale* Underberg infected bovine. Table 4-1b shows which antiserum was used for each individual lane. The arrows indicate the molecular weight (in kDa) of the major protein bands.

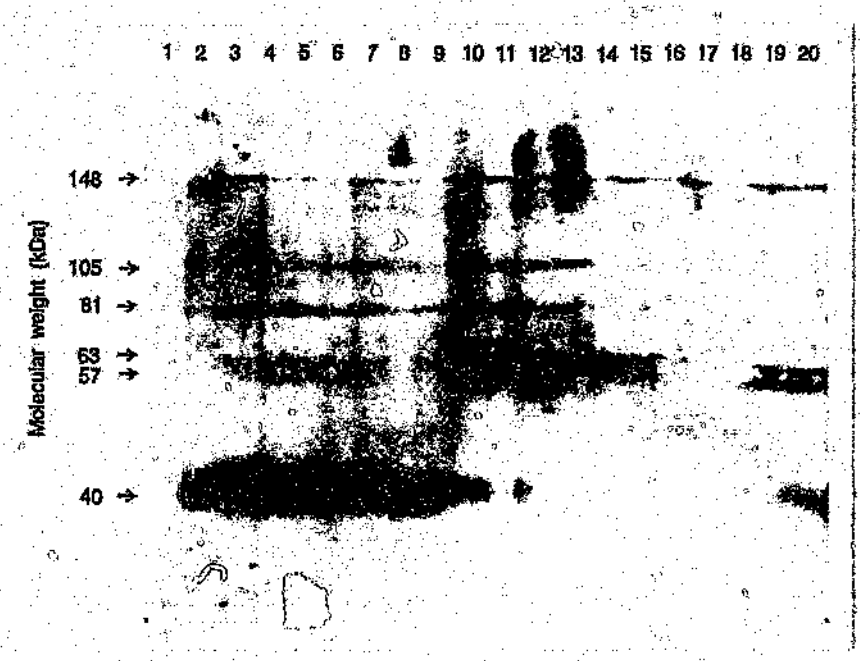

Fig. 4-8. *A. centrale* proteins immunoblotted with antisera from the *A. marginale* Barkly West infected bovine. *A. centrale* proteins separated by electrophoresis (on a 10% gel) and treated as described for Fig. 4-1 but the strips were immunoblotted with antisera collected during the course of infection of the *A. marginale* Barkly West infected bovine. Table 4-2 shows which antiserum was used for each individual lane. The arrows indicate the molecular weight (in kDa) of the major protein bands.

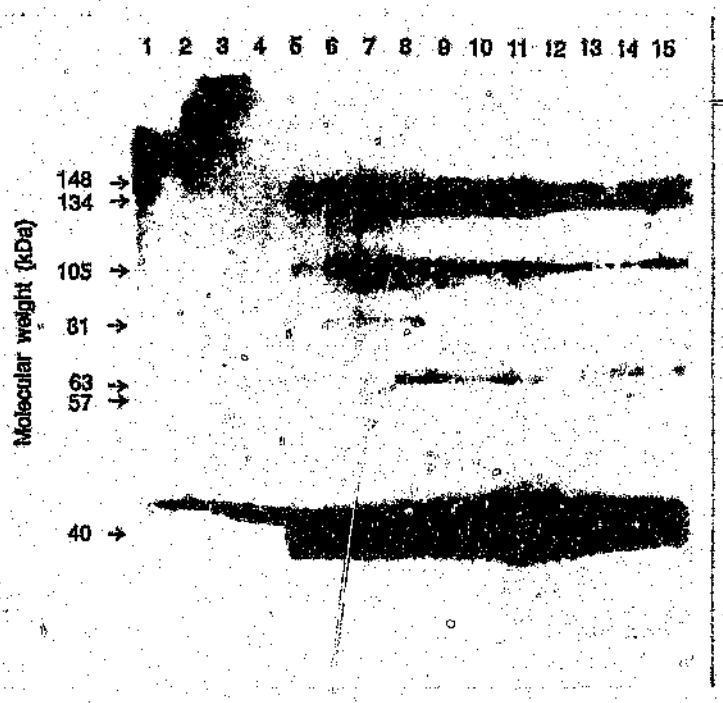

Fig. 4-9. *A. centrale* proteins immunoblotted with antisera from the *A. centrale* infected bovine. *A. centrale* proteins separated by electrophoresis (on a 10% gel) and treated as described for Fig. 4-1 but the strips were immunoblotted with antisera collected during the course of infection of the *A. centrale* infected bovine. Table 4-3 shows which antiserum was used for each individual lane. The arrows indicate the molecular weight (in kDa) of the major protein bands.

TABLE 4-6. Molecular weights (in kDa) of *A. centrale* proteins immunoblotted with serum from *A. centrale*, *A. marginale* Underberg and *A. marginale* Barkly West infected bovines.

A. centrale proteins		
A. centrale **serum** fig. 4-9	**Underberg serum** fig. 4-7	**Barkly West serum** fig. 4-8
148	148	148
134	134	
127		
105	105	105
97		
81	81	81
	67	
63	63	63
57	57	57
40*	40*	40*
	31	

#: Very broad band ranging from 38 to 41 kDa.
Most prominent bands are in bold letters.

Erythrocyte proteins were probed with all the serum samples used and some signals were obtained after extended exposure times (Figs. 4-10, 4-11 and 4-12). The *A. marginale* Underberg antiserum recognised 40, 80 and 116kDa proteins early in the infection (Fig. 4-10, lanes 5, 6 and 7) corresponding to days 28 to 47 post infection. The intensity of the 80kDa protein band increased from day 97 (lanes 10 to 15) when the 22kDa protein was first detected. The *A. marginale* Barkly West antiserum recognised a protein in the 80kDa region (Fig. 4-11, lane 6 to 19) from day 55 onwards. No protein bands were detected by the *A. centrale* antiserum (Fig. 4-12). The bovines were infected with blood stabilates and could therefore have mounted an antibody response against foreign bovine erythrocyte proteins.

Table 4-7 shows a comparison of the proteins recognised by the homologous antisera of each of the isolates. The 105 and 40kDa proteins were common to all three isolates: the 57kDa protein was also common but in *A. marginale* Barkly West the signal was very faint. *A. marginale* Underberg and *A. marginale* Barkly West had more immunogenic proteins in common than either isolate had with *A. centrale*. *A. centrale* had high molecular weight proteins (148, 134 and 127kDa) that were not detected in the two *A. marginale* isolates (Figs. 4-3 and 4-6) but which were recognised by their antisera (Figs. 4-7 and 4-8).

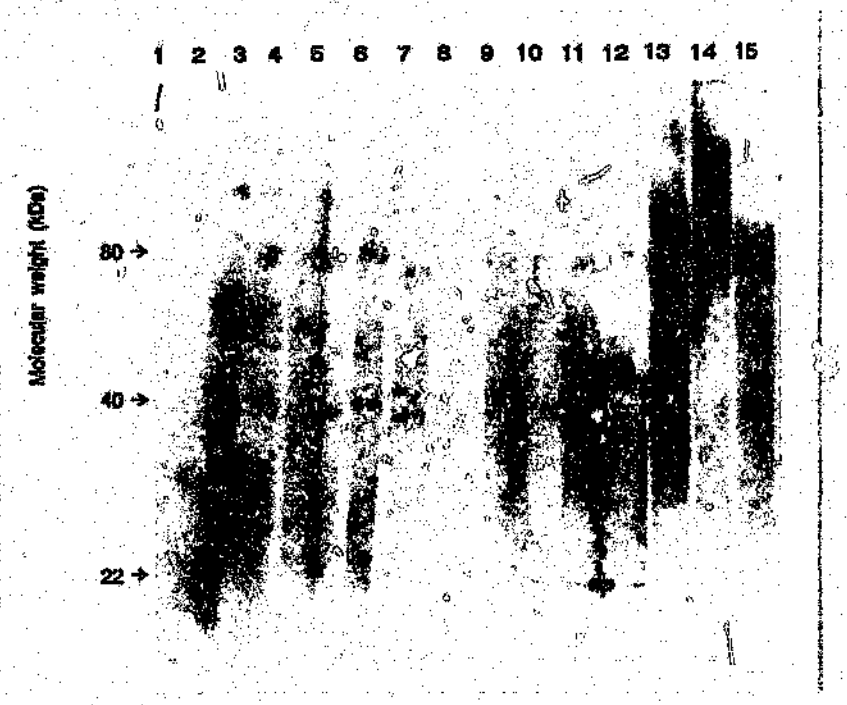

Fig. 4-10. Uninfected erythrocyte proteins immunoblotted with antisera from the *A. marginale* Underberg infected bovine. Uninfected erythrocyte proteins separated by electrophoresis (on a 7%-17.5% gradient gel) and treated as described for Fig. 4-1 but the strips were immunoblotted with antisera collected during the course of infection of the *A. marginale* Underberg infected bovine. Table 4-1a shows which antiserum was used for each individual lane. The arrows indicate the the molecular weights (in kDa) of the bands.

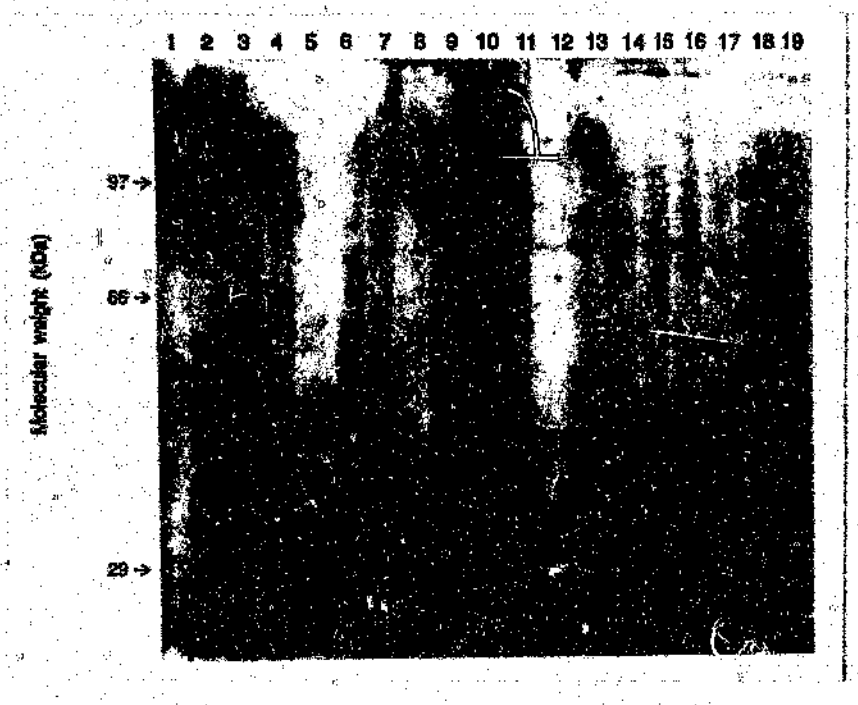

Fig. 4-11. Uninfected erythrocyte proteins immunoblotted with antisera from the *A. marginale* Barkly West infected bovine. Uninfected erythrocyte proteins separated by electrophoresis (on a 10% gel) and treated as described for Fig. 4-1 but the strips were immunoblotted with antisera collected during the course of infection of the *A. marginale* Barkly West infected bovine. Table 4-2 shows which antiserum was used for each individual lane. The arrows indicate the position of the molecular weight markers (in kDa).

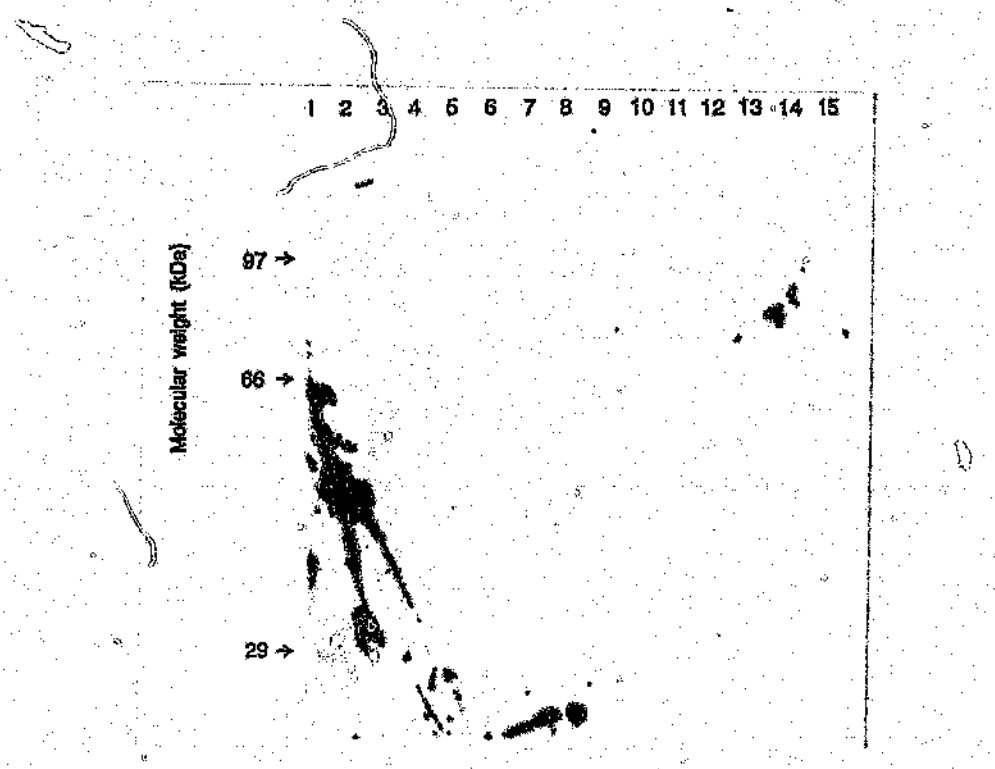

Fig. 4-12. Uninfected erythrocyte proteins immunoblotted with antisera from the *A. centrale* infected bovine. Uninfected erythrocyte proteins separated by electrophoresis (on a 10% gel) and treated as described for Fig. 4-1 but the strips were immunoblotted with antisera collected during the course of infection of the *A. centrale* infected bovine. Table 4-3 shows which antiserum was used for each individual lane. The arrows indicate the position of the molecular weight markers (in kDa).

TABLE 4-7. Table comparing *A. marginale* Underberg, *A. marginale* Barkly West and *A. centrale* protein molecular weights (in kDa) recognised by each isolate's homologous antisera.

A. marginale Underberg	*A. marginale* Barkly West	*A. centrale*
		148
		134
		127
	114	
105	105	105
		97
92	92	
86	89	
82		81
76	75	
73		
69	69	
		63
57	57*	57*
53	53	
51	51	
46	46	
40#	40#	40#
32	33	
30	29	
28		
26		
22		

* Faint signals.
\# Very broad band ranging from 38 to 41 kDa.

4.4. DISCUSSION

The kinetics of antibody production in bovines infected with two *A. marginale* isolates and *A. centrale* was studied over a period of more than 3 months. Serological cross-reactions between these *Anaplasma* isolates were also shown in this study. The major *Anaplasma* proteins identified with bovine antisera on immunoblots, using ECL as the detection system, were 22, 40, 46, 76, 82, and 105kDa in size as well as proteins in the 51 to 63kDa region which varied between the *Anaplasma* isolates.

Proteins and antisera from *A. marginale* Underberg, *A. marginale* Barkly West and *A. centrale* cross-reacted with each other. However, antibodies developed later during *A. centrale* infection and did not react with as many proteins as the two *A. marginale* isolate antisera. During the *A. centrale* infection antibodies started to appear at day 28, a few days after *A. centrale* organisms were first detected in the blood smears (day 21), whereas in the case of *A. marginale* Underberg both antibodies and parasites appeared on day 7. This suggests that *A. centrale* induces a lower level of antibodies than the *A. marginale* isolates. *A. centrale*, which is used as a live blood vaccine against anaplasmosis in South Africa, produces milder disease symptoms and induces a lower antibody response than *A. marginale* Underberg and *A. marginale* Barkly West. Wilson *et al.* (1980) found that a good antibody response was related to resistance to challenge which might explain why the *A. centrale* vaccine does not always provide adequate protection (Potgieter and Van Rensburg, 1983).

It is difficult to identify one protein that cross-reacts with the sera of all the isolates for the time period investigated, but isolate-specific and isolate-common proteins could be identified. In *A. marginale* Underberg the 86, 73, 51 and especially the 32, 28 and 26kDa proteins are only recognised by the homologous antiserum and seem to be isolate-specific. The Underberg serum does not cross-react strongly to any protein of the similar size in all three isolates. In the Barkly West infection the 46kDa protein, and for *A. centrale* the 40kDa protein, are both strongly recognised by the Underberg antiserum.

A 22kDa band is strongly recognised by the *A. marginale* Underberg antiserum in both *A. marginale* Underberg and *A. marginale* Barkly West infections, thus a protein of 22kDa is present in Barkly West although the Barkly West antiserum does not appear to strongly recognise a band of this size. Thus in *A. marginale* Barkly West the protein with cross-reactive epitopes to the *A. marginale* Underberg 22kDa protein must be of a different size. In the *A. centrale* experiments the smaller proteins ran off the gel so the 22kDa was not detected. This 22kDa protein may be equivalent to the 19kDa protein identified in the Florida isolate, which was present in all isolates of *A. marginale* in the USA, Israel and Zimbabwe, also in *A. centrale* and *A. ovis*. This protein showed no size variation

between the isolates and is currently being studied for its possible role in immune protection against anaplasmosis and its use as antigen in serological diagnosis (Visser *et al.*, 1992).

In *A. marginale* Barkly West the 92 and 29kDa proteins are only recognised by the homologous antiserum and the 92kDa protein increases in intensity only after the third cycle of parasite replication in the animal. The 105, 53, 46 and 40kDa proteins are common to all the isolates with the 105 and 40kDa proteins being the most prominent.

A species-specific diagnostic assay for *A. centrale* could be very useful in determining whether animals have antibodies against *Anaplasma* due to vaccination or due to exposure to the disease in the field. Unfortunately we have detected no *A. centrale*-specific proteins recognised only by the homologous antiserum. The 105, 81, 63, 57 and 40kDa proteins are common to all three isolates tested. The 40kDa protein was the most prominently recognised; antibodies for each isolate persisted during the course of the infection with the homologous antiserum but decreased in the case of the heterologous antisera. Antisera from the *A. centrale* infected animal were only collected up to day 98 but up to days 125 and 160 from the Underberg and Barkly West infected animals, thus the level of antibodies from *A. centrale* might also decrease in a similar fashion. Therefore the 40kDa protein would probably not be useful as antigen in detection of carrier animals because the antibody levels might be too low or eventually disappear. However it might play a role in protection; *A. centrale*-immunised animals are protected against *A. marginale* infections and Tebele *et al.* (1991) found that protection correlated to antibody titre. In this study the majority of antibodies reacted with proteins in the 40kDa region and it is possible that a cross-protective protein in this region might be identified.

Using chromogenic substrates, proteins in the 108 to 91, 47 to 38 and 27kDa regions (Adams *et al.*, 1986; Adams *et al.*, 1989) or only in the 38 to 40kDa region (Nakamura *et al.*, 1991) have been reported. Shkap *et al.* (1991) used ^{125}I-protein A to detect bovine antibodies which was a more sensitive detection method, and again the 33 to 36 and 86kDa were the major proteins identified. The proteins in the 47 to 38 (Adams *et al.*, 1986) and the 33 to 36kDa (Shkap *et al.*, 1991) regions are thought to be equivalent to the 36kDa protein identified by immunoprecipitation with both rabbit and bovine antisera (Palmer and McGuire 1984; Palmer *et al.*, 1988b). Proteins in the 40kDa region that were very prominent by immune detection in this study are probably also equivalent to the 36kDa protein of the USA Florida isolate of *A. marginale*. This 36kDa protein bears epitopes highly conserved among *A. marginale* isolates from Israel, Kenya and the USA (Palmer *et al.*, 1988b). The 86kDa protein identified by Palmer *et al.* (1988b) in bovine antisera from many USA isolates was not detected in the South African isolates studied, but proteins ranging from 75 to 97kDa were seen.

The 105 and 40kDa proteins were recognised by both *A. marginale* Barkly West and *A. centrale* to be species-common proteins and have been shown to induce some protection in the American isolates (Palmer *et al.* 1989. Palmer *et al.* 1988b). They are good candidates to study further for use as antigens in an improved serological test. Another possibility is a combination of the two *A. marginale* Underberg (40 and 46kDa) proteins.

GENERAL DISCUSSION.

Various methods were employed to compare the proteins of six South African isolates of *A. marginale* and *A. centrale*. All the methods showed that there are common and isolate-specific proteins, and that common epitopes occur on proteins of different molecular sizes.

Polyclonal rabbit antisera against *A. centrale* and *A. marginale* were used to immunoprecipitate radiolabelled *Anaplasma* proteins. Common proteins were precipitated but several proteins were different between the isolates. The common proteins identified by immunoprecipitation (154, 148, 105, 75, 63 and 42kDa) were similar to those which were found with the USA isolates. Palmer and McGuire (1984) identified proteins with molecular weights of 105, 86, 61, 36 and 31kDa by immunoprecipitation with rabbit antisera prepared in a manner similar to the rabbit antisera used in this study. Similar proteins were identified with rabbit antisera made against the local isolates in our study: Palmer and McGuire's 61kDa protein could be equivalent to our 63kDa protein and their 36kDa equivalent to our 42kDa. Owing to the reaction of the 105, 75 and 42kDa proteins with the control rabbit antisera the results were difficult to interpret and so alternative approaches were explored.

Immunoblotting with antisera made against *A. marginale* Underberg was used to show immunogenic differences among the *Anaplasma* isolates. The two monospecific antisera (anti-42U and anti-82U) that were produced did not recognise *Anaplasma* common epitopes. Anti-82U only reacted weakly with proteins of *A. marginale* Barkly West and Escourt and not at all with *A. centrale*, and anti-42U did not react with these three isolates. Anti-82U and anti-42U cross-reacted with proteins of varying molecular weights in the heterologous isolates. This confirms previous reports that *Anaplasma* proteins with similar epitopes vary in size (Oberle *et al.*, 1988; McGuire *et al.*, 1984).

The kinetics of antibody production by bovines infected with *Anaplasma* and serological cross-reactivity between *Anaplasma* isolates were investigated by immunoblotting. The major common proteins between *A. marginale* Underberg, *A. marginale* Barkly West and *A. centrale* had molecular weights of 105, 57 and 40kDa, the 57kDa protein showing relatively weak signals. *A. centrale* confers a degree of protection against *A. marginale* infection so one of these proteins might be of importance in the protection of bovines. The 105 and 36kDa (probably equivalent to 40kDa in this study) of the Florida isolate have been shown to contain neutralising-sensitive epitopes (Palmer and Maguire, 1984). *A. marginale* Underberg showed several isolate-specific proteins whereas *A. centrale* showed no isolate-specific proteins.

It is known that USA isolates of *A. marginale* differ in morphology, protein structure, antigenicity and in ability to induce cross-protection (Palmer *et al.*, 1988a). This study confirms that the antigenic proteins of the South African isolates of *A. marginale* as well as *A. centrale* also differ. Because the isolates differ to a certain degree it is important to take into account all the possibilities when looking for candidate epitopes for subunit vaccines and antigens for serological tests.

It is difficult to identify one protein that cross-reacts with the sera of all the isolates for the time period investigated, but the 22, 40, 46, 76, 82 and 105kDa proteins all occur in at least two of the three isolates and antibodies are still prevalent in the animals infected with the respective isolates long after infection. It is shown in chapter 3 that some *Anaplasma* proteins with similar epitopes vary in size, so in this study with the bovine antisera only cross-reacting proteins of identical size could be identified. It must be kept in mind that the immune response of only one bovine per isolate was studied: more animals should be investigated to confirm these results and more isolates should be included in the investigations. The proteins mentioned above look promising for further investigation and monospecific antiserum or, even better, monoclonal antibodies should be made against them to show conclusively the relatedness of the proteins between the isolates. The proteins should be better separated, as was done with the 40kDa region in chapter 3 so that all the proteins can be studied separately. Monospecific or monoclonal antibodies can be used to screen existing DNA expression libraries for the coding regions of these proteins. Large amounts of protein can be obtained when cloned genes are expressed in an *in vitro* system and the specific proteins can then be used in protection studies or as antigen in serological diagnosis. This study lays the groundwork for further studies on *Anaplasma* proteins of the South African isolates.

Table showing antisera produced and shortened name of each antiserum.

Serum identity	Inoculum
CTRL	Uninfected erythrocytes
anti-ACE	*A. centrale* infected erythrocytes
anti-ACI	*A. centrale* initial bodies
anti-AME	*A. marginale* infected erythrocytes
anti-AMI	*A. marginale* initial bodies
anti-82U	*A. marginale* Underberg 82kDa protein
anti-42U	*A. marginale* Underberg 42kDa protein

REFERENCE LIST

ADAMS, J.H., SMITH, R.D. and KUHLENSCHMIDT, M.S., 1986. Identification of antigens of two isolates of *Anaplasma marginale* using a western blot technique. *American Journal of Veterinary Research*. 47,501-506.

ADAMS, J.H. and SMITH, R.D., 1988. Differential extraction of antigens of *Anaplasma marginale*. *American Journal of Veterinary Research*. 49,257-260.

ADAMS, J.H., SHIELS, I.A. and DE VOS, A.J., 1989. Heterologous antibody responses of calves to *Anaplasma centrale* and *Anaplasma marginale*. *Veterinary Parasitology*. 31,7-11.

ALLEN, P.C., KUTTLER, K.L. and AMERAULT, T.E., 1981. Clinical chemistry of anaplasmosis: Comparative changes elicited by attenuated and virulent *Anaplasma marginale* isolates. *American Journal of Veterinary Research*. 42,326-328.

ALLRED, D.R., McGUIRE, T.C., PALMER, G.H., LEIB, S.R., HARKINS, T.M., McELWAIN, T.F. and BARBET, A.F., 1990. Molecular basis for surface antigen size polymorphisms and conservation of a neutralization-sensitive epitope in *Anaplasma marginale*. *Proceedings. National Academy of Sciences. USA*. 87,3220-3224.

BARBET, A.F. and ALLRED, D.R., 1991. The *msp*1β multigene family of *Anaplasma marginale*: Nucleotide sequence analysis of an expressed copy. *Infection and Immunity*. 59,971-976.

BARBET, A.F., ANDERSON, L.W., PALMER, G.H. and McGUIRE, T.C., 1983. Comparison of proteins synthesized by two different isolates of *Anaplasma marginale*. *Infection and Immunity*. 40,1068-1074.

BARBET, A.F., PALMER, G.H., MYLER, P.J. and McGUIRE, T.C., 1987. Characterization of an immunoprotective protein complex of *Anaplasma marginale* by cloning and expression of the gene coding for polypeptide Am105L. *Infection and Immunity*. 55,2428-2435.

BARRY, D.M. and VAN NIEKERK, C.H., 1990. Anaplasmosis in improved goats in South Africa artificially infected with *Anaplasma ovis*. *Small Ruminant Research*. 3,191-197.

BUDDEN, J.R. and DIMOPOULLOS, G.T., 1977. Finite purification of *Anaplasma marginale*: Serological inactivity of the anaplasma body. *American Journal of Veterinary Research*. 38,633-636.

BUENING, G.M. 1973. Cell-mediated immune responses in calves with anaplasmosis. *American Journal of Veterinary Research*. 34,757-763.

CARSON, C.A., SELLS, D.M. and RISTIC, M., 1976. Cell-mediated immunity in bovine anaplasmosis and correlation with protection induced by vaccination (a review). *Veterinary Parasitology*. 2,75-81.

CARSON, C.A. and BUENING, G.M., 1979. The immune response of cattle to live inactivated anaplasma vaccines and response to challenge. *Journal of the South African Veterinary Association*. 50,330-331.

CORRIER, D.E., WAGNER, G.G. and ADAMS, L.G., 1981. Recrudescence of *Anaplasma marginale* induced by immunosuppression with cyclophosphamide. *American Journal of Veterinary Research*. 42,19-21.

DAVIS, W.C., TALMADGE, J.E., PARISH, S.M., JOHNSON, M.I. and VIBBER, S.D., 1978. Synthesis of DNA and protein by *Anaplasma marginale* in bovine erythrocytes during short-term culture. *Infection and Immunity*. 22,597-602.

DIMOPOULLOS, G.T. and FINERTY, J.F., 1976. Cross reactivity between *Anaplasma marginale* and two *Plasmodium* species as demonstrated by passive hemagglutination. *Journal of Veterinary Research*. 37,693-695.

DU PLESSIS, J.L., BERCHE, P. and VAN GAS, L., 1991. T cell-mediated immunity to *Cowdria ruminantium* in mice: the protective role of Lyt2⁺ T cells. *Onderstepoort Journal of Veterinary Research*. 58 171-17.

DZURIS, J.L., HART, L.T., TODD, W.J. and McDONOUGH, K., 1987. A simple method for separating viable *Anaplasma marginale* from bovine erythrocytes. In: Abtracts of the Annual Meeting of the American Society of Microbiology. B-83.

EMERY, D.L., 1981. Adoptive transfer of immunity to infection with *Theileria parva* (East Coast fever) between cattle twins. *Research in Veterinary Science*. 30,364-367.

EMERY, D.L., MACHUGH, N.D. and MORRISON, W.I., 1988. *Theileria parva* (Muguga) infects bovine T lymphocytes *in vivo* and induces coexpression of BoT4 and BoT8. *Parasite Immunology*. 10,379-391.

ERIKS, I.S., PALMER, G.H., McGUIRE, T.C., ALLRED, D.R. and BARBET, A.F., 1989. Detection and quantitation of *Anaplasma marginale* in carrier cattle by using a nucleic acid probe. *Journal of Clinical Microbiology*. 27,279-284.

FRIEDHOFF, F.T., 1990. Interaction between parasite and tick vector. *International Journal for Parasitology*. 20,525-535.

GERSHONI, J.M. and PALADE, G.E., 1983. Protein blotting: principles and applications. *Analytical Biochemistry*. 131.1-15.

GIULIAN, G.G., MOSS, R.L. and GREASER, M., 1984. Analytical isoelectric focusing using high-voltage vertical slab polyacrylamide gel system. *Analytical Biochemistry*. 142,421-436.

GODDEERIS, B.M., MORRISON, W.I., TEALE, A.J., BENSAID, A. and BALDWIN, C.L., 1986. Bovine cytotoxic T cell clones specific for cells infected with the protozoan parasite *Theileria parva*: parasite strain specificity and class I major histocompatibility complex restriction. *Proceedings. National Academy of Sciences. USA*. 83,5238-5242.

GOFF, W.L., JOHNSON, L.W. and KUTTLER, K.L., 1986. *Anaplasma marginale, Eperythrozoon wenyoni*: Lectin reactions with bovine erythrocytes. *Experimental Parasitology*. 61,103-113.

GOFF, W.L., BARBET, A., STILLER, D., PALMER, G.H., KNOWLES, D., KOCAN, K., GORHAM, J. and McGUIRE, T.C., 1988. Detection of *Anaplasma marginale*-infected tick vectors by using a cloned DNA probe. *Proceedings. National Academy of Sciences. USA*. 85,919-923.

HANCOCK, K. and TSANG, V.C.W., 1983. India ink staining of proteins on nitrocellulose paper. *Analytical Biochemistry*. 133,157-162.

HARLOW, E. and LANE, D.P., 1988. Antibodies. A laboratory manual. Cold Spring Harbour Laboratory, Cold Spring Harbour, NY, USA.

HART, R.T., LARSON, A.D., DECKER, J.L., WEEKS, J.P. and CLANCY, P.L., 1981. Preparation of intact *Anaplasma marginale* devoid of host cell antigens. *Current Microbiology*. 5,95-100.

HERBERT, W.J., WILKINSON, P.C. and STOTT, D.I., Eds. 1985. Dictionary of Immunology. Third edition. Blackwell Scientific Publications.

HIDALGO, R.J., JONES, E.W., BROWN J.E. and AINSWORTH, A.J., 1989a. *Anaplasma marginale* in tick cell culture. *American Journal of Veterinary Research*. 50,2028-2032.

HIDALGO, R.J., PALMER, G.H., JONES, E.W., BROWN, J.E. and AINSWORTH, A.J., 1989b. Infectivity and antigenicity of *Anaplasma marginale* from tick culture. *American Journal of Veterinary Research*. 50,2033-2036.

KESSLER, S.W., 1975. Rapid isolation of antigens from cells with a Staphylococcal protein A-antibody adsorbent: parameters of the interaction of antibody-antigen complexes with protein-A. *Journal of Immunology*. 115,1617-1624.

KOCAN, K.M., 1986. In: Morphology, physiology and behavioral biology of ticks. Ellis Horwood Limited. Sauer, J.R. and Hair, J.A. Eds. 473-505.

KOCAN, K.M., EWING, S.A., HOLBERT, L. and HAIR, J.A., 1982. Morphologic characteristics of colonies of *Anaplasma marginale* Theiler in midgut epithelial cells of *Dermacentor andersoni* Stiles. *American Journal of Veterinary Research*. 43,586-593.

KOCAN, K.M., YELLIN, T.N., CLAYPOOL, P.L., BARRON, S.J., EWING, S.A. and HAIR, J.A., 1990. Development and infectivity of *Anaplasma marginale* in *Dermacentor andersoni* nymphs. *American Journal of Veterinary Research*. 51,1292-1294.

KUTTLER, K.L., 1967. A study of the immunological relationship of *Anaplasma marginale* and *Anaplasma centrale*. *Research in Veterinary Science*. 8,467-471.

KUTTLER, K.L., 1983. Influence of a second *Anaplasma* exposure on the success of treatment to eliminate *Anaplasma* carrier infections in cattle. *American Journal of Veterinary Research*. 44,882-883.

KUTTLER, K.L., ZAUGG, J.L. and JOHNSON, L.W., 1984. Serological and clinical responses of premunized, vaccinated and previously infected cattle to challenge exposure by different two *Anaplasma marginale* isolates. *American Journal of Veterinary Research*. 45,2223-2226.

KUTTLER, K.L. and ZAUGG, J.L., 1988. Characteristics of an attenuated *Anaplasma marginale* of deer origin as an anaplasmosis vaccine. *Tropical Animal Health and Production*. 20;85-91.

LAEMMLI, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. 227,680-685.

LOGAN, T.M., KOCAN, K.M., EDWARDS, W., HAIR, J.A., CLAYPOOL, P.L. and EWING, S.A., 1987. Persistence of colonies of *Anaplasma marginale* in overwintering *Dermacentor variabilis*. *American Journal of Veterinary Research*. 48,661-663.

LOTZE, J.C. and YIENGST, M.J., 42. Studies on the nature of *Anaplasma*. *American Journal of Veterinary Research*. July, 312-320.

LUTHER, D.G., COX, H.U. and NELSON, W.O., 1980. Comparisons of serotests with calf inoculations for the detection of carriers in anaplasmosis- vaccinated cattle. *American Journal of Veterinary Research*. 41,2085-2086.

MAZZOLA, V., AMERAULT, T.E. and ROBY, T.O., 1976. Survival of *Anaplasma marginale* in *Aedes albopictus* cells. *American Journal of Veterinary Research*. 37,987-989.

MAZZOLA, V. and KUTTLER, K.L., 1980. *Anaplasma marginale* in bovine erythrocyte cultures. *American Journal of Veterinary Research*. 41,2087-2088.

McGUIRE, T.C., PALMER, G.H., GOFF, W.L., JOHNSON, M.I. and DAVIS, W.C., 1984. Common and isolate-restricted antigens of *Anaplasma marginale* detected with monoclonal antibodies. *Infection and Immunity*. 45,697-700.

McGUIRE, T.C., DAVIS, W.C., BRASSFIELD, A.L., McELWAIN, T.F. and PALMER, G.H., 1991. Identification of *Anaplasma marginale* long term carrier cattle by detection of serum antibody to isolated MSP-3. *Journal of Clinical Microbiology*. 29,788-793.

MINAMIDE, L.S. and BAMBURG, J.R., 1990. A filter paper dye-binding assay for quantitative determination of protein without interference from reducing agents or detergents. *Analytical Biochemistry*. 190,66-70.

MORRIS, H., RISTIC, M. and LYKINS, J., 1971. Characterization of opsonins eluted from erythrocytes of cattle infected with *Anaplasma marginale*. *American Journal of Veterinary Research*. 32,1221-1228.

MORRISON, W.I., GODDEERIS, B.M., TEALE, A.J., GROOCOCK, C.M., KEMP, S.J. and STAGG, D.A., 1987. Cytotoxic T cells elicited in cattle challenged with *Theileria parva* (Muguga): evidence for restriction by class I MHC determinants and parasite strain specificity. *Parasite Immunology*. 9,563-78.

MURPHY, F.A., OSEBOLD, J.W. and AALUND, O., 1966. Kinetics of the antibody response to *Anaplasma marginale* infection. *Journal of Infectious Diseases*. 116,99-111.

NAKAMURA, Y., SHIMZU, S., MINAMI, T. and ITO, S., 1988. Enzyme-linked immunosorbent assay using solubilised antigen for the detection of antibodies to *Anaplasma marginale*. *Tropical Animal Health and Production*. 20,259-266.

NAKAMURA, Y., KAWAZU, S. and MINAMI, T., 1991. Analysis of protein compositions and surface protein epitopes of *Anaplasma centrale* and *Anaplasma marginale*. *Journal of Veterinary and Medical Science*. 53,73-79.

NORDELO, M.A. and YSERN-CALDENTEY, M., 1982. Abnormal bovine erythrocyte membrane proteins and glycoproteins during and after infection with *Anaplasma marginale*. *Biochemical and Biophysical Research Communications*. 104,664-672.

NORMAN, B.B., 1973. Current status of vaccination for the control of bovine anaplasmosis. In: Proceedings of the Sixth National Anaplasmosis Conference. Ed: Jones, E.W., Heritage Press, Stillwater, OK, 103.

NORVAL, R.A.I., 1983. Arguments against intensive dipping. *Zimbabwe Veterinary Journal*. 14,19.

OBERLE, S.M., PALMER, G.H., BARBET, A.F. and McGUIRE, T.C., 1988. Molecular size variations in an immunoprotective protein complex among isolates of *Anaplasma marginale*. *Infection and Immunity*. 56,1567-1573.

PALMER, G.H., 1989. Anaplasma vaccines. In: Veterinary Protozoan and Hemoparasitic vaccines. Ed: Wright, G., 1-29.

PALMER, G.H. and McGUIRE, T.C., 1984. Immune serum against *Anaplasma marginale* initial bodies neutralizes infectivity for cattle. *The Journal of Immunology*. 133,1010-1015.

PALMER, G.H., KOCAN, K.M., BARRON, S.J., HAIR, J.A., BARBET, A.F., DAVIS, W.C. and McGUIRE, T.C., 1985. Presence of common antigens, including major surface protein epitopes, between the cattle (intraerythrocytic) and tick stages of *Anaplasma marginale*. *Infection and Immunity*. 50,881-886.

PALMER, G.H., BARBET, A.F., DAVIS, W.C. and McGUIRE, T.C., 1986a. Immunization with an isolate-common surface protein protects cattle against anaplasmosis. *Science*. 231,1299-1302.

PALMER, G.H., BARBET, A.F., KUTTLER, K.L and McGUIRE, T.C., 1986b. Detection of an *Anaplasma marginale* common surface protein present in all stages of infection. *Journal of Clinical Microbiology*. 23,1078-1083.

PALMER, G.H., BARBET, A.F., MUSOKE, A.J., KATENDE, J.M., RURANGIRWA, F., SHKAP, V., PIPANO, E., DAVIS, W.C. and McGUIRE, T.C., 1988a. Recognition of conserved surface protein epitopes on *Anaplasma centrale* and *Anaplasma marginale* isolates from Israel, Kenya and the United States. *International Journal for Parasitology*. 18,33-38.

PALMER, G.H., OBERLE, S.M., BARBET, A.F., GOFF, W.L., DAVIS, W.C. and McGUIRE, T.C., 1988b. Immunization of cattle with a 36-kilodalton surface protein induces protection against homologous and heterologous *Anaplasma marginale* challenge. *Infection and Immunity*. 56,1526-1531.

PALMER, G.H., BARBET, A.F., CANTOR, G.H. and McGUIRE, T.C., 1989. Immunization of cattle with the MSP-1 surface protein complex induces protection against a structurally variant *Anaplasma marginale* isolate. *Infection and Immunity*. 57,3666-3669.

PIPANO, E., MAYER, E. and FRANK, M., 1985. Comparative response of Friesian milking cows and calves to *Anaplasma centrale* vaccine. *British Veterinary Journal*. 141,174-178.

POTGIETER, F.T., 1979. Epizootiology and control of anaplasmosis in South Africa. *Journal of the South African Veterinary Association*. 50,367-372.

POTGIETER, F.T. and BESTER, J.B., 1981. Freeze-drying of *Anaplasma marginale*. *Onderstepoort Journal of Veterinary Research*. 48,179-180.

POTGIETER, F.T., SUTHERLAND, B. and BIGGS, H.C., 1981. Attempts to transmit *Anaplasma marginale* with *Hippobosca rufipes* and *Stomoxys calcitrans*. *Onderstepoort Journal of Veterinary Research*. 48,199-122.

POTGIETER, F.T. and VAN RENSBURG, L., 1983. Infectivity, virulence and immunogenicity of *A.centrale* live blood vaccine. *Onderstepoort Journal of Veterinary Research*. 50,29-31.

POTGIETER, F.T., KOCAN, K.M., McNEW, R.W. and EWING, S.A., 1983. Demonstration of colonies of *Anaplasma marginale* in the midgut of *Rhipicephalus simus*. *American Journal of Veterinary Research*. 44,2256-2261.

POTGIETER, F.T. and VAN RENSBURG, L., 1987. Tick transmission of *A.centrale*. *Onderstepoort Journal of Veterinary Research*. 54,5-7.

RISTIC, M., 1981. Anaplasmosis. In: Diseases of cattle in the tropics. Ed: Ristic, M. and McIntyre, I. The Hague, Martinus Nijhoff. 327-344.

RYFF, J.F., WEIBEL, J.L. and THOMAS, G.M., 1964. Relationship of ovine to bovine anaplasmosis. *Cornell Veterinarian*. 54,407-414.

SHKAP, V., BIN, H., ENGAR-WARON, H. and PIPANO, E., 1990. An enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies to *Anaplasma marginale*. *Veterinary Microbiology*. 25,45-53.

SHKAP, V., PIPANO, E., McGUIRE, T.C. and PALMER, G.H., 1991. Identification of immunodominant polypeptides common between *Anaplasma centrale* and *Anaplasma marginale*. *Veterinary Immunology and Immunopathology*. 29,31-40.

SIMPSON, C.F., KLING, J.M. and LOVE, J.N., 1967. Morphologic and histochemical nature of *Anaplasma marginale*. *American Journal of Veterinary Research*. 28,1055-1065.

STEWART, C.G., 1987. Specific immunity in farm animals to heartwater. *Onderstepoort Journal of Veterinary Research*. 54,341-342.

STICH, R.W., KOCAN, K.M., PALMER, G.H., EWING, S.A., HAIR, J.A. and BARRON, S.J., 1989. Transstadial and attempted transovarial transmission of *Anaplasma marginale* by *Dermacentor variabilis*. *American Journal of Veterinary Research*. 50,1377-1380.

STILLER, D., KOCAN, K.M., EDWARDS, W., EWING, S.A., HAIR, J.A. and BARRON, S.J., 1989. Detection of colonies of *Anaplasma marginale* in salivary glands of three *Dermacentor* spp infected as nymphs or adults. *American Journal of Veterinary Research*. 50,1381-1385.

TEBELE, N., McGUIRE, T.C. and PALMER, G.H., 1991. Induction of protective immunity by using *Anaplasma marginale* initial body membranes. *Infection and Immunity*. 59,3199-3204.

TEBELE, N. and PALMER, G.H., 1991. Crossprotective immunity between the Florida and a Zimbabwe stock of *Anaplasma marginale*. *Tropical Animal Health and Production*. 23,197-202.

THEILER, A., 1910. 'Anaplasma marginale'. A new genus and species of the protozoa. *Transactions of the Royal Society of South Africa*. II,69-72.

THEILER, A., 1911. Further investigations into anaplasmosis of South African cattle. *1st Report of the Director of Veterinary Research*, Union of South Africa. 7-46.

TOWBIN, H., STAEHELIN, T. and GORDON, J., 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proceedings. National Academy of Sciences. USA*. 76,4350-4354.

TRUEBLOOD, E.S., McGUIRE, T.C. and PALMER, G.H., 1991. Detection of *Anaplasma marginale* rickettsemia prior to onset of clinical signs by using an antigen capture enzyme-linked immunosorbent assay. *Journal of Clinical Microbiology*. 29,1542-1544.

VISSER, E.S., AMBROSIO, R.E. and DE WAAL, D.T., 1991. An *Anaplasma centrale* DNA probe that differentiates between *Anaplasma ovis* and *Anaplasma marginale* DNA. *Veterinary Microbiology*. 28,313-325.

VISSER, E.S., McGUIRE, T.C., PALMER, G.H., DAVIS, W.C, SHKAP, V., PIPANO, E. and KNOWLES, D.P., 1992. The *Anaplasma marginale msp5* gene encodes a 19-kilodalton protein conserved in all recognized *Anaplasma* species. *Infection and Immunity*. 60:5139-5144.

WEISBURG, W.G., BARNS, S.M., PELLETIER, D.A. and LANE, D.J., 1991. 16S Ribosomal DNA amplification for phylogenetic study. *Journal of Bacteriology*. 173,697-703.

WILSON, A.J., PARKER, R. and TRUEMAN, K.F., 1980. Experimental immunization of calves against *Anaplasma marginale* infection: observations on the use of living *Anaplasma centrale* and *Anaplasma marginale*. *Veterinary Parasitology*. 7,305-311.

ZAUGG, J.L. and KUTTLER, K.L., 1984. Bovine anaplasmosis: In utero transmission and the immunologic significance of ingested colostral antibodies. *American Journal of Veterinary Research*. 45,440-443.