The development of an ELISA test for the detection of *Mycoplasma bovis* antigen in milk.

A thesis submitted for the degree of Master of Veterinary Medicine in the Faculty of Veterinary Medicine of the University of Glasgow.

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Declaration.

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Summary.

The objective of the work presented in this thesis was the construction of an ELISA test for the detection of Mycoplasma bovis antigen in milk samples. Consequently, monoclonal antibodies to M. bovis were prepared for use in the ELISA. Whole cell antigen was used to immunise two BALB/c or BALB/c hybrid mice from which serum and spleen cells were harvested. Fusion was carried out with NSO myeloma cell lines and 441 hybridomas were produced. One hundred and fifty-one of these produced antibody to an M. bovis antigen at levels twice background. The test used for detection and screening of hybridomas was an ELISA constructed with a membrane antigen of approximately 30 kD. Antigen for these M. bovis screening tests was prepared by the ultrasonic disruption of whole cell suspensions of M. bovis grown in media containing mouse serum. The membrane fractions were harvested by ultracentrifugation at 34 000 g for 30 minutes, resuspended in 10 mM TRIS-HCl buffer, pH 7.8, treated with the chaotropic agent guanidinium thiocyanate (6.0 M) and dialysed against 6.0 M urea. The resulting solubilised antigen was designated antigen "i.p." and used in the screening ELISA . Six antibody-producing hybridomas were cloned further to produce 210 stage-1 clones from which 6 monoclonal antibody lines were produced. Monoclonal antibody (MAb) "5G4" proved to be directed against media constituents but the remaining 5 were directed against M. bovis antigen. MAb "5A10" contained IgG1 with a titre in excess of $^{1}/_{1000}$ and was selected for use in the final ELISA.

The final ELISA was constructed using MAb "5A10" as capture

antibody at a dilution of $^{1}/_{1000}$. The same antibody was biotinylated for use at $^{1}/_{500}$ as the developing antibody. O-phenylenediamine dihydrochloride was used as the substrate and 2.5 M sulphuric acid as the stopping agent. It was evaluated against cultures of 34 isolates from 20 mycoplasma species. Absorption greater than twice background was only noted in the M. bovis and one M. agalactiae isolates. The occurrence of the same antigen in the two species does not prevent the use of this ELISA in the detection of M. bovis in bovine mastitis as M. agalactiae does not occur in this species.

Serial dilutions of broth cultures of M. bovis sc38, the strain against which the monoclonal antibodies were produced, were examined by ELISA and culture. The level of detection was found to be 10^8 cfu/ml. Milk samples from cows which had been infected experimentally with an East German isolate of M. bovis were supplied by Dr H Pfutzner, Jena, in frozen form together with information about the numbers of mycoplasmal cfu present at the time of sampling. 78 milk samples from 6 cows were examined for M. bovis antigen using the ELISA, and for N-acetylglucosaminidase (NAGase) using an ELISA available at Stormont. The ELISA detected M. bovis in milks which had an original concentration of 10^7 cfu/ml. ELISA results were positive for milks taken between days 3 and 21 (Cow 1); 7 and 38 (Cow 2); 7 and 10 (Cow 3); 7 and 38 (Cow 8); and 7 and 10 (Cow 9). Cow 7 was negative both by ELISA and culture. The NAGase results were positive over a longer period than the M. bovis ELISA results.

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Chapter One.

Literature Review

1. Introduction.

A review of the current literature relating to *M. bovis* was undertaken, and is presented below. To date, no incidents of bovine mastitis caused by *M. bovis* have been recorded in Northern Ireland, and the purpose of this study was to develop a rapid test for the detection of the organism. Consequently, the review details the general properties of the organism and the standard protocols for its identification. Details of the clinical presentation and pathology of bovine mastitis caused by *M. bovis* are documented, including pertinent aspects of the immunopathological characteristics of the disease syndrome.

1.1 General properties

Mycoplasmas have no cell wall, are membrane bound, and are very small. They are the smallest micro-organisms which can multiply independently outside the cell. They can pass through bacterial filters, have a small genome and the guanine and cytosine ratio of their DNA (22 - 40 mol%) is generally lower than in bacteria (3).

1.11 Characteristics of the mycoplasmas as a group.

The classification is based largely on serology and biochemical properties. Within the genera, the species are identified by their biochemical characteristics and especially by their surface

antigens which are demonstrated by growth inhibition and epifluorescence.

The single most important characteristic that distinguishes the mycoplasmas from other prokaryotes is their total lack of a cell wall, due to their inability to synthesise peptidoglycan and its precursors. Demonstration of a single membrane with a trilaminar structure in properly fixed and sectioned cells is an essential requirement for defining a new isolate as a mycoplasma (4). This anatomical feature is responsible for most of the peculiar properties of the mycoplasmas as a group:-

Pleomorphology and plasticity;

Sensitivity to lysis by osmotic shock, alcohols, organic solvents, detergents, antibody and complement;

Filterability through 0.45 um pore-diameter filters;

Fried-egg colony shape;

Total resistance to penicillin and other antibacterial substances that degrade or specifically inhibit peptidoglycan synthesis.

Many of these features are shared by the L-phase variants (L-forms) of bacteria, in which the cell wall is either defective or is totally missing. The L-phase variants, which are usually laboratory artifacts, can revert to the wall-covered bacterial forms once the inducing substance (penicillin, lysozyme, antibody) is removed from the growth medium. The L-phase variants, unlike mycoplasmas, are capable of synthesising precursors of bacterial cell wall polymers and penicillin-binding proteins (3).

The lack of a cell wall constitutes the basis for the inclusion of the mycoplasmas in a separate class: Mollicutes (mollis - soft; cutis - skin). The trivial term mycoplasmas has been used rather loosely to denote any species in the class Mollicutes. The mycoplasmas are the smallest self-replicating prokaryotes. Mycoplasma cultures contain coccoid cells that have diameters as small as 300 nm and are still capable of reproduction.

Mycoplasmas usually exhibit an extremely simple ultrastructure. The mycoplasma cell is bounded by a plasma membrane, and the enclosed cytoplasm contains ribosomes and a circular double-stranded DNA molecule. The mycoplasma genome is typically prokaryotic with semiconservative replication. The protein-synthesising machinery in mycoplasmas is also prokaryotic in nature so that antibiotics such as chloramphenicol and the tetracyclines inhibit protein synthesis and growth. The mycoplasma membrane resembles the plasma membranes of other prokaryotes in being composed of approximately 2/3 protein and 1/3 lipids. The mycoplasma lipids are mostly typical prokaryotic phospholipids and glycolipids, but the sterol-requiring strains contain, in addition, significant quantities of cholesterol. Membrane proteins, glycolipids, and lipoglycans exposed on the cell-surface are major antigenic determinants in mycoplasmas. Antisera containing antibodies to these components inhibit mycoplasma growth amd metabolism and deform cell shape and, in the presence of complement, cause lysis of the organisms. These phenomena serve as the basis for a variety of serological tests used to differentiate mycoplasma species.

1.12Morphology

The absence of a cell wall and the enclosure of the cell by a thin, 3-layered cytoplasmic cell membrane permit considerable pleomorphism. Granular, rod and filamentous forms are all recorded (3).

1.13 Cultural characters and the isolation of mycoplasmas.

Mycoplasmas require isotonic media, containing growth factors which they are unable to synthesise, like cholesterol, fatty acids, some amino acids and certain components of their DNA. Basic media to which yeast extract, horse serum, DNA and other substances have been added must be used in order to satisify the requirements of the mycoplasmas (3).

1.14 Media for the isolation of mycoplasmas.

Mycoplasmas require cholesterol (contained in serum) for their growth, acholeplasmas do not. Antibiotics directed towards the bacterial cell wall (e.g. penicillin) are usually added to the media for selective culture of mycoplasmas. Many mycoplasmas will only grow, or grow better, under micro-aerophilic conditions. As a general principle, primary cultures are incubated 48 hours at $37^{o}C$, in an atmosphere of 5 % CO_2 (3).

The assessment of solid-media colonies is done under the dissecting microscope with a magnification of x40. Typical

pinpoint colonies show a more or less dense, darker granular centre which grows embedded in the agar and this is surrounded by a thinner, lighter veil-like surface zone ("fried-egg" form). One can assume that mycoplasma colonies are present if the typical colony picture is seen, there is no growth on serum-free media (Acholeplasmas are the exception), and the colonies adhere to the medium.

1.15 Identification of Mycoplasmas

A number of different procedures are required in order to identify mycoplasmas. The following are of practical importance (3):

1. Identification to family:

This relies upon the demonstration of the growth-inhibiting effect of digitonin (cholesterol analogue) on Mycoplasmataceae and on the demonstration of cholesterol dependence, which is tested in serum-free media containing fatty acids, albumin and cholesterol.

2. Identification to genus:

Mycoplasmas are urease negative: ureaplasmas can be distinguished from mycoplasmas by their ability to hydrolyse urea. This property is tested in media containing phenol red and 0.1% urea: if ureaplasmas are present, the colour changes within 24 - 48 hours from yellow to red.

3. Identification to species:

This may be carried out using biochemical tests in which a suitable medium, such as mycoplasma broth pH 7.0 - 7.3 , is supplemented with glucose and 0.2% L-arginine, with phenol red as indicator of fermentation and arginine hydrolysis.

Biochemical characteristics are useful during isolation. Species identification relies on serological detection of specific antigens by the epifluorescence and growth inhibition tests. Epifluorescence is the technique of visualising the presence of antigen in tissue sections which have been incubated with specific antisera and subsequently developed using a fluorescent conjugate. Examination is then carried out using a fluorescence microscope. In the growth inhibition test, the absence of colony development around an area containing species specific mycoplasma antisera allows identification to species level.

1.16 Incidence and veterinary significance of mycoplasma.

The mycoplasmas of veterinary significance parasitise the mucus membranes(42). They are found mainly in the respiratory and urogenital tracts, in the udder and joints, and they exhibit a relatively strict host specificity(37). The pathogenicity of the different species of mycoplasma for animals varies considerably. Some cause recognised diseases, e.g. Contagious Bovine Pleuropneumonia (C.B.P.P.), whilst others cause no clinical signs, e.g. Mycoplasma gallinarum. This thesis is concerned with Mycoplasma bovis (M. bovis) - therefore the remainder of the review is devoted to consideration of the role of M. bovis in

bovine mastitis, and to a review of the methods by which it can be detected(53,54).

Mastitis is the most important condition caused by M. bovis - this is followed in frequency by catarrhal pneumonia and refractory arthritis of calves and young cattle (31, 67). It has been implicated as a cause of abortion (63). The mere statement that mycoplasmas have been demonstrated in a lesion is of little clinical value, because many species are entirely or largely non-pathogenic, even if they are isolated from a pathogenic lesion. Further identification is therefore often required in order to determine the significance and pathogenicity of an isolate and a multiplicity of sera are required for their serological identifications (3).

2.1 Aetiology of Mycoplasma bovis mastitis.

2.11 Characteristics of M. bovis.

Order	Mycoplasmatales
Family	Mycoplasmataceae
Genus	Mycoplasma
Species	Mycoplasma bovis

Previously Mycoplasma agalactiae subsp. bovis, then Mycoplasma bovimastitidis -elevated to species rank 1976 on the basis of serology and nucleic acid homology studies (4, 5).

Coccoid and very short filamentous forms;

Growth of typical Mycoplasma "fried egg" colonies (0.2 mm) optimum about 37^{o}C no growth at 28^{o}C;

mol % G + C of the DNA is 27.8 - 32.9;

the DNA/DNA homology between the type strain of this species and the type strain of Mycoplasma agalactiae is about 40%

Glucose catabolism	-
Mannose catabolism	-
Arginine hydrolysis	-
Phosphatase	+
Tetrazolium reduction (Ae/An)	+/+
Mol % G + C of DNA	27.8 - 32.9

2.12 History and Distribution

The first recognised outbreak of mastitis due to the species now called M. bovis, the most frequent cause of bovine mycoplasmal mastitis, occurred in Connecticut in 1961 (14, 20, 21). Because of the apparent similarity of this outbreak to that of contagious agalactia in goats and sheep caused by M. agalactiae, it was suggested tentatively that the organism be called M. agalactiae var bovis (39). In recognition of serological differences, M. bovimastitidis was suggested as a more appropriate designation (39). Based upon indirect epifluorescence, growth inhibition, metabolic inhibition, and nucleic hybridization observations, it was later recommended that these strains be regarded as a separate species, and that the new species be named M. bovis (40). Shortly following the original outbreak in Connecticut, mycoplasma mastitis was described in 16 herds in New York: the organism was demonstrated to be serologically similar to the Connecticut strain now called M. bovis (4, 5, 39). An outbreak of

mycoplasma mastitis occurred in Israel that was apparently due to M. bovis (20, 39): mastitis due to M. bovis occurred in 1964 in a large herd in California (39, 41). Since this time, mastitis due to M. bovis or isolation of M. bovis from milk has been reported from Australia (20), England (39), Bulgaria (39), Canada (39), France (65), Denmark (29), Switzerland (70), Hungary (39), Germany (66), Italy (20), Netherlands (20, 21), Spain (20), Portugal (20) and Yugoslavia (20). It would appear that the disease is spreading to, or being identified in, more countries. Most of the reported cases have been in the United States, where it has been widely distributed(22).The greatest prevalence appears to be in California, where 104 herds have been involved in clinical mycoplasma mastitis outbreaks (43): M. bovis was involved in 59 of the outbreaks. M. alkalescens, M. arginini, M. bovigenitalium, M. bovirhinis, M. canadense, and `strain ST-6' (M. californicum) were also involved in these problem herds.

In 2 statewide surveys of bulk tank milk samples in California, pathogenic mycoplasmas were recovered from about 4% of the samples (39, 43). About 59% of the pathogenic isolates from the bulk tank milk samples were M. bovis.

Five of the pathogens found in samples from clinical cases were found in bulk tank samples and in somewhat the same frequency, i.e. 52% of the herds with bulk tank samples that were positive for pathogenic mycoplasmas were also positive for M. bovis.

Based on continuing observations it appears that mycoplasma mastitis is becoming more widespread in areas such as California, which has had the disease for some time, and in other places such

as England, where mycoplasma mastitis due to M. bovis was described for the first time in 1978 (39, 76).

2.13 Economic aspects.

The economic impact of M. bovis mastitis can be relatively mild if discovered when only a few cows are involved and if control is instituted immediately. 10% of the herd is usually infected by the time a diagnosis is made and usually not more than 25 - 30% of the cows are infected before the endemic disease is controlled (39). Morbidity of 10 - 70% and salvage sales of 1/3 of the cows from 14 herds with M. bovis mastitis have been recorded.

Additional cost following M. bovis mastitis arises because the return to milk production may be quite variable in terms of both time and production. Consequences of infection listed below have been recorded:

a return to production after 3 - 4 weeks at much reduced yields (20);

reduced yield after 8 - 12 weeks if the onset was early in lactation, or drying off if the onset was more than 12 weeks into lactation (39);

no saleable milk for the entire lactation with recovery the next lactation, recovery after several months and apparent recovery, followed by relapse (39);

50% loss of yield (39); poor yields in the subsequent lactation from cows found infected during the dry period (39);

massive decrease in production or agalactia (39);

generally poor performance in succeeding lactations (39).

The total economic impact of M. bovis mastitis varies greatly, but can be considerable, depending upon the number and value of the animals involved, severity, milk loss, etc(26).

3.1 Clinical Characteristics.

Mycoplasma mastitis should be suspected whenever the following clinical signs are present:

1. A rapidly spreading increase in severe purulent mastitis cases that resist treatment, in cows that show little other evidence of illness;

2. Mastitis cases that typically involve more than 1 quarter (often all quarters);

3. Marked loss of production in affected quarters, most of which show severe mastitis, but some of which may simply cease lactating;

4. Abnormal secretions with tannish or brownish discolouration and sandy or flaky sediments in watery or serous fluid. Particles may rise to the top of the tube early in the clinical disease but usually sink to the bottom. After a few days the entire secretion may be seropurulent (40).

Variable degrees of oedematous swelling and tenderness usually accompany M. bovis mastitis (35). Although the inflammatory swelling is extensive in some cows, in others it is very mild with agalactia predominating as a sign and quarters drying up. Often all 4 quarters are found to be infected (20, 39, 40, 41, 56). Infection spreads from one infected quarter to the other quarter on the same side and then to the opposite quarters over a period of 1 - 3 weeks (39, 40, 41).

Mycoplasma can be demonstrated in large numbers in milk from infected quarters for 2 or 3 days before clinical signs or abnormal milk can be detected. For 3 or 4 days thereafter the milk may be thin or watery and flakes or sandy sediment may adhere to the sides of the sample tube, settle out upon standing, or rise to the top with the cream. Tannish discolouration is common at this time and milky fluid is gradually replaced by a seropurulent secretion during the next few days which may persist for weeks or months. Sometimes the secretion may be thick and resemble colostrum. M. bovis can survive and multiply in milk with high somatic cell counts and infection occurs frequently in quarters already infected with other pathogens (39).

There is great variation in severity and duration of the disease between and within affected herds. Some cows shedding mycoplasma in their milk are never observed to have clinical signs. Cows that appear to recover after several days or weeks can shed mycoplasma for a variable period of time, perhaps intermittently, for at least 13 months (39). This variable duration of clinical signs and shedder status contributes to difficulty in predicting

the outcome of disease in infected quarters and determination of full bacteriological recovery. For this reason, cows that have been diagnosed positive for M. bovis mastitis should be considered as positive possibly for life. In some outbreaks culture of the mammary secretions of dry cows is sometimes positive for mycoplasma. Most of these cows will not develop clinical disease until parturition, or possibly several days after parturition. Non-immune herds newly infected with M. bovis are likely to experience a high percentage of very severe cases. In some cows that are culturally positive there is often no history of past or present clinical mastitis (39).

The major cellular change in milk infected with M. bovis is a very marked leucocytosis due primarily to neutrophils. Cell counts from natural M. bovis infections vary from 0.86 - 80.0 x 10^6 cells/ml in countable samples. In contrast to other forms of mastitis, casein nitrogen is greatly increased (39).

3.2 The demonstration of M. bovis in milk.

M. bovis has been isolated from milk within 12 - 18 hours following experimental infection with 10^5 - 10^{10} organisms. The usual excretion period is considered to be 2 months: it is known that substantially longer or shorter excretion periods may occur - a 13-month excretion period has been reported (39).
The mycoplasma survive in milk for over 2 years at -20°C, 63 days at 4°C, 14 days at 20°C and 7 days at 37°C (83).

Giemsa staining of milk films is not a reliable diagnostic method because the organisms may not be recognised even though present or particles resembling mycoplasma may be present in apparently uninfected milk. One staining technique for milk smears uses polychrome methylene blue. The organisms are metachromatic, appearing reddish purple against the blue background (39). Another is to stain clinical milk samples with 0.01% acridine orange at pH 3.0 (38).

The literature cites the ELISA detection of bovine serum antibody specific to M. bovis for use in epidemiological field studies of bovine mastitis (17, 33, 36, 64, 77, 80, 82). Varying degrees of serological cross-reactivity to seven heterologous bovine mycoplasmal species were demonstrated: cross-reactivity was minimized by preincubation of cattle sera with suspensions of heterologous mycoplasma antigens, prior to measuring serum antibody to solid-phase antigen (36). Heterologous absorption improved the immunological specificity of the assays while avoiding the need to prepare species-unique antigens (23).

Cultural demonstration of M. bovis initially involves inoculation of 2.5 ml mycoplasma broth and incubation under standard conditions for up to 48 hours (48). The broth cultures are checked routinely after 24 hours for growth by placing a drop of the inoculated broth on solid `Friis' agar, the latter itself being incubated overnight and then examined under a dissecting microscope for typical colonies after 24 hours incubation.

Methods for the demonstration of M. bovis are further reviewed below (3).

3.31 Pathogenesis and Pathology

The mechanism by which M. bovis infection spreads from quarter to quarter is considered to be haematogenous, given the demonstration of mycoplasmaemia. Dipping teats in infected secretion once for 10 sec before milking was sufficient for infection, illustrating the high infectivity of this pathogen and its capacity for spread (39).

Gross and histological changes of acute, purulent, parenchymal mastitis are observed in affected quarters within 17 - 60 hours post intracisternal innoculation of M. bovis cultures (39). At day 6 p.i. the infected udder quarters were swollen, their cut surface was yellowish-brown and granular. At 8 days p.i., the lymph nodes were swollen and the interalveolar connective tissue was infiltrated by lymphocytes, plasma cells and neutrophilic granulocytes. By 14 days p.i., the interalveolar connective tissue had undergone proliferation and showed a diffuse infiltration by plasma cells, lymphoid cells and granulocytes. The glandular alveoli had ceased to secrete milk. At 28 days p.i., both the infected and uninfected udder quarters were tough, firm, and the mucous membrane of the milk cistern was thickened and uneven. A variable amount of swelling and firmness of the quarter is almost always noticed early in the course of the disease. Later the swelling usually disappears and the udder tends to become flabby and slack (18). At this time agalactia is marked and involution and atrophy of alveoli have taken place (39). In cases that proceed to agalactia and involution without

protracted or especially severe inflammatory reactions, permanent fibrotic lesions are probably minimal. In general, more severe and destructive inflammatory processes may be expected following infection during early lactation as contrasted with mid- or late lactation, when the tendency is more toward early agalactia, which persists until the next calving. Multiple abscess formation is not uncommon. When cut, the infected gland usually seems to be hard due to increased fibrosis (39).

M. bovis is also associated with bovine arthritis and pneumonia (31). One may speculate that mycoplasmas might spread to the udder from other body or environmental sites without the necessity for udder-to-udder transmission. Clinical mastitis accompanied by mycoplasmaemia has been produced in cows following intracisternal inoculation with various M. bovis strains (18). Haematogenous spread from the respiratory tract is a logical possibility, given the demonstration of mycoplasmaemia (39, 78).

3.32 Microscropic Pathology

The pathology, as with mastitis due to other pathogens, may vary considerably from lobule to lobule depending upon the presence of infection and the stage of the process. Neutrophils and some macrophages are found very early within the alveoli and ductal epithelium. Eventually cellularity decreases and mature fibrous tissue becomes prominent and may be interspersed with numerous abscesses of varying size. Fluorescent microscopy revealed the mycoplasma to be present in discrete colonies throughout the gland. In some areas all alveoli were replaced by an invasive and obliterative fibrosis within fibrotic interstitial tissue

(39). Lymphocytes from calves inoculated with M. bovis antigens showed suppressed lymphoblastogenesis, suggesting that mycoplasma cell membrane morphology is similar to that of mammalian cells, and mycoplasma have been shown to incorporate environmental proteins, including antibodies, onto their surface membranes: it might be that the T-lymphocyte suppressor cells are functioning due to the similarity to host-tissue and/or the camouflage of mycoplasma-specific determinants by host antigens (39).

4.1 Resistance and Immunity.

It is common in infected herds to find shedding of mycoplasmas by animals with no history of typical mastitis. Herds in which the disease has been endemic for a long time may experience a milder form of disease and occasionally an outbreak may be almost entirely composed of mild clinical or non-clinical infections. Immunological resistance may be involved in such cases. Short-term resistance to mycoplasma re-infection is mycoplasma-species-specific. Infections of the bovine mammary gland due to M. bovis usually elicit substantial systemic antibody responses. However, increased indirect haemagglutinating (IHA) titres from previous naturally occurring M. bovis mastitis infections, although indicative of recent exposure to the organism, do not prevent reinfection from experimental challenge exposure (11). Therefore, bovine mammary infections with mycoplasma are somewhat analogous to mycoplasmal respiratory infections in that circulating antibodies do not appear to provide protection to current infection or against reinfection(15).

5.1 Epidemiology.

Many new outbreaks of *M. bovis* mastitis can be traced to purchase or introduction of infected cows (20, 21, 39). Outbreaks also occur in "closed" herds, as recorded in some instances (20). Recent introduction of infected animals is not always a precursor to mycoplasma mastitis outbreaks. A high prevalence of nasal carriers of *M. bovis* occurs in young calves from farms having, or recently having had, *M. bovis* mastitis problems (43, 67).

Spread also occurs from treatment at drying-off or during lactation in infected herds. A common history is for cows to be diverted to a "hospital" group for treatment of conventional mastitis, from which they recover and then return a week or so later with mycoplasma mastitis. *M. bovis* mastitis infection has been observed in non-lactating cows. Some become clinical while non-lactating; others were clinical upon calving. In such instances infected cows could sometimes be grouped according to certain drying-off treatment dates. Spread was believed to occur during this therapy.

Apparently recovered animals may remain continuous or intermittent shedders for many months, and even into the next lactation (39). Newly infected cows may shed mycoplasma in large numbers for several days prior to showing clinical signs. Such cows often serve as a possible source of infection in a regular group of milking cows with spread within the herd being facilitated by the milking machine contamination inevitably resulting.

A study of California dairy herds for the presence of pathogenic mycoplasma in bulk tank milk samples revealed a strong association with large herd size and a lesser association with higher percentage of cows leaving the herd (culled) (43). Herd size is important as a factor determining the prevalence of pathogenic mycoplasma in bulk tank milk. Other factors possibly related to epidemiology include stress, cold, inferior feeding, and airborne and genital spread (39).

Diagnosis.

In a situation where clinical signs and history are present as described above, and routine bacteriological examination of milk samples have not produced any isolations, mycoplasmal mastitis due to M. bovis may be suspected. Confirmation of the diagnosis is achieved using the special techniques outlined above to demonstrate and identify the organism (3).

5.2 Therapy.

Investigators in most countries have concluded that there is no satisfactory treatment for M. bovis mastitis. Intramammary therapy generally gives disappointing results. Italian workers claim good results from intramuscular injection of 5 g tylosin, 3 doses at 3-day intervals. As with most mycoplasma infections, the sooner after infection that treatment begins the better the prospects of a good response, and in infections more than 10 - 12 days old, results tend to be very poor. The potential of the recently developed antibiotics danofloxacin and enrofloxacin have

not yet been evaluated in a field outbreak of M. bovis mastitis (32).

5.3 Control.

The general principles of barrier control techniques have been applied to the few areas presently free of M. bovis. Statutory screening of all possible sources prior to entry to the country is presently achieved by cultural examination of semen etc. At the level of the individual herd a closed breeding policy prevents the introduction of the organism from bought in animals.

Once mycoplasma mastitis is diagnosed in a herd, identification of the specific cause is recommended since the clinical expectations may differ between mycoplasma species. Samples from all newly calved cows and all clinical mastitis cases in infected or suspect infected herds should be cultured for mycoplasma. Bulk tank milk should be monitored by frequent cultures for mycoplasma. Usually it is necessary to culture all the cows in the herd using composite samples in order to locate infected cows. If the outbreak is actively spreading, it is likely that some newly infected cows will not be discovered at the initial culture. Investigators have consistently recommended segregation or culling of infected animals (12, 20, 39).

6.1 The development of tests for the detection of mycoplasmas.

A review of the epidemiology of M. bovis mastitis reveals the highly contagious and pathogenic nature of the organism. Consequently, any control measures rely on the rapid, accurate

identification of infected individuals. Another epidemiological feature which must be addressed by diagnostic tests is that 1 intermittent shedding cow can give a cultural isolation from a bulk tank sample for the whole 300 cow herd. Thus the reasons for the development of the test are:

1. To confirm the identity of infected animals at the begining of a mastitis outbreak.

Effective control measures rely on the earliest possible identification of infected individuals, and their immediate isolation, so preventing spread throughout the herd.

2. To confirm carrier animals in outbreaks of mastitis.

Given the 13-month duration of intermittent shedding which is possible, regular routine screening of bulk milk samples is necessary to maintain herd surveillance.

3. To confirm the infected status of purchased, quarantine stock.

Preventing entry of infection to the herd is necessary by screening quarantined animals. The number of animals dealt with may be the individual dairy herd. Given the current situation in Northern Ireland, the disease security of the national herd may be maintained by preventing the introduction of infection by importation - either of infected individuals, or of infected material such as semen(62).

The literature cites several strategies used to detect M. bovis involvement in clinical mastitis outbreaks:

1. detection of specific serum antibodies, assayed by complement fixation (CF), indirect haemagglutination (IHA) and

ELISA (15, 17, 83). The origin of infection giving rise to the serum antibody may not necessarily be from the current mastitis challenge. Also given the intrinsic delay in production of an immune response, detection of serum antibody, even if specific, does not fulfil the demands of an effectively early diagnosis.

2. direct isolation from samples (3);

The previously described isolation protocols take 3 - 7 days for colony emergence - the species identification by serological and immunofluorescence techniques must then follow. Again, this delay is not diagnostically satisfactory, especially in dealing with the early stages of an outbreak.

3. detection of M. bovis antigens by crude ELISA (19, 63);

There is a recognised cross-reactivity problem in mycoplasma diagnosis, which may contribute to poor test sensitivity - as such, false negatives may occur if the test is crude, with obviously serious implications.

In this respect, the advantages of an antigen capture ELISA were suitable for the development of a diagnostic test:

very high sensitivity and specificity potential of this assay technique (especially appropriate in the context of the recognised cross-reactivity problem of Mycoplasma);

rapid, simple assay;

feasible under field conditions;

exploits full potential of MAb technology.

Monoclonal antibodies, developed against a species-specific non-cross-reacting epitope, were used to give maximum test specificity.

Chapter 2.

Antigen preparation.

2.1 Introduction.

This chapter describes the preparation of M. bovis antigens used for the construction of the ELISA and the development of monoclonal antibodies to antigenic determinants of M. bovis as described in Chapters 3 and 4. The mycoplasma antigens used in classification of the organisms and in their detection are those associated with the cell membrane (39).

A variety of methods have been used to obtain mycoplasma antigens for use in ELISAs (16, 45, 57, 61). The lack of a cell wall makes mycoplasmas fragile and pliable organisms, susceptible to lysis by more gentle means than are usually suitable for disrupting bacteria with cell walls. Special attention must be given to harvesting and washing procedures in order to obtain a suspension of washed intact cells. Combined with the absence of intracytoplasmic membranes, this facilitates the fractionation of organisms into cytoplasmic and membrane fractions, eliminating the need for separation of the bacterial cell wall from the plasma membrane (16).

The antigenicity of mycoplasmas varies with the age of the culture: for antigen preparation it is therefore preferable that organisms be in the logarithmic phase of growth. The antigens expressed on the mycoplasma membrane consist of proteins, carbohydrates and glycoproteins. All these types of antigen can theoretically be employed for use in the construction of ELISA tests - however, carbohydrate antigens may present problems in

that they do not adsorb easily to polystyrene, the most commonly used carrier surface. For this reason protein antigens, or those composed primarily of protein, were used in this study.

Purification of Bacterial Membrane Proteins.

Proteins are major constituents of biological membranes. Although some membrane proteins have been successfully extracted and purified, most integral membrane proteins have not been accessible to standard techniques of biochemical fractionation (66). In large part this has been due to the tendency of these proteins to aggregate in aqueous solvents. To facilitate the study of hydrophobic proteins, "chaotropic" agents have been used, which promote the solubilisation of many hydrophobic compounds in aqueous solvents. The order of effectiveness of these agents is:

thiocyanate (SCN^-) > guanidinium$^+$ > urea > chloride (Cl^-)

The combination of the most efficient chaotropic anion and cation in the form of guanidinium thiocyanate is an effective solubilizer of membrane proteins (58).

In this study the three main methods employed were "Sephadex" gel chromatography, ion exchange chromatography and guanidinium thiocyanate treatment (34, 58, 71).

Chromatography is the separation of solutes by percolation of a mobile phase through a stationary phase which selectively retards the solutes of the mobile phase. There are chromatographic techniques which achieve separation on the basis of various protein properties.

Protein antigens from mycoplasma membranes can be separated by chromatography using techniques which operate using particular properties of the antigens - they are listed below:

Property	Chromatographic technique
Size	Gel permeation
Charge	Ion exchange
Solubility	Reverse phase
Immunological Activity	Affinity

Ion exchange chromatography and gel filtration were used in this study. In ion exchange chromatography separation is obtained by reversible adsorption of the membrane fractions, the polarity of which depends on the eluting buffer. Ion exchange experiments are performed in two main stages:

1. Sample application and adsorption.

The sample is applied to the top of the column suspended in starting buffer and unbound substances are then washed from the exchanger bed using a column volume of starting buffer.

2. Elution and separation.

The separation is obtained since different substances have different affinities for the ion exchanger due to differences in their charge. These affinities can be controlled by varying conditions such as ionic strength and pH.

When using guanidinium thiocyanate treatment, crude membrane preparations are solubilised and used as an antigen for screening monoclonals produced by hybridomas. They were not used to induce

monoclonal antibodies. The production of antigens from M. bovis by these methods is described below.

2.2 Materials and Methods.

2.21 Recovery of Whole Mycoplasma Cells.

The M. bovis isolate used in these studies was of Scottish origin, designated the "sc38" strain. It was used for all subsequent antigen preparations, having been originally identified at the V.R.L. Belfast, and maintained by storage at -70°C. 20 ml of an actively growing culture were inoculated into 500 ml mycoplasma broth: incubation was for 48 hours at 37°C, in an atmosphere of 5% CO_2, at which stage a slight change in the phenol red indicator of the medium reflected maximum growth. The protocol for collection of this material was:

1. The cells were harvested by cold (4°C) centrifugation for 20 minutes at 12 000 g.

2. The culture fluid was carefully discarded.

3. The cell pellet was re-suspended in cold isosmotic 0.25 M NaCl solution, to 2% of the volume of the growth medium.

4. The re-suspended cells were collected by centrifugation for 20 min at 12 000 g, at 4°C.

5. The cell pellet was washed twice more with cold isosmotic 0.25 M NaCl. 0.2 ml aliquots of this material were stored frozen at -20°C for future use.

2.22 Preparation of Cell Fractions by Ultrasonic Oscillation.

Rupture of mycoplasma cells by ultrasonic oscillation was the most effective way of preparing mycoplasma-cell fractions and was carried out as follows:

1. Organisms in the logarithmic phase of growth were harvested and washed as described above. Protein determination was performed (51) on the washed cell pellet suspended in 0.025 M TRIS buffer, pH 7.5.

2. The suspension, diluted to 0.1 mg cell protein/ml was immersed in crushed ice and sonicated for 1 minute, at 20 kilocycles/second, using an M.S.E. 150W ultrasonicator. The process was repeated twice, separated by 1 - 2 minute cooling periods.

3. The membranes were collected by centrifugation for 30 minutes at 34 000 g: the supernatant was collected and stored at -20^{o}C as the **"soluble cytoplasmic fraction"**("C") for use in screening monoclonals (Chapter 4).

4. The membrane was resuspended in 2.0 ml standard buffer containing 10 mM TRIS-HCl, pH 7.8).

5. The solubilised membrane suspension was stored at -70^{o}C to prevent degradation of membrane phospholipids and proteins by endogenous phospholipases and peptidases.

The mycoplasma membrane contains numerous proteins capable of acting as antigens. An attempt was made to separate these into groups using chromatographic techniques (gel chromatography, ion exchange chromatography) or exposure to a chaotropic agent. The aim of this was to reduce competition between different antigens

in coating wells for the ELISA, thereby increasing the chances of obtaining monoclonal antibodies to more than one antigen type.

2.23 Purification of Membrane Proteins.

1. Gel filtration.

This was carried out by column chromatography, using a 100 cm column of 25 mm diameter, packed with Sephadex G100 (Pharmacia) or Sephadex G200 (Pharmacia) equilibrated with standard buffer at room temperature (71). Fractions were collected using an LKB-bromma ultrorac II fraction collector linked to an LKB-bromma 2138 uvicord S spectrophotometer.

The ultrasonicated membrane fraction (in standard buffer from step 4 above) was solubilised by the addition of 10% Tween 20 (Sigma). 3 - 4 ml of the suspension was loaded onto the column and elution fractions were collected at an absorbance of 280 nm (61).

2. Anion Exchange Chromatography.

This was carried out using the column described above, packed with DE-52 anion exchange resin (Pharmacia) equilibrated with standard buffer. 3 - 4 ml of the ultrasonicated membrane suspension, solubilised with 10% Tween 20 (Sigma), were applied to the column.

After applying the sample, the columns were washed with 250 ml of the starting buffer. This was followed by: (i) a stepwise elution using 0.1 M, 0.2 M, 0.3 M, 0.4 M, 0.5 M and 1.0 M NaCl containing the TRIS component of the standard buffer.

(ii) a continuous gradient from 0 - 0.5 M NaCl containing the TRIS component of the standard buffer, delivered from a gradient maker. The column was finally eluted with 200 ml of 1.0 M NaCl in the TRIS component of the standard buffer.

3. Guanidinium thiocyanate treatment.

Solid guanidinium thiocyanate was added to the membrane suspension to achieve a final concentration of 6.0 M. The degree of solubilisation is proportional to the concentration of guanidinium thiocyanate - maximal solubilisation was achieved at 6 M of this "chaotropic" agent. This turbid suspension became clear during gentle agitation at room temperature for 30 minutes. The entire mixture was centrifuged for 30 minutes at 4000 g, 4^{o}C. The supernatant fluid was immediately dialysed at 4^{o}C overnight against 100 volumes of standard buffer containing 6 M urea until the pH of the solubilised cell membranes was equal to that of the standard buffer in 6M urea. The dialysate was then centrifuged for 30 minutes at 4000 g, 4^{o}C and the pellet obtained by this step was combined with the earlier pellet and re-extracted with guanidinium thiocyanate in a similar fashion. The second extraction has been shown to solubilise an additional 5 - 10% of the protein of the membrane suspension (58). Solubilisation may be determined by direct measurement of protein in the supernatant solution (51). In this study protein concentration was determined by spectrophotometry at 280 nm using an LKB-bromma 2138 uvicord S. The majority of the polypeptides appeared to remain in solution when the guanidinium thiocyanate was removed by dialysis against 6M urea.

2.3 Results

1. Recovery of whole mycoplasma cells.

The method described above was found to yield 1 - 2 ml of cells per litre of culture. The system was found to be repeatable and two batches of cells were produced. 1 batch of 2 aliquots of whole cells was stored frozen for future use.

2. Preparation of cell fractions.

No problems were experienced with the method. A total of 10 x 0.5ml aliquots of ultrasonicated cell membrane were produced for further work from the two batches of culture, and designated "M". 10 aliquots of soluble cytoplasmic fraction were also produced, designated "C".

3. Results of separation of membrane antigens.

(i) Gel filtration.

The results of filtration using a Sephadex G200 (Pharmacia) column are shown in Figure 1i. A major peak between fractions 3 and 4 was the most obvious feature, but a subsidiary peak also occurred between fractions 4 and 5. All fractions were tested as antigen in the direct ELISA against hyperimmune serum described in Chapter 4.

Filtration using a Sephadex G200 (Pharmacia) column (Figure 1ii) also produced 2 major peaks. A repeat of this at a slower pump speed (Figure 2) also produced 2 major peaks, yielding fractions 9 - 15 which were used as antigen along with fraction 19 from the summit of the second peak.

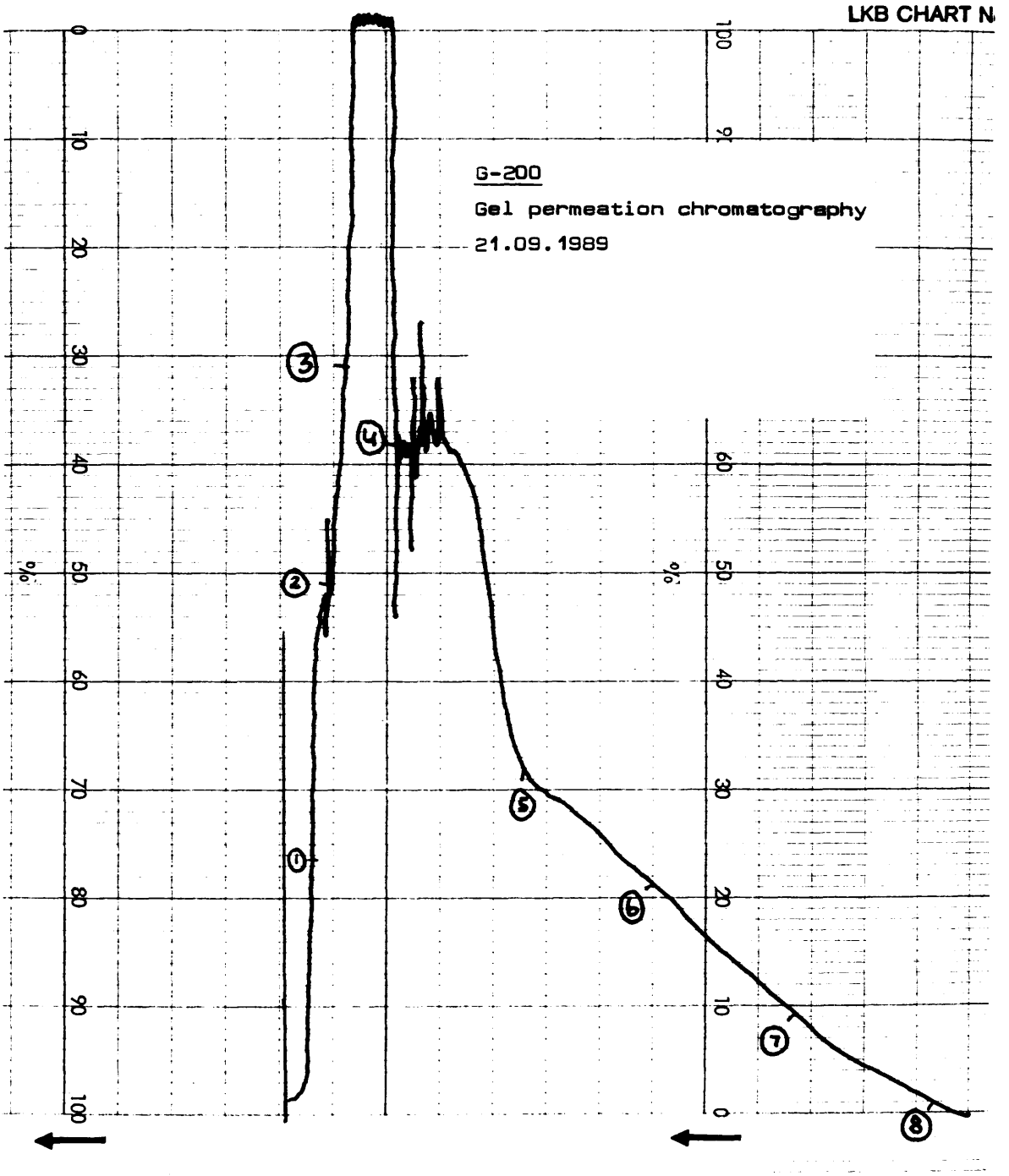


Figure 1i.

Absorption at 280 nm of the eluate from a G200 Sephadex column loaded with M. bovis membrane antigens.

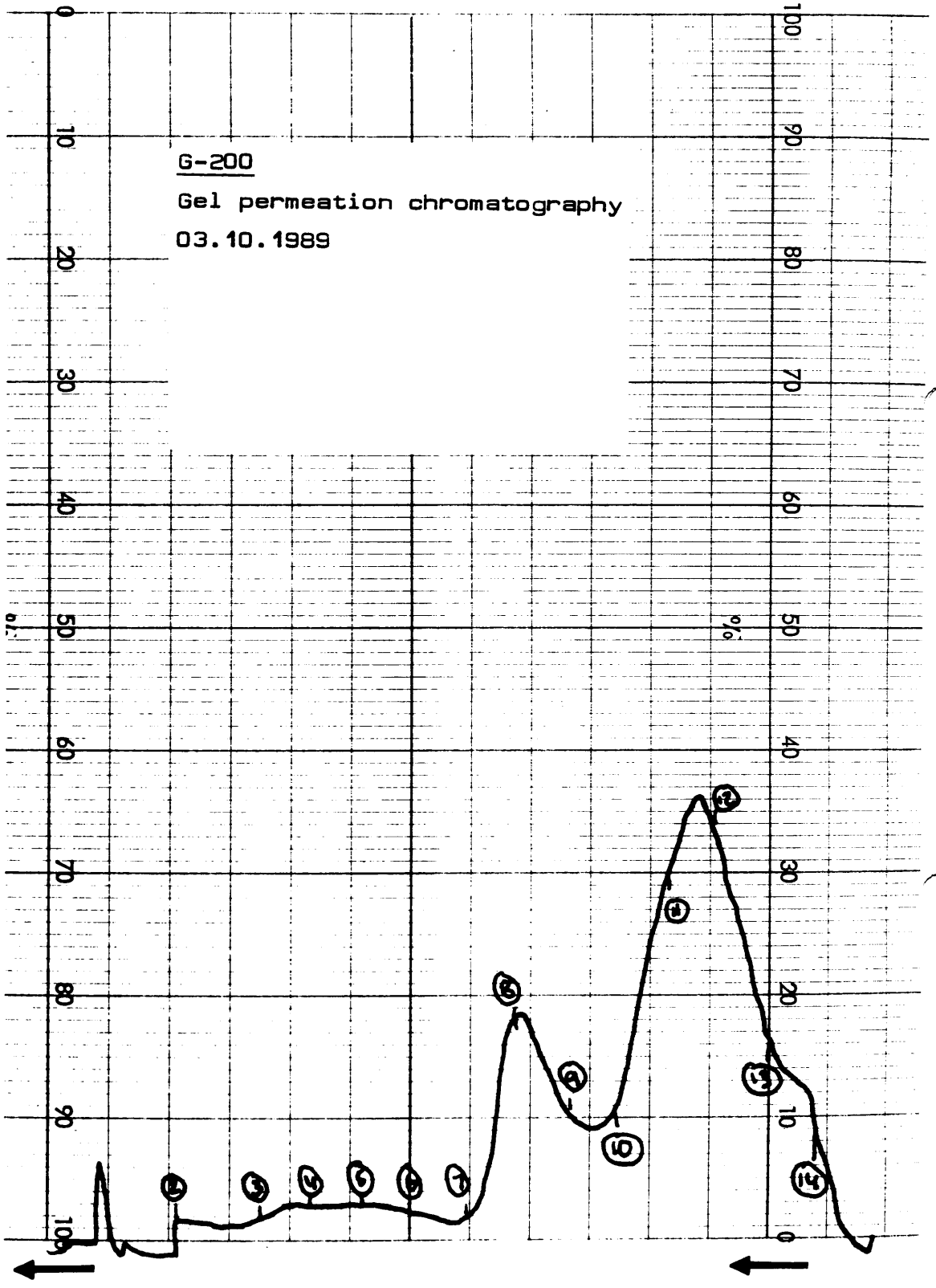


Figure 1ii.
Absorption at 280 nm of the eluate from a G200 Sephadex column loaded with M. bovis membrane antigens.

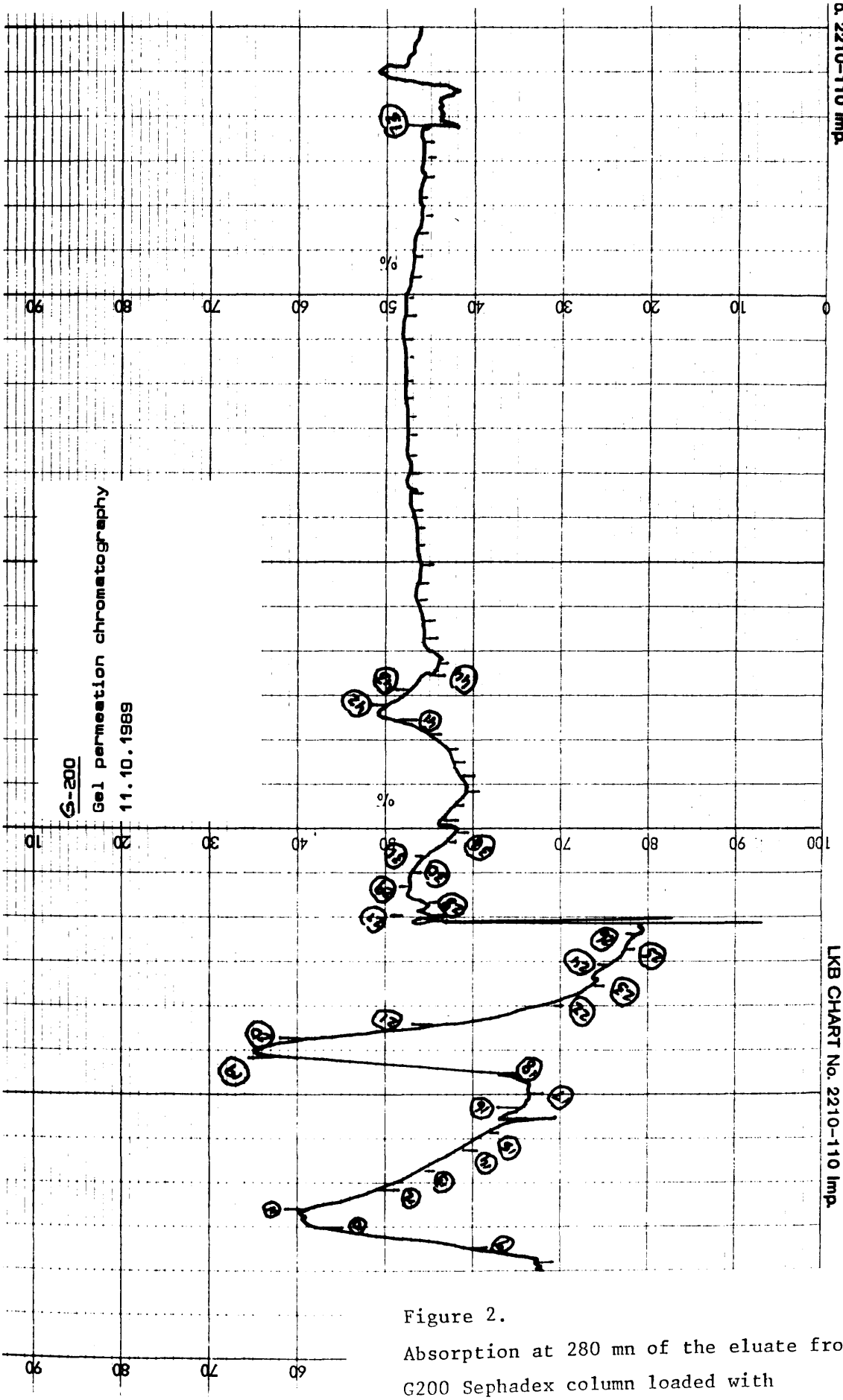


Figure 2.

Absorption at 280 mn of the eluate from a G200 Sephadex column loaded with M. bovis membrane antigens.

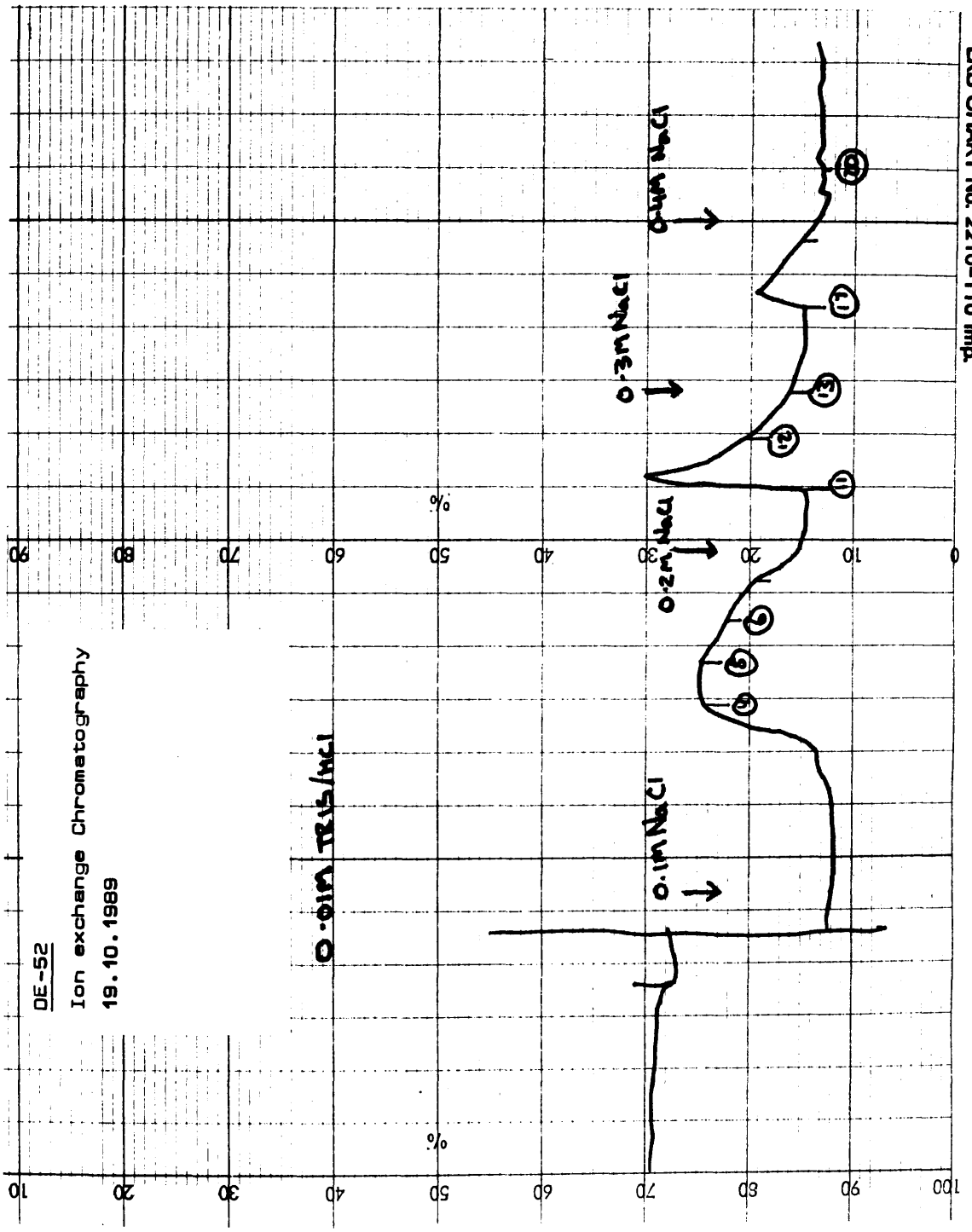


Figure 3i.

Absorption at 280 nm of the eluate from a DE-52 anion exchange column loaded with M. bovis membrane antigens.

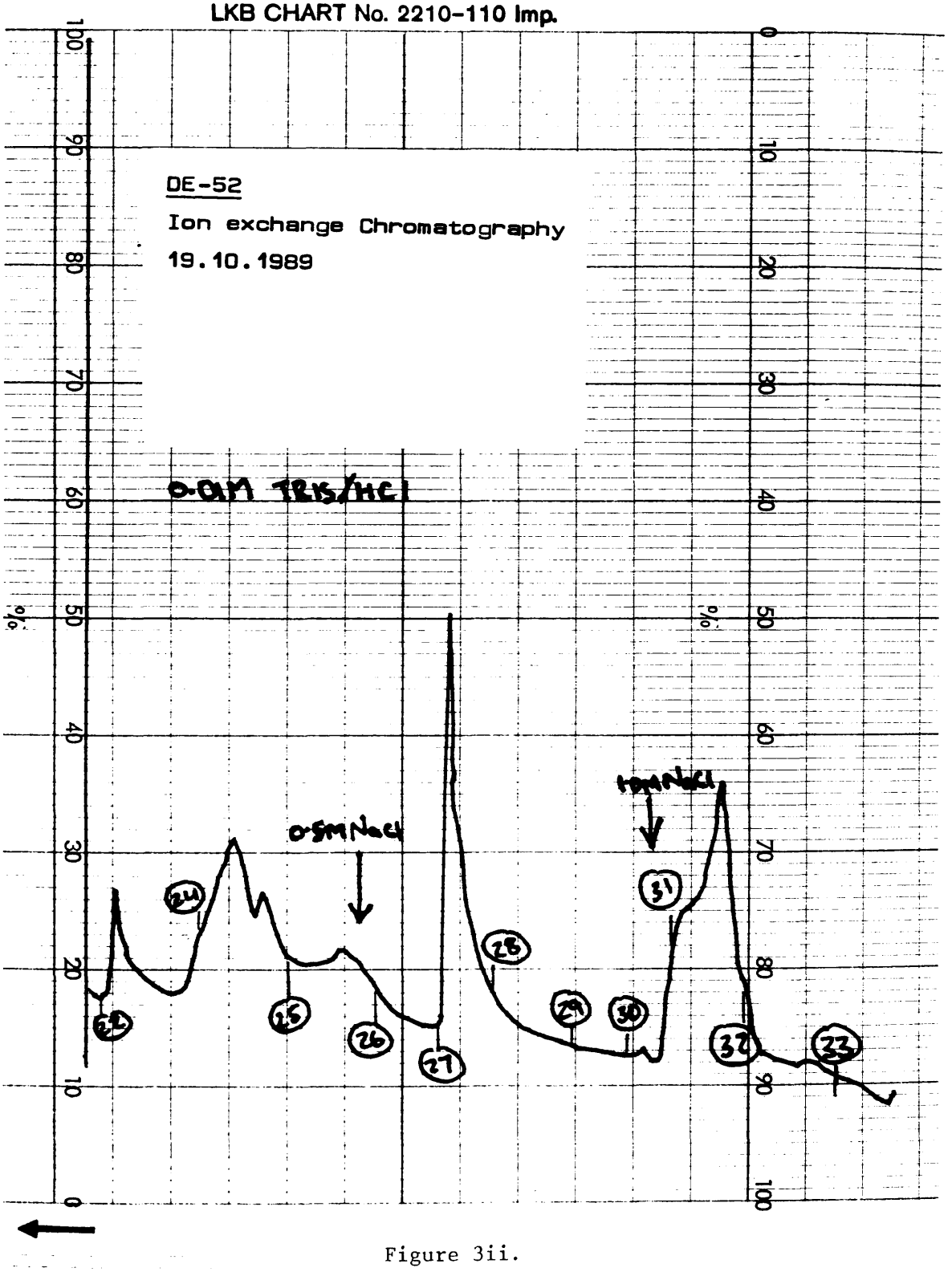


Figure 3ii.

Continuation of recording Figure 3i.

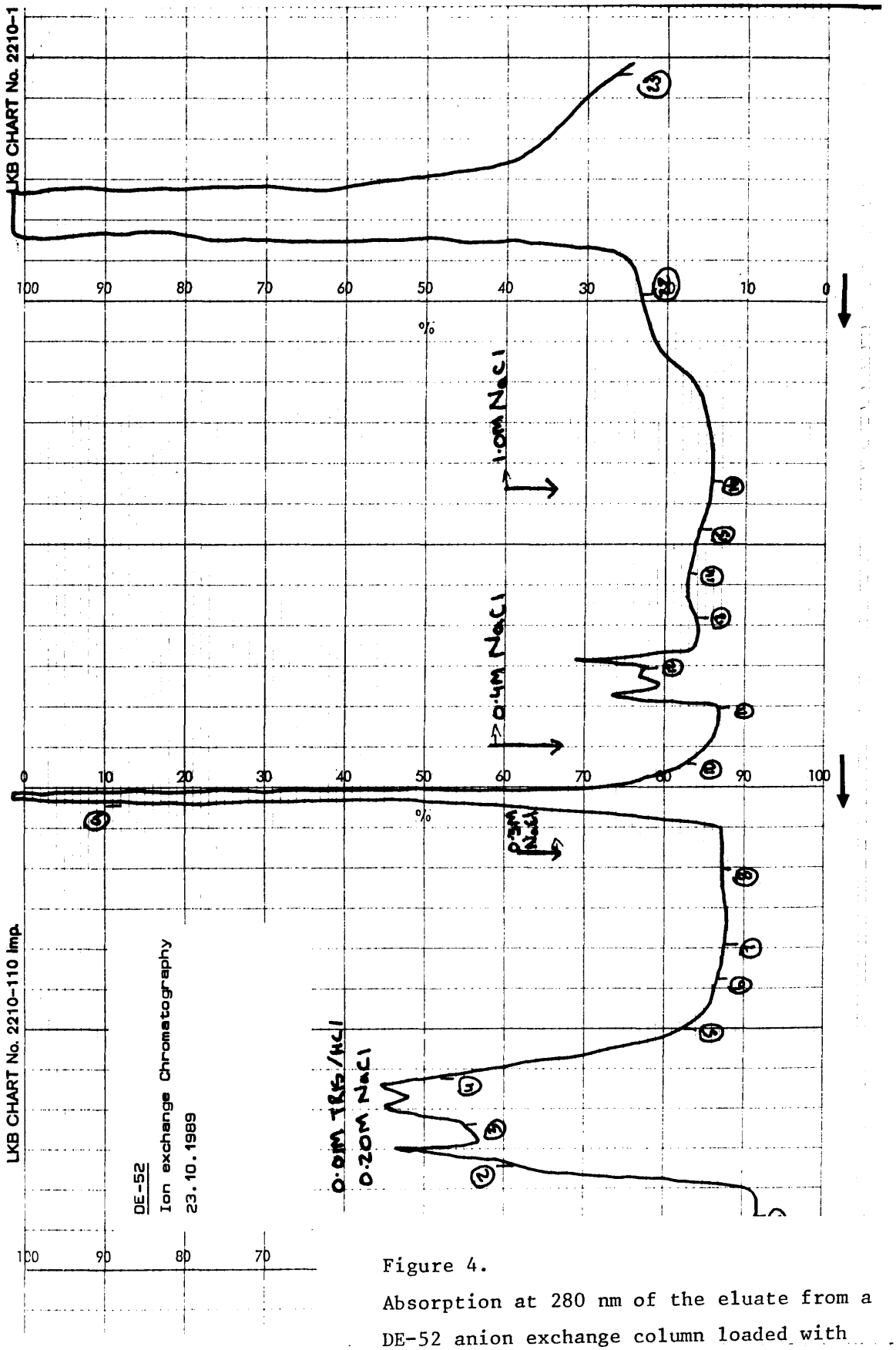


Figure 4.

Absorption at 280 nm of the eluate from a DE-52 anion exchange column loaded with M. bovis membrane antigens.

Step-wise elution

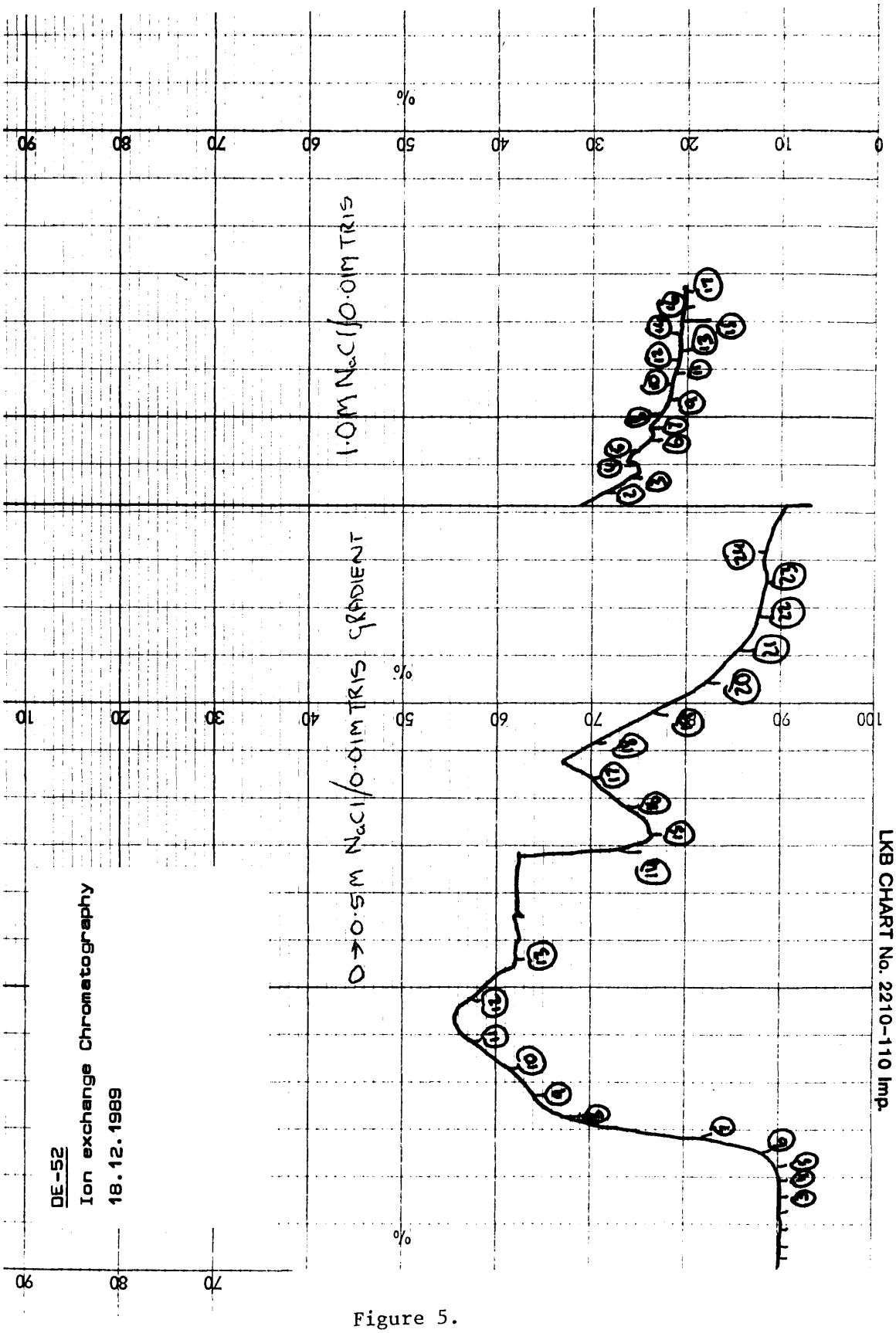


Figure 5.

Absorption at 280 nm of the eluate from a DE-52 anion exchange column loaded with M. bovis membrane antigens.

Gradient elution technique.

(ii) Anion exchange chromatography.

The results of stepwise chromatography are shown in Figures 3i and 3ii. Single peaks were demonstrated following each stepwise dilution, with the exception of the 0.4 M step which produced 2 peaks. All fractions were tested as antigen in the ELISA described in Chapter 4. A repeat of this preparation (Figure 4) showed similar peaks following the stepwise inoculation of buffer. All fractions were tested for use as antigen.

The results of continuous chromatography are shown in figure 5. An initial peak is followed by a plateau and then by a second peak. All fractions shown were tested as antigen.

(iii) Guanidinium treatment.

Two 2 ml aliquots of treated material were produced and labelled antigen "i.p."

2.4 Discussion.

2.41 Recovery of whole mycoplasma cells.

The yield of cells was considered to be satisfactory. The ease with which the whole cells were processed in this study was fortunate. Highly viscous cellular DNA may be released from some of the cells during washing. Centrifugation may enhance the rupture of the cell membranes and release DNA. The released DNA is responsible for stickiness and aggregation of the cell pellet. Addition of deoxyribonuclease to the washed cell suspension can be used to overcome this difficulty (16), but was not required in this study.

2.42 Preparation of cell fractions.

The crude cell fractions prepared by ultrasonication (cell membrane "M" and soluble cytoplasmic "C") may not be sufficiently pure for use as antigens without further processing. Ultrasonic oscillation is not recommended in enzyme location studies because the membrane is disintegrated into minute particles, some of which may not be sedimented even at very high gravitational forces. Therefore, the technique may cause contamination of the cytoplasmic fraction with minute membrane particles (16, 72) and render it unsuitable as a source of antigen. Similar questions arise about the purity of the membrane fraction. The criteria for membrane purity are arbitrary rather than absolute. A good membrane preparation should be as free as possible of unbroken cells, cytoplasmic constituents (ribosomes, DNA), and components of the growth medium (16).

The growth medium used in this study contained mouse serum, thus reducing one of the major sources of antigenic contamination. The use of chromatography was intended to produce protein antigens of higher purity on a repeatable basis free from unbroken cells and cytoplasmic constituents.

Gel permeation was successful in producing two main peaks of protein, but this was less specific than the use of stepwise anion exchange chromatography which, in turn, produced more peaks than gradient elution. Stepwise elution (using different salt molarities or pH changes) is technically simpler but has its disadvantages. Substances eluted by a sharp change in pH or ionic strength tend to elute close together and so peaks have

sharp fronts and pronounced tailing since they contain more than one component. Tailing may lead to the appearance of false peaks if a buffer change is introduced too early. Linear salt or pH gradients help substances to be eluted in symmetrical peaks and give better resolution. There was no evidence that this problem occurred in this study.

Gradient elution generally produced good resolution. This is because during elution "zone-sharpening" occurs. In the absence of a gradient, one of the factors which would contribute to poor resolution would be band spreading as a result of diffusion. In gradient elution, the leading edge of a peak is retarded if it advances ahead of the concentration or pH required to elute it. Thus the trailing edge of the peak has a relatively higher speed of migration, resulting in zone sharpening. The total volume of eluent in the gradient used here was about x5 the bed volume of the ion exchanger. Longer gradients may lead to excessive band spreading and dilution, whilst shorter gradients may not give adequate separation. It is not clear whether the plateau following the first peak shown in Figure 5 was of this nature.

2.43 Guanidinium thiocyanate treatment.

Effective solubilisation of tightly bound membrane proteins was achieved by the use of the chaotropic agent, guanidinium thiocyanate. Further protein fractionation is difficult in the presence of the high ionic concentrations used. The polypeptides remain soluble when guanidinium thiocyanate was replaced by urea (58). This would make possible the use of fractionation techniques depending on charge differences of the different

polypeptides, such as ion exchange chromatography and polyacrylamide gel electrophoresis. The antigen produced here was used for these purposes but the information is not included in this thesis as the "i.p." fraction produced was used directly.

Chapter Three.

Production of Polyclonal and Monoclonal Antibodies to *Mycoplasma bovis*.

3.1 Introduction.

This chapter describes the production of the immunological reagents used in the study. Both hyperimmune sera and monoclonal antibodies were produced, the latter both as ascites fluid and as tissue culture supernatants.

The reasons for the use of monoclonal antibodies in the ELISA detection of mycoplasma antigens in milk were given in Chapter 1, but are restated here: monoclonal antibodies are specific to individual epitopes and therefore avoid the cross-reactions so common in polyclonal sera to mycoplasmas; sensitivity, of particular importance for the detection of carriers and production of the large quantities necessary for a herd-screen type test. Monoclonal antibodies are produced from splenic B-lymphocytes of mice which have been stimulated by priming with antigen *in vivo*. B-lymphocytes can be activated to produce antibody specific for an antigen, but are very short lived in culture. Myeloma lines grow indefinitely *in vitro* but have lost their ability to produce immunoglobulin. The development of monoclonal technology by Kohler and Milstein (47) solved the two main problems:

1. Developing lines of myelomas that did not secrete any antibody of their own;
2. The development of a system of separating fused hybrids from

unfused myeloma and spleen cells. In their original paper, Kohler and Milstein used Sendai virus as the fusion agent. This virus has the ability to cause the two membranes to fuse. Later it was found that polyethylene glycol (PEG) would do the same thing but was much easier to handle, and it is now accepted as the fusion agent ("fusogen") of choice.

The variables that are involved during a fusion are cell ratio, medium, conditions for achieving contact, the fusion agent, time, temperature and processing after fusion.

The method developed for the separation of fused hybrids from unfused cells makes use of myelomas defective in the enzyme hypoxanthine-phosphoribosyl-transferase (HPRT): unfused cells die in medium that contains hypoxanthine, aminopterin and thymidine ("HAT" medium). The aminopterin blocks the main pathway of DNA synthesis, and the rescue pathway using exogenous hypoxanthine and thymidine depends on the presence of HPRT. Spleen cells die in culture and the unfused myeloma cells die in HAT medium: the only cells that will survive are the hybrids in which the myeloma provides the ability to grow in culture, and the spleen cell provides the HPRT.

3.11 Antibody Purification.

Proteins are made up of an assortment of 20 amino acids. The choice, repetition and arrangement of these amino acids is unique to each protein. In the process of protein formation, as the primary chain of amino acids is assembled, so the secondary

conformation (an alpha-helix, or beta-pleated sheet), is assumed. The folding of these sequences in relation to each other forms the tertiary structure. Non-protein groups added to the final protein give glycoproteins in the instance of carbohydrate chains. The primary structure is held together by the covalent peptide bonds between the amino acids. The covalent bonds stabilise the tertiary structure. The secondary and tertiary structure is further stabilised by hydrogen bonds and electrostatic forces. These interactions are dependent on the environmental conditions - changes in pH, ionic strength and temperature will interfere with them, thus changing the tertiary structure and changing the biological activity. Therefore when working with proteins, the conditions should be such that their tertiary structure is not irreversibly interfered with, so that they are able to maintain their natural, active form (1).

Purification of murine MAbs from ascites fluid or culture fluid is an essential step in the scheme of MAb production and characterisation, so producing an homologous antibody class, directed against a specific epitope (52).

3.12 Purification of IgG by Protein A Affinity Chromatography.

Protein-A (SpA) is a cell-wall constituent of Staphylococcus aureus, which has gained increasing importance as a tool in both quantitative and qualitative immunological techniques because of its ability to interact with immunoglobulins, mainly IgG, from mammalian species. Such applications mainly employ SpA in the

insoluble state as SpA-Sepharose. Fractionation on SpA-Sepharose provides a simple way to produce murine IgG1 uncontaminated by other murine IgG subclasses (50, 71).

The SpA molecule is divided into 2 structurally and functionally different regions: a region consisting of highly homologous immunoglobulin-binding units, and a non-immunoglobulin binding unit covalently linked to the cell wall. SpA remains markedly stable on exposure to high temperatures, low pH and denaturing agents, e.g. guanidine-HCl (50).

SpA interacts with IgG via the Fc part of the immunoglobulin. Differences in the affinity for SpA of different subclasses of IgG is reflected by the different pHs needed for elution from SpA-Sepharose columns, which have been utilised in separating different subclasses from each other (34).

The value of SpA as a reagent in laboratory techniques is lessened due to some defects in its Ig-binding reactivity:

mouse immunoglobulins have a low affinity for SpA;

lack of Fc reactivity with IgG 3, a subclass constituting approximately 8% of total IgG (30).

3.13 Protein-G.

Protein-G (SpG) is the IgG-binding protein of group C and G streptococci. The molecule binds IgG of different subclasses from most mammalian species and thereby has a broader IgG-binding capacity than SpA. SpG also has a higher affinity for murine IgG than SpA (1, 2, 13, 73).

The unique IgG-binding properties displayed individually by SpA

and SpG are combined in the chimaeric Protein-AG consisting of the IgG-binding domains of both SpA and SpG. SpA and SpG have complementary binding patterns - the chimaeric receptors retain the binding capacities of both the parental constituents. The advantages are:

the binding spectra toward polyclonal immunoglobulins are broader than the individual parent proteins;

individual subclasses of mouse monoclonals are more likely to possess strong affinity to the chimaeras than to SpA or SpG itself (27, 28).

In 1960 it was reported that under acidic conditions, plasma proteins could be differentially precipitated by short chain fatty acids, such as caprylic acid (n-octanoic acid) (25,68). Based on this observation a procedure was developed in 1969 to isolate IgG from mammalian sera. It has been adapted for the isolation of monoclonal antibodies from mouse ascites fluid (69, 74).

3.2 Materials and Methods

Both hyperimmune murine serum and hybridomas for monoclonal antibody production were produced from the same two mice.

3.211 Production of immune mice

Two BALB/c or BALB/c-hybrid mice from a colony known to have no serum antibody to *M. bovis* were used. They were immunised with *M. bovis* sc38 whole cell antigen which consisted of washed

cells, 50 - 100 fold concentrated from a broth culture using 20% murine serum, disrupted by ultrasonication, as described in Chapter 2.

100 ug of washed cell antigen in Complete Freund's Adjuvant was injected into the peritoneal cavity, followed by 100 ug of washed cell antigen in Incomplete Freund's Adjuvant 14 days later, again intraperitoneally. This was followed 2 months later by an intrasplenic boost of 100 ug of washed cell antigen alone. Hyperimmune serum was prepared from blood harvested by tail bleeding from the immunised mice, and stored at -20°C until required (59).

3.212 Harvesting of primed splenic cells

Three days after the final, intrasplenic, inoculation the mouse was killed by cervical dislocation and placed on its right side so the spleen was uppermost. The body was swabbed with 70% ethanol. An incision was made in the body wall to expose the spleen, without puncturing either the gut or the surrounding splenic arteries and veins. The spleen was removed, placed in a sterile petri dish and transferred to a sterile hood. Using sterile 21 G needles, the spleen was teased apart, in 2 ml of PBSJ medium, to release the cells. As the medium became cloudy it was pipetted off into a 10 ml centrifuge tube. The whole organ was broken down in this way.

Once the debris settled out, the supernatant was pipetted off into another tube. This was made up to 10 ml with more PBSJ and the cells counted: 50 ul of supernatant-cell- suspension + 450

ul 1% acetic acid in PBS, were added to 500 ul 0.2% Trypan Blue. The mixture was counted using a Neubauer haemocytometer. The counting chamber was divided up into 9 large squares: the centre square was subdivided into 25 whilst the surrounding 8 squares are subdivided into 16. All the viable cells in one of the large squares were counted. Non-viable cells are blue while cells lysed by the 1% acetic acid are red. The number of cells counted was multiplied by 10^4 and then by 20 (50 ul added to 950 ul), giving the number of cells in 1 ml of the original suspension. The cells were washed twice in serum-free medium with 30 mM HEPES buffer. The cells were centrifuged at 1500 rpm/500 g for 10 min between washes (75).

3.213 Preparation of the myeloma cells

The NS0 myeloma cells (BALB/c-derived myeloma line) used were removed from liquid nitrogen and thawed out at least 1 week before the fusion. The myeloma cells were taken from the storage vessel and rapidly thawed out in a waterbath at 37^oC. 5 ml of 5% foetal calf serum (FCS) was then added to the tube a drop at a time over a period of about 5 mins whilst the tube was shaken. The cells were then washed once in medium and put into a 500 ml flask. The medium used was RPMI 1640 Origen (IGEN, USA). The cells were then split the day before the fusion and resuspended in 250 ml flasks to 3×10^5 cells/ml. The next day they were at the exponential phase of growth and ready for fusion.

3.214 MAb Fusion.

Three days after the final intrasplenic inoculation, the spleen cells were fused with exponential-growth-phase NSO myeloma cells, and the hybridoma cells maintained in RPMI 1640 medium supplemented with 20% gamma-globulin free horse serum (59). The myeloma cells were harvested and counted using Trypan Blue. They were washed twice in serum-free HEPES buffered RPMI. The spleen and myeloma cells were mixed in a 10 ml tube at a ratio of 10 viable spleen cells: 1 viable myeloma cell and spun down. The supernatant was removed and the tube placed in a 37°C waterbath. 1 ml warm polyethylene glycol (PEG) was slowly added to the cells over a period of 1 min, with continual stirring. The cells must not be in contact with the PEG for more than 2 min. 1 ml of serum-free medium was immediately added, a further 4 ml serum-free medium over 3 - 4 min run down the side of the tube. 20 ml serum-free medium was added followed by 20 ml medium + 15% FCS. This medium was buffered with sodium bicarbonate. This mixture was left at 37°C for 1 - 2 hours. 0.5 ml of a suspension of macrophages were added per 25 ml of hybridoma cell suspension as feeders (46).

3.215 HAT Selection.

24 hours later, 1 ml of medium with x2 [HT] + 10% FCS was added to each well. 1 week later, 1 ml was removed from each well and replaced with 1 ml medium with x1 HAT + 10% FCS. 2 weeks later, 1 ml was removed from each well and replaced with 1 ml medium with x1 HT + 10% FCS. Every week after this the medium was changed

using RPMI 1640 + 10% FCS + sodium bicarbonate. During this period the unfused spleen and myeloma cells died in the HAT medium. Usually the hybridomas started to grow by the second week and grew rapidly once the HT was added. The medium turned yellow when the hybrids were growing well, and they were screened at that point.

3.216 Cryopreservation of Cells.

Sometimes hybridoma cells ceased antibody secretion for no apparent reason, so it was important to be able to go back to a vial that was frozen down soon after cloning Cells of second clone hybrids, secreting to high titre, were frozen down at the earliest opportunity to guard against clone contamination or incubator failure. The earliest time for consistently successful freezing is known to be after the first cloning. Subsequent aliquots of cells were frozen at regular intervals and stored in liquid nitrogen. The freezing mixture used was:

FCS 90%

DMSO 10%

The mixture was kept on ice, as DMSO is toxic to cells at temperatures > $4^{o}C$. The cells were harvested from 2 confluent `Linbro' wells, and spun for 5 min at 1000 rpm. The cells were counted and resuspended to 0.1 - 5.0 x 10^{6} viable cells/ml in freezing mixture, and 1 ml aliquots were pipetted into cryostat tubes and placed in the freezing machine immediately. The cooling profile was:

$0^{o}C$ to $-30^{o}C$ at $1.0^{o}C$/min

$-30^{o}C$ to $-60^{o}C$ at 2.0^{o} C/min

with subsequent transfer into liquid nitrogen. When required, a $37^{o}C$ waterbath was used to thaw the cells rapidly. 10 ml (1640 RPMI + 10% FCS) was added quickly to dilute out the DMSO as it is toxic to the cells at temperatures above 4^{o} C. The cells were then spun down and the supernatant decanted, removing the DMSO. The cells were re-suspended in fresh medium (59).

3.217 Hybridoma Screening.

The hybridomas produced by the method above were screened for antibody production. The 2 most suitable assays for soluble antigens are the ELISA and "Dot Blot" assays. The ELISA method is more suitable for quantitative investigation and Dot Blot or Immunoblot techniques for qualitative assay. Soluble antigen is bound to either the plastic of a 96-well plate (ELISA), or to nitrocellulose paper (Dot Blot). Monoclonal supernatants are incubated with this bound antigen and those containing specific antibody will bind to it. An enzyme-linked secondary antibody with specificity for the monoclonal immunoglobulin (anti-mouse Ig) is used to detect wells/dots where the primary monoclonals have bound. This binding is then visualised by the addition of an appropriate enzyme substrate which yields a coloured product after reaction with the enzyme. Where peroxidase-linked antibodies are used: the substrate opDD (0-phenylenediamine dihydrochloride) is used for ELISA (soluble reaction product), and DAB (Appendix) for Dot Blots (insoluble reaction product).

There are several parameters to be determined before the assay can be used:

1. The antigen titration required to produce an optimal reaction (colour change). For ELISA, this is usually in the range of 5 - 50 ug/ml of protein adsorbed onto the plate. Dot Blots usually require a more concentrated antigen solution.

2. A blocking step may be required. For Dot Blots, blocking is essential, but for ELISA it will depend on the antigen. Insufficient antigen will not saturate all the binding sites and non-specific antibody-plastic binding will occur, constituting a false-positive.

3. The optimal dilution of second layer, conjugated antibody must be determined to minimise background levels. These initial parameters are determined from a titration of antigen against a known antibody - either a polyclonal antibody specific for the antigen, or the serum from the immunised mouse diluted to an optimum working dilution (about 10 ug/ml) protein as in a hybridoma supernatant (46).

3.218 ELISA Construction.

An ELISA was used to screen hybridoma supernatants. It was constructed and used as follows (19). The ELISA was carried out in microtitre trays using 100 ul volumes of each reagent per well. The optimum dilution of each reagent was established by titration. The plates were coated at 4^{o} C overnight with a $^{1}/_{4000}$ dilution of the "i.p." antigen All incubations were at 37^{o}C and after each incubation stage, the wells were washed with

5 changes of 0.01 M PBS pH 7.2, containing 0.05% Tween 20. The stages following coating were as follows:

1. test sample for 1 hour;

2. peroxidase-labelled goat-antimouse immunoglobulin (GAM);

3. substrate: 0.4 mg O-phenylenediamine dihydrochloride/ml + 0.4 ul/ml (v/v) 30% H_2O_2 in 0.1 M citrate/phosphate buffer, pH 5.5, for 15 min;

5. The substrate reaction was stopped by the addition of 50 ul 2.5 M H_2SO_4 per well;

6. Absorbance measured at wavelength 492 nm on an ELISA plate-reader (Titertek).

3.220 Cloning techniques.

The cloning of the selected wells is necessary because although positive, it is likely that there will be more than one clone of cells in the well. There are 3 techniques available for cloning:

1. "Limiting Dilution" - where the cells are diluted out in the wells of a 96-well microtitre plate until they are diluted down to a theoretical 0.3 cells/well after the second and third cloning. The antibody from a well should then be the result of a single hybrid alone.

2. "Soft Agar Cloning" - where the cells from the positive well are mixed in agar and allowed to grow. The agar stops the cells from moving around so the little clumps of cells that form from a single clone may be visualised by a microscope. These cells are then removed from the agar and cultured.

3. "Single-cell Manipulation" - where the cells are diluted out

and single cells picked up and plated out.
Of the 3 methods, "Limited Dilution" is the easiest and quickest, but the single cell manipulation is probably more accurate and need only be done once (46).

3.221 Cloning by Limiting Dilution.

There are two methods that can be used for limiting dilution. Either the cells can be diluted to a set concentration and plated out in a microtitre plate to give a theoretical 0.3 cells/well, or, starting with a higher concentration of cells, they may be titrated across a microtitre plate so that only the last 2 columns will be at less than 1 cell/well. This latter method is easier to perform and also eliminates the risk of losing a clone due to the slow growth that sometimes occurs when the cells are plated out at a very low density. Not all the wells will contain single clones, even when they are titrated to such low concentrations. The only way to be absolutely certain of a pure clone is inspection of the wells regularly as they start to grow up. This is very time consuming, but it does eliminate the necessity of repeating the cloning yet again. It is important to second-clone as early as possible to avoid the clone being overgrown by a non-producer.

3.222 Cloning: Limiting Dilution by Set Concentration.

This was the technique chosen for this study. The hybridoma cells were distributed by dilution in standard medium (RPMI 1640 + 20% serum) to achieve 0.5 cells/well, i.e. 1 cell every other

well. The well containing hybridoma cells secreting ELISA-positive supernatant was harvested and counted. This was done by adding 50 ul of the cell suspension to 50 ul Trypan Blue and pipetting some of the mixture into a Neubauer haemocytometer (the counting chamber is divided into 9 large squares; the centre square is subdivided into 25, whilst the surrounding 8 squares are subdivided into 16; all the cells in one of the large squares are counted). The number of cells (or an average of 4 counted squares) is multiplied by the following factors:

x 10^4 for 1 mm^2 area

x 2 50:50 cells:Trypan blue dilution

Cloning 2 plates required 100 cells in 40 ml standard medium (RPMI 1640 + 20% serum).

Counting gives, for example, a cells/mm^2

= (a) x (2) x (10^4) cells/ml

= (20a) x (10^3) cells/ml

10 ul of a suspension containing 20a x 10^3 cells/ml were added to 10 ml standard medium to give 20a cells/ml, thus making pipetting off 100 cells manageable and accurate. 100/20a ml of the cell suspension were added to 40 ml standard medium, thus achieving 0.5 cells/well, covering 2 plates with 100 cells/40 ml.

A macrophage suspension was added to the cells as a feeder layer (0.5 ml macrophage suspension/96-well plate). 200 ul of the cell/macrophage mix was added to each well of the plate. ELISA screening of the supernatant fluid of these `**stage-1 clones**' started 2 weeks later. Wells selected as positive were cloned again by the same protocol (46).

3.223 Bulk production of Monoclonal Antibody (MAb).

There are 2 ways of producing large volumes of MAb:

1. Simply grow the cells in large flasks/bags and harvest the supernatant produced. Culture supernatants have only 10 ug/ml antibody, which may be concentrated in a variety of ways.
2. The hybridomas may be used to produce ascites fluid, in which the antibody concentration is usually 10 - 20 mg/ml.

Both methods were used in this study.

3.224 Production of Sac Fluid

This method of producing large amounts of antibody is achieved by growing the cells in "Costar" plates which have larger wells, then transfering the growing cells to an osmotic tube bathed in growth medium and incubated under standard conditions. The monoclonal antibody is secreted into the tissue culture supernatant.

3.225 Production of Ascites Fluid.

Hybridomas will grow in the peritoneal cavity of animals of the same strain as the tumour cell- line donor and spleen cell-donor, and secrete monoclonal antibody into the ascites fluid formed within the peritoneal cavity. The ascites fluid will also contain a proportion of immunoglobulins from the recipient animal.

Each MAb cell line was injected (0.5 ml, 2.0 x 10^6 cells/ml) intraperitoneally (i/p) into a single BALB/c mouse 4 days after

the priming of the mouse with 0.5 ml Freund's incomplete adjuvant, "Pristane" (4,6,10,14-tetramethylpentadecane)(92). The developed ascites fluid was removed as the abdomen swells and clarified by centrifuging 10 min at 500 g and stored at -20^{o}C. The MAb produced in ascites fluid can be purified to 10 mg/ml by the same methods as for the cell culture supernatant. The mouse was exsanguinated at euthanasia to for allow serum antibody collection (59, 75).

3.231 Purification of IgG by Protein A Affinity Chromatography.

This method makes use of the fact that Protein A from Staphylococcus A will bind the Fc portion of a number of IgG's of animal origin at high pH but not at low pH. Protein A is linked to Sepharose CL 48 beads with a low pH buffer - this type of separation is termed affinity chromatography (30). Ascites or hybridoma supernatant must first be purified by salt fractionation.

Salt fractionation was carried out using 10 M TRIS to adjust the pH of the Ig to 8.0 - 8.5. The Protein-A column was equilibrated with phosphate buffer, pH 8.0. After collecting the first elution volume, 0.1 M citrate butter, pH 4.0,was added to the top of the column. The second IgG (ph 4.0) elution fraction was collected. The non-bound (pH 8.0) fraction constituted the first elution volume. To avoid low pH damage to the IgG, the bound fractions were neutralised with 1.0 M TRIS. The sample was desalted using a G25 column to remove the salt ions, and transferred to PBS, pH 7.2.

3.232 Partial purification of Ig by Salt Fractionation.

This method constitutes the first, basic clarification of any antibody. As the salt concentration of the medium is raised, there is interference with the interaction of water molecules with the charged polar groups on protein molecules, rendering them less hydrophilic. This allows a greater hydrophobic interaction between protein molecules and so they eventually become insoluble. The salt concentration at which each protein precipitates is different, but between closely related molecules such as immunoglobulins, the difference is not sufficiently great to give a high grade purity of precipitate. However, it is useful as a first step as many unwanted serum proteins, such as albumin, will remain in solution when immunoglobulins are precipitated (52).

An alternative method to the long process of dialysis is to use a G25 column - it will allow salt ions to enter the beads but will exclude the much larger antibody (M.W. 150 000). As the path through the beads is much further that the path around them, the antibody will be eluted in PBS and so will have been dialysed. After dialysis, a Protein-A column may be used to purify the IgG fraction.

Using caprylic acid, the majority of plasma proteins are precipitated without affecting IgG. After caprylic acid treatment of ascites fluid or spent culture medium, the immunoglobulin may still contain an undesirable amount of albumin which can be removed by DEAE-cellulose chromatography. However

each immunoglobulin elutes from DEAE-cellulose at an ionic strength characteristic for that particular MAb.
MAb was purified from mouse ascites fluid using a combination of caprylic acid and ammonium sulphate treatments. Ascites fluid was treated with caprylic acid (n-octanoic acid) (69) while adjusting the volume of each reagent proportionately depending on the starting volume of pooled ascites fluid.

Ammonium sulphate was used, at 4°C, to precipitate immunoglobulins. The MAb was dialysed overnight against PBS, pH 7.2. The saturated ammonium sulphate solution was added very slowly at 4°C while stirring to a final 50%. The mixture was stirred at 4°C for a further 30 min, then centrifuged for 30 min at 3000 rpm. The supernatant was discarded and the precipitate resuspended in a very small volume of distilled water and dialysed twice agains PBS (52).

3.233 Purification of monoclonal antibody from ascites fluid.

1. Serum or ascites fluid was diluted with 4 vols. of acetate buffer (60 mM, pH 4.0), and the pH adjusted to 4.5.
2. Caprylic acid (25 ul/ml of diluted sample) was slowly added dropwise with thorough mixing: the solution was stirred for an additional 30 min. The MAb-containing supernatant was separated (from insoluble material: albumin and other non-IgG proteins) by centrifugation at 10 000 g for 30 min at 5 C and dialyzed against 100 vols of phosphate-buffered saline (137 mM NaCl, 2.6 mM KCl, 10 mM sodium-potassium phosphate buffer, pH 7.4,

containing 0.2 mM EDTA) at 5°C.

3. The supernatant was mixed with 10 x concentrated PBS (10 parts supernatant to 1 part 10 x PBS). The pH was adjusted to 7.4 with 1.0 N NaOH.

4. The supernatant was cooled to 4°C and fractionated with ammonium sulphate (0.277 g/ml to give 45% saturation). The sample was stirred for 30 min before the precipitated IgG was collected by centrifugation (5000 g for 15 min).

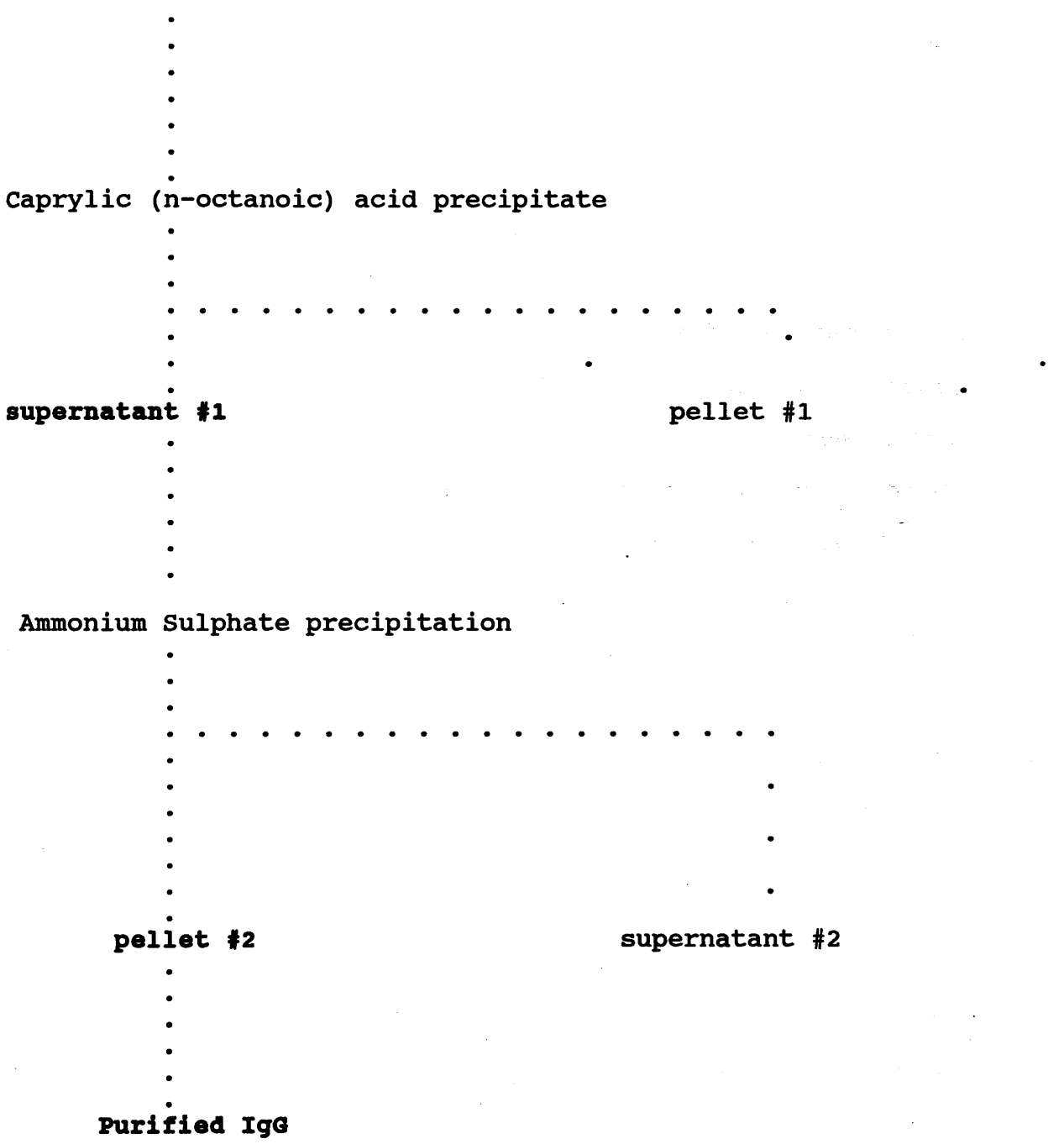
5. The supernatant was discarded, and the IgG pellet resuspended in a small volume of PBS (about 1/10 of the original volume of serum or ascites fluid was routinely used).

6. The resuspended IgG was dialyzed overnight against 100 vols. PBS.

7. Finally, insoluble material (usually a negligible amount) was separated from the purified immunoglobulin by centrifugation at 8000 g for 15 min.

8. Purified MAb were stored at -20°C.

3.234 Outline of the procedure to purify IgG.



3.241 Qualitative evaluation of the monoclonal antibodies produced to M.bovis

This was carried out by Immunoblotting (81). Membrane antigen was prepared as described in Chapter 2 (24).

3.242 (SDS-PAGE).

SDS is a detergent which efficiently solubilizes bacterial membrane proteins. In addition a reducing agent, 2-mercaptoethanol (2-ME), can be added to break the disulphide bridges. Charge differences between proteins are negated, and they move in the gel only on the basis of their molecular weight - the other individial differences due to their tertiary and quaternary structure are rendered unimportant. SDS-PAGE using a modified Laemmli discontinuous buffer system can be performed on a slab gel. In slab-gel electrophoresis, the gel is prepared between 2 plates. A 2-stage system is used - proteins initially run into a "stacking-gel", allowing the proteins to concentrate into a single tight band before enterig the stacking gel. This gives good resolution in the separating gel. Since the gel actually fractionates the proteins by their molecular weight, the choice of polyacrylamide concentration greatly influences the range of molecular weights that can be fractionated. 10% is the commonest concentration of polyacrylamide - lower concentrations are more useful for the separation of high molecular weight proteins and higher concentrations are used for the separation of proteins and peptides of lower molecular weight. The technique

is useful to test the purity of Ig after ammonium sulphate precipitation and Protein-A column purification (16).

3.243 "Western Blotting".

This is a method of characterising antigens with MAbs, which is simpler than immunoprecipitation and does not involve using radioactivity. The technique involves transferring the proteins from the gel onto nitrocellulose paper,sections of which can be incubated with the monoclonals and finally developed using anti-mouse-Ig Horse Radish Peroxidase (Sigma).

SDS-PAGE was carried out with mycoplasma fractions on 10% slab gels with 5% stacking gel. Strips were removed for protein staining and the remainder of the gel applied to a nitrocellulose sheet and blotted overnight at 17 mA/cm. Strips of nitrocellulose sheet from each fraction were blocked with 1% bovine-serum-albumin in 0.1 M PBS with 0.001 M EDTA and 0.5% Tween 80, and then incubated with dilutions of the MAbs followed by rabbit anti-mouse IgG conjugated with horseradish peroxidase. Each incubation was at 37^{o}C and the strips were washed between reagents as described above. The peroxidase substrate used was 0.5 mg 3,3'-diaminobenzidine tetrahydrochloride/ml in 0.02 M TRIS/HCl buffer, pH 7.3, with 0.3 ul H2O2 (30% solution)/ml (82).

3.3 Results

3.31 Cell Fractions.

ELISA reactivities of M. bovis fractions with hyperimmune sera raised in mice to M.bovis sc38 whole cells were determined. The objective of this assay was to optimise the antigen-coating prior to screening the MAbs.

Table 1. Results of ELISA screening of M.bovis sc38 hyperimmune mouse serum with M. bovis sc38 antigens.

		Serum dilution		
		1000	2000	4000
Antigen reciprocal dil.				
W (whole cell)	1000	>2.0	>2.0	>2.0
	2000	>2.0	>2.0	>2.0
	4000	>2.0	1.8	1.1
C (cytoplasmic)	500	>2.0	1.8	1.2
	1000	1.7	1.2	0.8
	2000	0.8	0.7	0.5
M (membrane)	500	>2.0	1.3	0.8
	1000	0.8	0.6	0.4
	2000	0.4	0.3	0.2

Results are expressed in spectrophotometer (Titertek) absorption units at 480 nm.

Conjugate ($^1/_{6000}$) control - 0.6; Serum control - 0.09

The results indicate that the optimum concentration of each antigen coating for screening MAbs was:

Whole $^{1}/_{4000}$

Cytoplasmic $^{1}/_{1000}$

Membrane $^{1}/_{500}$

This ELISA format was repeated for conjugate optimisation, with the following results:

Table 2. Results of ELISA tests for conjugate optimisation.

		Conjugate dilution	
		2000	4000
Antigen dil			
W	4000	>2.0	>2.0
C	1000	1.7	1.2
M	500	>2.0	>2.0

Results are expressed in spectrophotometer (Titertek) absorption units at 480 nm.

The conjugate dilution of $^{1}/_{4000}$ was considered optimal.

3.32 MAb hybridoma screening.

The supernatant fluid from actively growing hybridomas was screened by an ELISA test, performed in microtitre plates (Dynatec) coated with the M. bovis "i.p." antigen. After the second cloning those producing significant ELISA readings (> X2 background) selected as positive and were transferred to 24-well

"Costar" plates to grow up in bulk. Aliquots of cells were frozen as soon as possible. 151 positive (> x2 background) hybridomas were selected for "stage 1" cloning. The antigen-panel used for screening the stage-1 clones were:

cytoplasmic #;

"Sephadex G-200" #21;

Pig serum;

"i.p." antigen.

A total of 441 hybridomas were developed, of which 151 had significant ELISA activity, from which the reserve bank of frozen hybrids was created. Of the 151 original hybridomas, 14 were retested using the above panel of antigens: those 6 with the best ELISA-screening profile were selected for cloning.

Table 3. ELISA results selecting stage-1 MAb clones 08/01/1990

Clone	Antigen	ELISA value
3G3	S	1.590
2H1	S	1.853
4F2	C	1.271
4H9	C	1.307
5A10	S	>2.0
5G4	S	>2.0

Results are expressed in spectrophotometer (Titertek) absorption units at 480 nm.

Control : 0.10

Antigen dilution: 1/2000

Cloning these 6 original hybridomas produced a total of 210 hybridoma-lines (`stage-1 clones'). Again 6 of the latter were selected for re-cloning: 6 `stage-2 clones' were selected on the basis of ELISA retesting.

The monoclonal antibody (MAb) class of the 6 stage-2 clones was determined by double immunodiffusion against rabbit antisera to mouse immunoglobulin isotypes (Nordic, Sweden). Each MAb cell line was cultured to larger volume by dialysis sac growth.

3.33 Immunoblotting.

The "i.p." antigen was subjected to PAGE, then "Western Blotted" onto nitrocellulose paper and immunoblotted with the 6 cloned MAbs, to characterise the antigen(s) against which they were directed. The results shown in Figure 6 illustrate the common antigenic epitope against which the "5A10" MAb is directed. The estimated molecular weight is 30 kD.

3.34 MAb "5G4".

The affinity chromatography profile produced during the purification of MAb 5G4 is shown. The fractions were collected and assayed for antibody activity on 29.03.90. This resulted in 3 batch-pools:

1. #8;
2. #7/9/10/11/12;
3. #3/4/5/6.

Detailed results of hybridomas and clones screened positive by ELISA are presented below in Tables 4 - 6 inclusive.

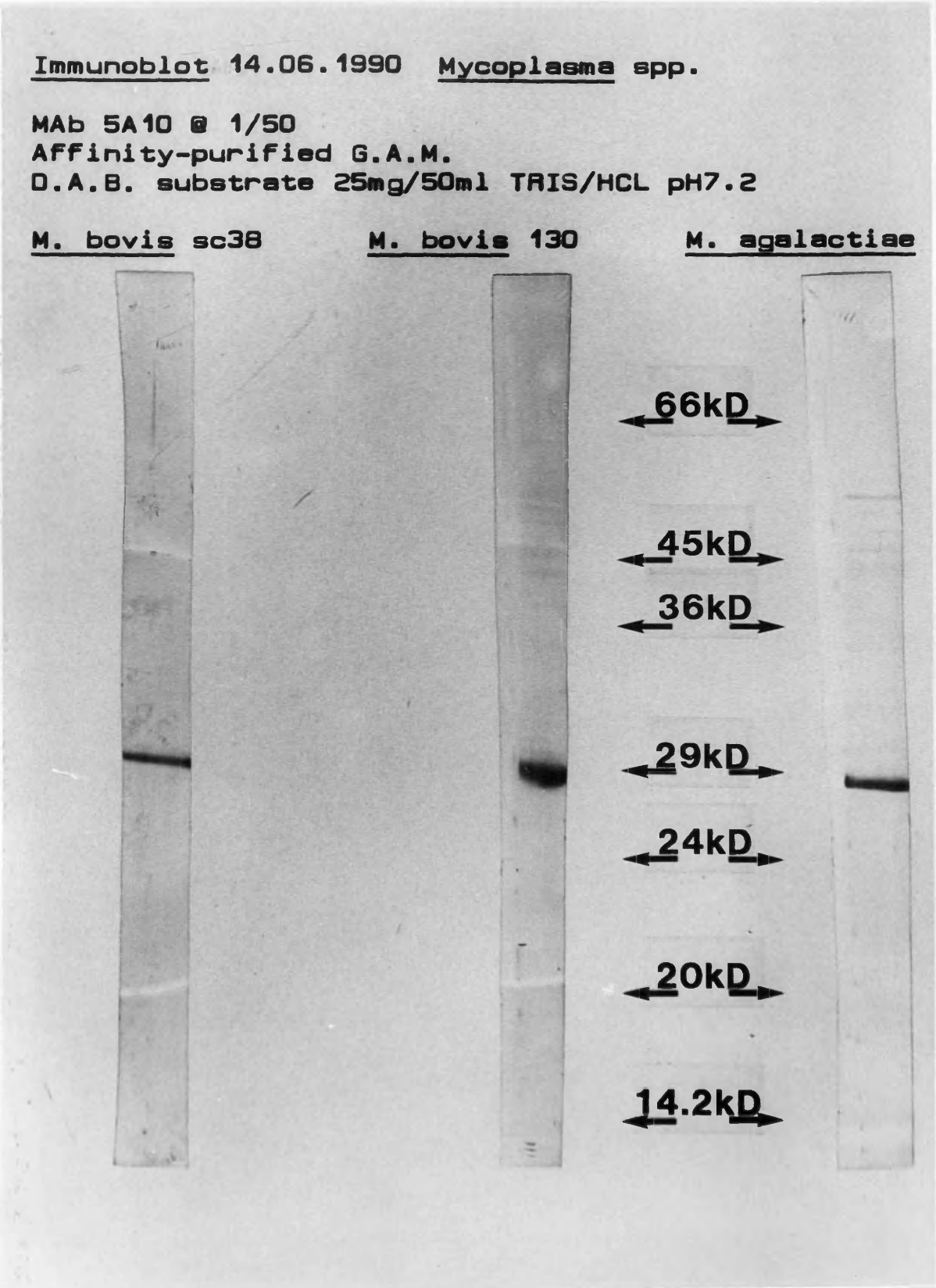


Figure 6.

Immunoblot of M. bovis sc38, M. bovis 130 and M. agalactiae with MAb 5A10.

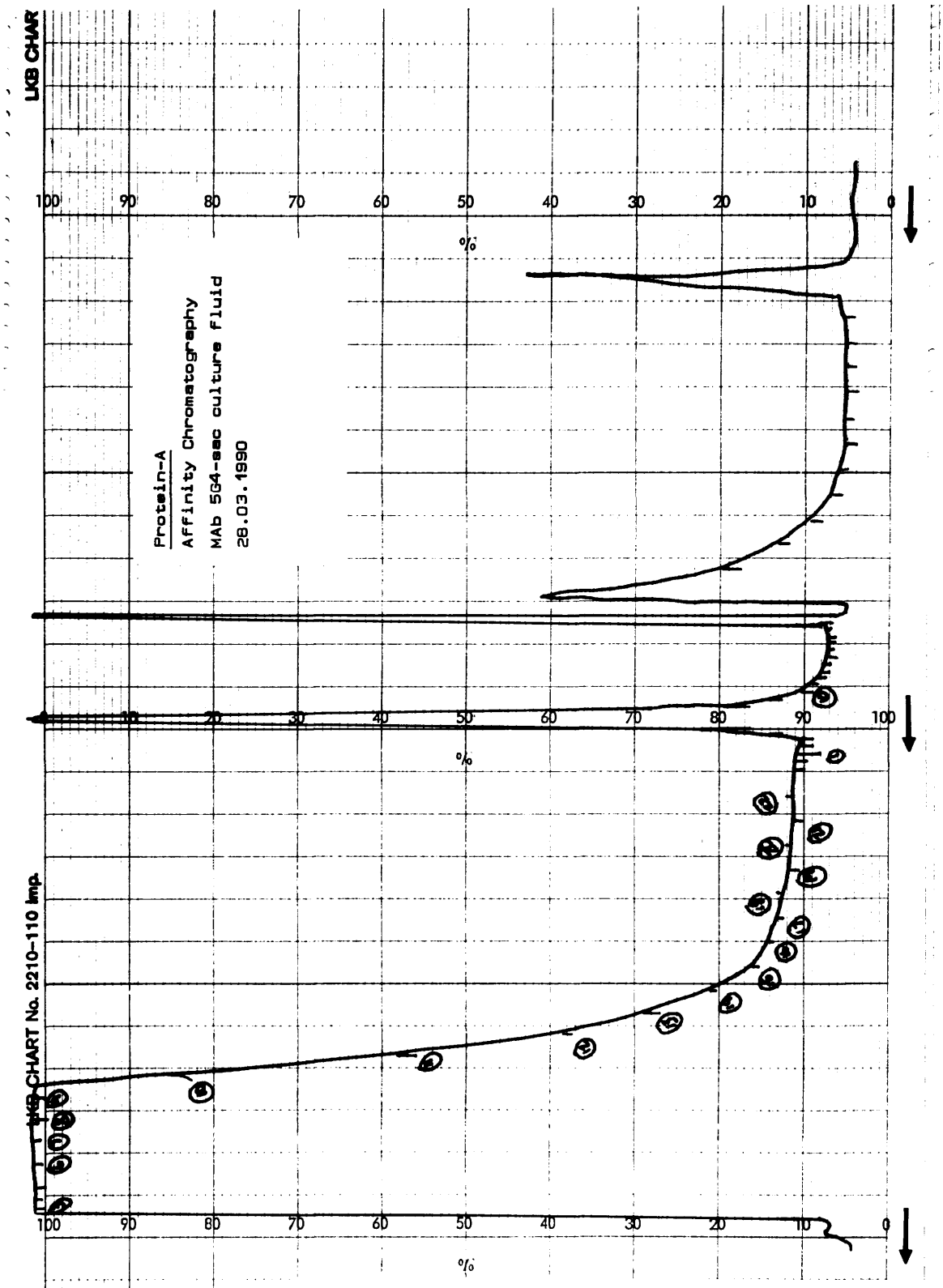


Figure 7.

Absorption at 280 nm of the eluate from a Protein-A column loaded with MAb 5G4.

Table 4. Detailed results of hybridomas screened positive.

Fusion 97: M. bovis 22.12.1989

Table 4a. 01.01.1990 (43)

Plate-1		P-2		P-3		P-4		P-5	
A9	03	A4	15	D1	36	A5	46	A7	60
A10	04	A12	16	D4	37	B2	47	B6	61
D9	09	B9	18	D12	38	B9	48	C2	63
F6	10	B11	19	E6	40	C4	49	C8	65
		C9	22	E11	41	D7	51	D2	66
		C10	23	G3	42	E4	52	D11	69
		D5	24	G6	43	E8	53	E8	71
		E7	25	H6	45	F4	54	F2	72
		G8	29			F5	55		
		G9	30			G5	57		
						H4	58		
						H5	59		

Table 4b. 02.01.1990 (53)

P-1		P-2		P-3		P-4		P-5	A2
21	F6	59	A8	66	B5	88	A10	14	
B4	25	H1	65	A9	67	B10	89	B10	16
B9	27			C1	71	C2	91	C10	21
D5	32			F11	78	C8	92	F4	34
E6	35					C12	93	G4	36
G10	42					D4	95	G11	38
H4	43					E3	97	H3	03
						F2	101		
						G3	106		
						H7	109		
						H9	110		
						H10	111		
						H11	112		
						H12	113		

P-6		P-7	E4	60	A2	67		
F4	62	A4	69					
G1	65	A7	70					
G8	66	A11	71					
D4	05	B3	73					
F7	09	C1	76					
		C2	77					
		C11	81					
		D5	82					
		D11	85					
		A12	13			B4	14	
		C4	16					

Table 4c. 03.01.1990 (14)

P-1		P-2	
A7	03	A7	54
A11	04	E1	72
B1	06	F9	83
C9	15		
C12	18		
E10	30		
F5	33		
F7	34		
G5	39		
G6	40		
G7	41		

Table 4d. 04.01.1990 (41)

P-1		P-2		P-3		P-4		P-5	
A9	01	A4	05	D1	15	A5	23	A7	35
A10	02	A12	06	D4	16	B2	24	C2	37
D9	03	B9	07	D12	17	B9	25	C8	39
		B11	08	E6	18	C4	26	D2	40
		C9	09	E11	19	D7	27	D11	42
		C10	10	G3	20	E4	28	E8	43
		D5	11	G6	21	F5	31	F2	44
		E7	12	H6	22	G5	32	H3	45
		G8	13			H4	33		
		G9	14			H5	34		

P-6		P-7	
F7	47	A12	48
		C4	50

Table 4e. 05.01.1990

P-1		P-2		P-3		P-4		P-5	
A2	1	A7	59	A8	10	B5	14	A10	27
B4	2	E1	60	A9	11	B10	15	B10	28
B9	3	F6	8	C1	12	C2	16	C10	29
D5	4	F9	61	F11	13	C8	17	F4	30
E6	5	H1	9			C12	18	G4	31
G7	58					D4	19	G11	32
G10	6					E3	20		
H4	7					F2	21		
						G3	22		
						H7	23		
						H9	24		
						H10	25		
						H11	26		

P-6		P-7	
E4	33	A2	37
F4	34	A4	38
G1	35	A7	39
G8	36	A11	40
		B3	41
		C1	42
		C2	43
		C7	44
		C11	45
		D5	46
		D11	47

Table 4f. 08.01.1990

3D1	1	3E6	2	3G3	3	4F4	4	5H3	5
6F7	6	7A12	7	2H1	8	3C1	9	4F2	10
4H9	11	5A10	12	5G4	13				

Table 5. Detailed results of stage-1 clones screened positive.

Table 5a. 19.01.1990

2H1

P-1		P-2	
A11	1	A2	13
A12	2	B12	14
B4	3	C11	15
C4	4	D7	16
D5	5	D11	17
D8	6	E3	18
D10	7	E4	19
E2	8	F4	20
E7	9	G8	21
F12	10	H3	22
G1	11	H10	23
H6	12		

3G3

P-1		P-2	
A11	24	A5	33
B5	25	A9	34
C1	26	A10	35
C12	27	B3	36
D11	28	B8	37
F7	29	G4	38
F12	30	G7	39
H8	31	H6	40
H11	32	H11	41

4F2

P-1		P-2	
A2	42	A1	6
A10	43	A4	62
B1	44	A6	63
B2	45	A10	64
C3	46	B7	65
C12	47	B9	66
D1	48	C2	67
D3	49	C3	68
D11	50	C12	69
E9	51	D11	70
F1	52	E2	71
F2	53	F6	72
F5	54	G4	73
G4	55	G8	74
G7	56	G9	75
H3	57	H9	76
H4	58		
H7	59		
H11	60		

Table 5b. 21.01.1990

2H1

P-1		P-2	
B2	1	A12	9
B5	2	B3	10
B6	3	B4	11
B11	4	C7	12
D3	5	E9	13
D11	6	F5	14
G4	7	F11	15
G7	8	F12	16
		G1	17
		G10	18
		G12	19

3G3

P-1		P-2	
B11	20	A1	29
C3	21	A11	30
C9	22	C12	31
D6	23	D7	32
E2	24	E9	33
E12	25	G3	34
F8	26	G10	35
F9	27		
G4	28		

4F2

P-1		P-2	
A6	36	A12	42
B9	37	C8	43
B11	38	C10	44
C5	39	D1	45
H2	40	D7	46
H10	41	E9	47

Table 5c. 23.01.1990

2H1		4F2
P-1	P-2	
D10	E4	C3
	G8	D1
		H4
		E9
		H3
		A1
		A4
		A6
		A10

Table 5d. 24.01.1990

2H1		4F2	
P-1	P-2		
B3		B9	C3
G12		B11	D1
C8	A6		
C10			
D1			

E9

Table 5e. 28.01.1990

4H9		5A10		5G4	
P-1	P-2	P-1	P-2	P-1	P-2
H9	B8	G10	B6	A5	B7
	E5		F8	A11	B11
			G12	A12	B12
				B4	C7
				B12	C10
				C4	D4
				C6	D5
				D10	D6
				E1	D8
				E5	D11
				G3	E1
				G10	E7
				H2	E10
				H6	E12
					F1
					G12

Table 5f. 29.01.1990

4F2	
P-1	P-2
C3	B9
D1	B11
A6	C8
	C10
	D1
	E9

Table 5g. 30.01.1990

4H9		5A10	
P-1	P-2	P-1	P-2
C9	A1	E4	A5
G8	B5	F2	A12
	C5	G5	C7
	E10	G7	F6
	G10		F8
			G12
			H4
			H8

Table 6. Detailed results of stage-2 clones screened positive.

Table 6a. 09.02.1990

2H1D10		4F2D1		5G4D8	
P-1	P-2	P-1	P-2	P-1	P-2
A11	A6	A2	A4	A2	A1
A8	A12	A12	B8	A5	A4
B2	B7	B6	C6	A8	A7
B8	B8	B11	C12	A11	B1
B10	D11	C8	D3	B2	B3
C11	D12	C11	F10	B5	B11
D4	E2	D8	G9	B12	C2
E2	E11	E5		C6	C7
E3	F3	E8		C11	C10
E6	F11	F7		C12	D2
E7	G2	F9		D4	D4
F3		G1		D5	D5
G11		G6		D7	D8
G12		H2		D9	D9
H10		H9		D10	E1
				E3	E3
				E4	E7
				E7	E11
				F3	F5
				F5	F6
				F10	F10
				F11	F11
				F12	F12
				G1	G2
				G3	G3
				G6	G9
				G11	G10
				H2	H2
					H7
					H8

Table 6b. 15.02.1990

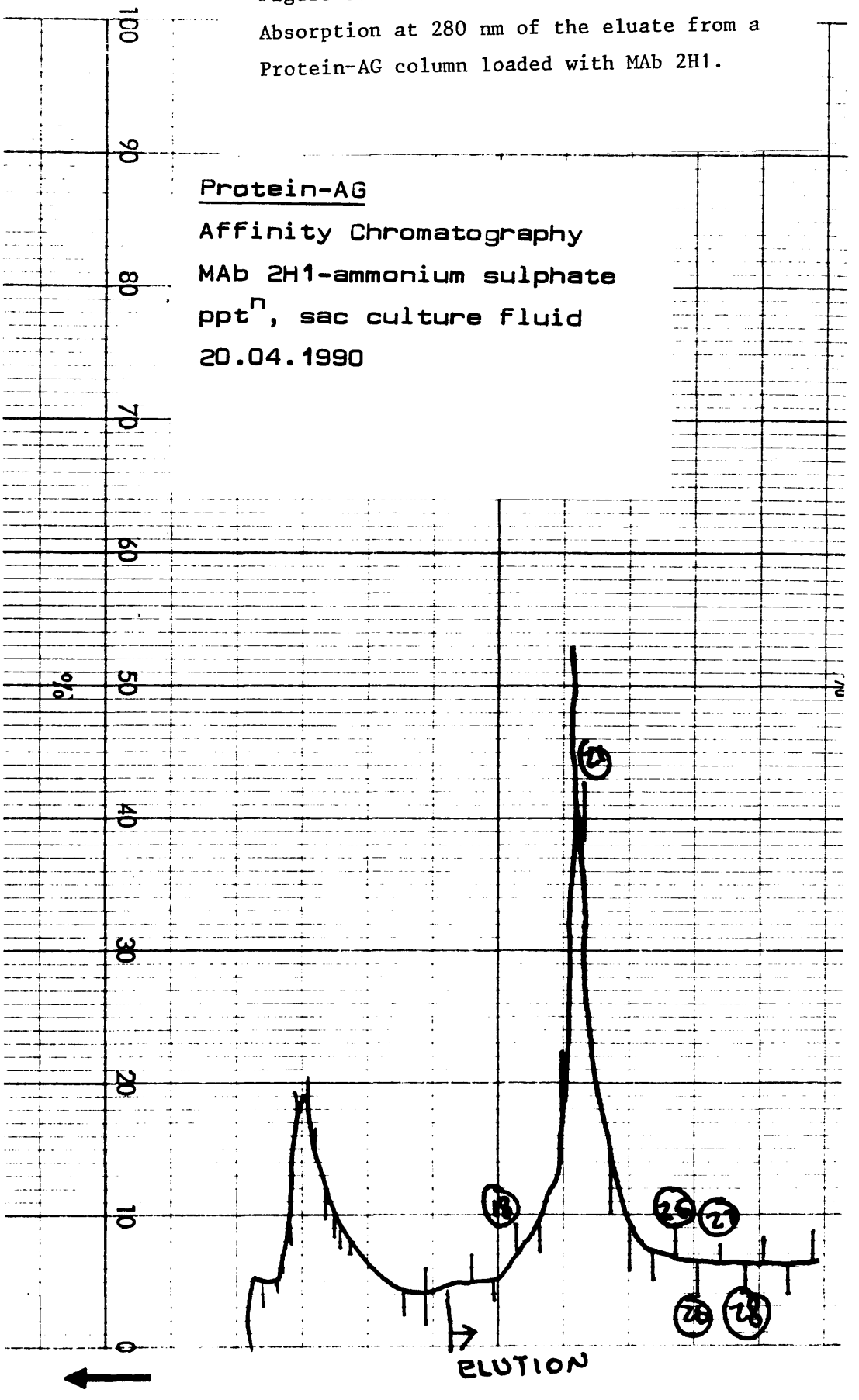
5A10
E4
F3

Table 6c. 19.02.1990

5A10E4	5A10F3
A1	B1
A2	B2

Figure 8.

Absorption at 280 nm of the eluate from a Protein-AG column loaded with MAb 2H1.



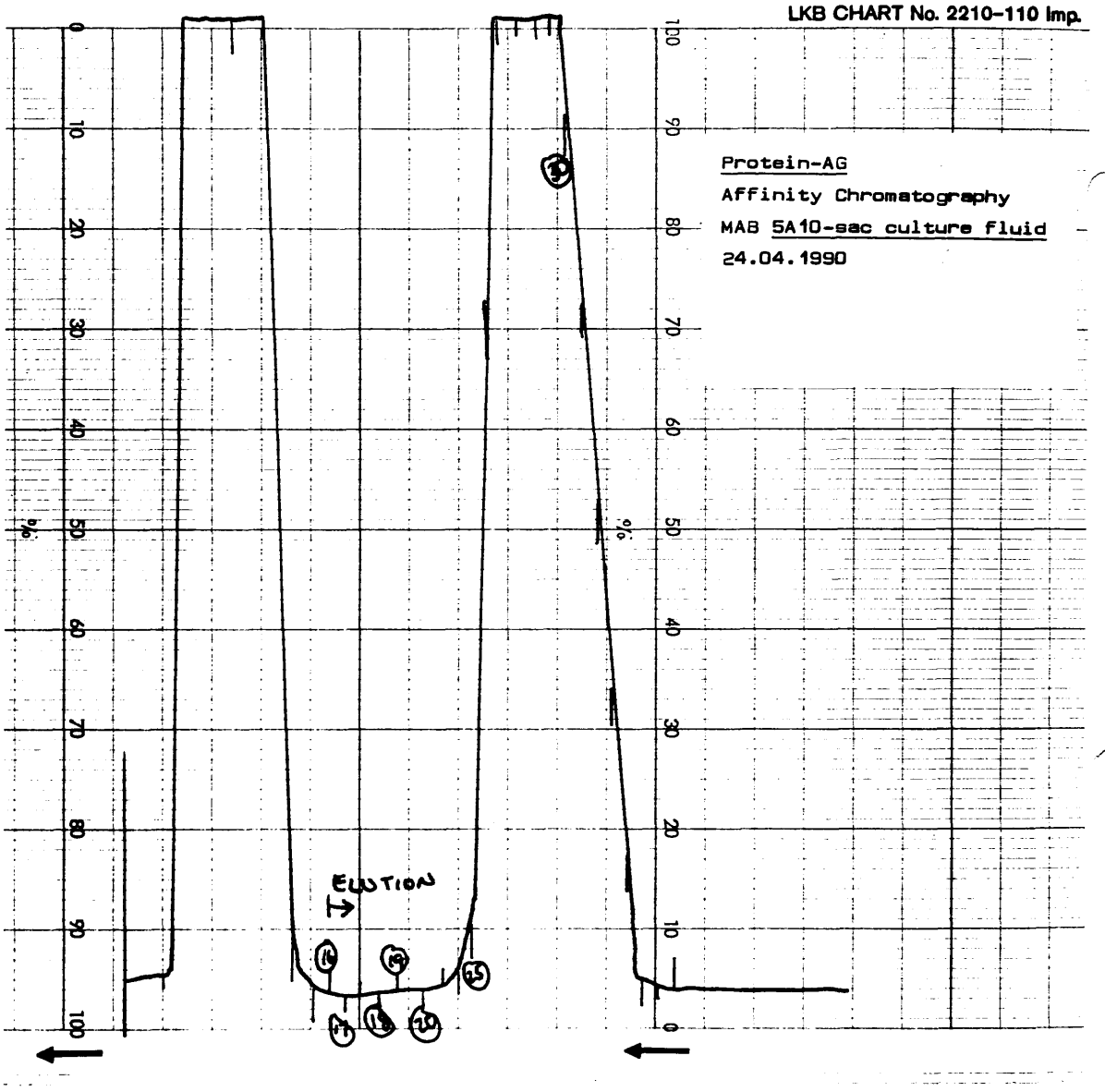


Figure 9.

Absorption at 280 nm of the eluate from a Protein-AG column loaded with MAb 5A10.

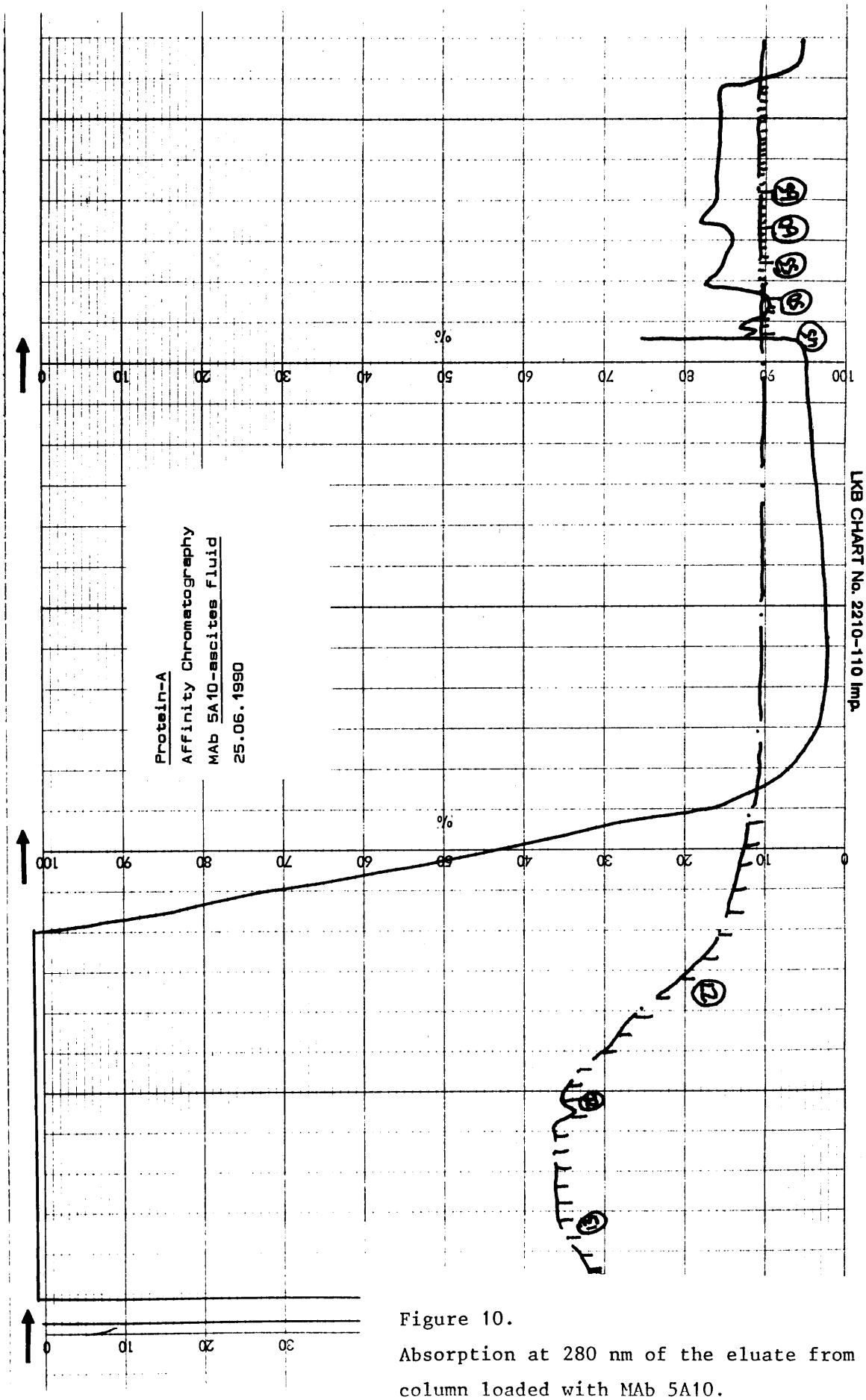


Figure 10.
Absorption at 280 nm of the eluate from a column loaded with MAb 5A10.

3.4 Discussion

All 441 hybrids were checked for growth, which appeared as small clumps of cells. The first screening resulted in some definitely positive wells, some possibly weakly positive and some definitely negative. The 290 negative hybridomas were discarded but it might have been worth keeping some of the intermediate wells. The decision to get rid of cells that do not show positivity is difficult. It may be that they are not producing very much antibody but are never-the-less positive, or the antigen (in the antigen preparation) may be in such low concentration that the well appears negative.

Retesting 14 of 151 active hybridomas gave a representative sample on which to base cloning selection, while reducing the workload at a very busy period of monoclonal development.

The number of the best original hybridomas selected for stage-1 cloning was decided on advice from those experienced in monoclonal antibody development. This was proven accurate, given the workload imposed in retesting the 210 stage-1 clones thus created.

The MAb prepared by the caprylic acid-ammonium sulphate method was highly purified. Treatment of ascites fluid with caprylic acid alone sometimes resulted in an unacceptable amount of albumin contamination (as evident on P.A.G.E. slab-gels). The precipitation of serum proteins by caprylic acid was markedly influenced by pH. Essentially, all non-IgG proteins were completely precipitated by caprylic acid at pH 4.2, and 4.5. At

pH 4.8, caprylic acid failed to precipitate certain globulins, whereas at pH 5.1 even albumin was incompletely precipitated. The use of ammonium sulphate after caprylic acid not only removed most of the residual albumin, but permitted concentration of the immunoglobulin (52).

The caprylic acid procedure described here is particularly applicable to situations when large amounts of many MAbs need to be purified from mouse ascites fluid. Several different pools of ascites can be treated at the same time since the procedure is simple, and each batch of ascites fluid is kept within one vessel. Compared to chromatographic methods, this method eliminates the need for manipulations of column fractions and reduces the chance of contamination of MAb due to the repeated use of the column. Also the expense of column matrices and column monitoring equipment are avoided. In addition, the capacity of this method is greater than that of most chromatographic techniques.

The ability to purify IgG1 antibodies with good recovery is an advantage over Protein-A Sepharose methods, since IgG1 is known to have poor affinity for Protein-A (30).

ELISA examination of the 6 stage-2 clones revealed one to be directed against media constituents, as reflected by the capture-ELISA values for uninoculated broth. Given the known incidence of adsorption of media constituents to the mycoplasma membrane, this finding is perhaps not entirely surprising (17, 23, 60, 79). The design of the screening protocols attempted to forestall such a problem developing from the porcine serum

component of the growth media, by substituting of murine serum - it was not possible to investigate which component was involved. Given the complex nature of the polypeptides constituting mycoplasma broth media, this might well be impossible in any case.

The occurrence of a monoclonal (MAb 5G4) apparently directed against a media constituent was of interest (79). The results of the ELISA test which initially showed this cross-reaction would appear at first glance to be anomalous. There was no problem indicated from the reading for the neat broth well, yet the 1/20 dilution produced the highest interference level. This may be explained by the absence of high pH carbonate coating buffer from the neat, undiluted broth which by inference did not coat the well of the plate. In contrast, the low dilution containing coating buffer did ensure binding of the antigen to the well, and thus a reaction.

The literature cites the cross-reactivity of bovine serum with mycoplasma antigens of bovine, caprine, avian, and environmental sources, with the strongest reaction between M. bovis and M. agalactiae (49).

Immunological cross-reactivity among members of the Mycoplasmatales is well documented (49). Cross-reactive antigens have been associated with culture medium components, with various members of the genus, and with other bacteria and viruses. Antibodies may be raised directed to medium components on the mycoplasmal cell. This could be investigated by comparing ELISA reactivity before and after adsorption with culture medium.

Adsorption is capable of removing reactivity with the medium (65.9 %) and removal of over 30 % of the reactivity with mycoplasmas of non-bovine habitats (M. agalactiae) (49).

The interfering influence of antibodies directed to an antigenic component adsorbable with culture medium obscures the specific ELISA reactivity between homologous antigen and antisera. The non-specific reactivity observed in ELISA will be hard to overcome using antisera raised against conventionally prepared mycoplasma cells grown on complex media. This problem has been somewhat alleviated in this project by using species-specific reagents (murine serum source in growth media) (60).

Chapter Four.

ELISA Construction and Field Testing.

4.1 Introduction

The need for a diagnostic test to detect M. bovis has been outlined in Chapter 1. This chapter describes the construction of the ELISA test for the detection of M. bovis antigen in milk and semen samples. The development of 6 monoclonal antibody lines has been described in Chapter 4: their evaluation for use in the final diagnostic format of the ELISA is outlined below.

The test format chosen for development was the capture ELISA type. This fulfilled the diagnostic requirements, particularly as the potential of this assay type for high specificity addressed the problem of cross-reactivity between different mycoplasma species (10).

Given the recognised cross reaction problem in serological diagnosis of mycoplasmal infection, extensive evaluation of the test against other mycoplasma species and media is necessary (9, 60, 80).

Having orientated the test development primarily toward specificity, the question of sensitivity is then of prime importance. The stoichiometry of the chemical reaction between streptavidin and biotin may be used as the signal produced by a positive reaction in the ELISA test.

Assessment of the test sensitivity was achieved by comparison of field test results with those obtained by conventional isolation techniques and from the NAGase mastitic milk ELISA, giving an overall impression of the sensitivity of the test developed. Testing serial dilutions of inoculated media was another method for assessing test sensitivity (8).

4.2 Materials and Methods.

The general protocol of the capture ELISA test is as follows:

1. Coat plates with MAb overnight at $4^{o}C$;
2. Capture of Mycoplasma sp. from broths incubated for 2 hours at $37^{o}C$;
3. Conjugation with biotinylated-MAb (BMAb) by incubation for 2 hours at $37^{o}C$;
4. Develop by the addition of Streptavidin-peroxidase and substrate.

4.21 Construction of Final ELISA.

The ELISA was constructed from testing various MAb/BMAb combinations by the general protocol outlined above against a 48 hour broth of *M. bovis* sc38, used at $^{1}/_{100}$ dilution. MAb "5A10" was chosen for use both as the coating monoclonal antibody and for biotinylation. The final format of ELISA was as follows:

1. Coat plate caprylic acid treated $^{1}/_{1000}$ MAb "5A10";
2. Capture broth-dilutions $^{1}/_{10}$ - $^{1}/_{10}{}^{-7}$;
3. Develop with $^{1}/_{500}$ B"5A10";
4. $^{1}/_{2000}$ Streptavidin.

4.22 MAb "5A10" Specificity.

MAb "5A10" was extensively tested against all Mycoplasma species known to be have common surface membrane antigens. The origin of the Mycoplasma species tested by capture ELISA are outlined below in Table 7. Cultures were taken from storage at - 70^{o}C and grown for 48 hours in standard mycoplasma broth, incubated at 37^{o}C in an atmosphere of 10% CO_2. 0.20 ml of this broth was then dispensed as the antigen in the ELISA test.

Table 7. Mycoplasma spp. tested by MAb "5A10" for serological cross-reaction.

Mycoplasma sp.	isolate	origin
M.californicum	162	Bovine semen
	M249	Bovine mastitis
	383/83	Bovine mastitis
M.bovigenitalium	49	Bovine vaginal swab
	M197	Bovine mastitis
	M314	Bovine mastitis
	MC961	Bovine vaginal swab
M.canadense	44	Bovine semen
	M128	Bovine mastitis
	M1043	Bovine vaginal swab
M.bovis	sc38	Bovine mastitis
	130	Bovine calf pneumonia
	Sh	Bovine mastitis
	10131	N.C.T.C.
	222/89	Bovine mastitis
	221/89	Bovine mastitis
	422/88	Bovine mastitis
M.agalactiae	10123	N.C.T.C.
M.synoviae	10127	N.C.T.C.
M.arginini	10129	N.C.T.C.
M.edwardii	10132	N.C.T.C.
	MM5d	Canine vaginal swab
M.alkalens	10135	N.C.T.C.
M.iners	10165	N.C.T.C.
M.bovoculi	10141	N.C.T.C.
M.verecundum	10145	N.C.T.C.
M.dispar	10125	N.C.T.C.
M.bovirhinis	10118	N.C.T.C.
M.anatis		Stormont
M.canis	MM50d	Canine nasal swab
	MM25d	Canine nasal swab
M.felis	MM40c	Feline nasal swab
M.fermentens	10117	N.C.T.C.
M.hyorhinis	MP621	Porcine lung
M.pulmonis	MM70R	Rat lung

4.23 Sensitivity Assay.

Mycoplasma broth was inoculated and incubated for 48 hours to achieve log phase growth. Serial ten-fold dilutions were made and colony counts performed on Friis agar plates. These dilutions were used as antigen for a capture-ELISA format.

4.24 N-acetylglucosamide.

The estimation of the level of N-acetylglucosamide (NAGase) in milk has been shown to be indicative of cell damage, and has been found to correlate well with milk cell counts (MCC). As such, this is an enzymatic method for the detection of mastitis. The test uses the enzymatic release of fluorochrome 4-methyl umbelliferone (6).

Milk samples.

A total of 78 milk samples were provided from Dr. H Putzner, Institute for Bacterial Animal Diseases, Jena, Germany. Each 5 ml sample was supplied in a heat-sealed glass vial, constituting a daily single-quarter sample from an individual cow which had been experimentally infected intracisternally with a local strain of *M. bovis*. The results of reisolation provided here are culture protocols performed at source. Similarly, the assessment of gross mastitic change in the milks was performed at collection and graded, if visible, as "slight" - +, or "gross" - ++.

NAGase examinations were carried out on the milks by Dr. H Ball at Stormont after the studies described here were completed. The

method is described here and the results presented. Duplicate 20 ul aliquots of milk and 40 ul of 2.25 mM 4-methyl umbelliferyl-N-acetyl-B-D glucosamide (MUFAG) in 0.25 M citrate buffer (pH 4.6) were dispensed into the wells of microtitre plates. Fluorescent standards for each batch of milk samples were 7.5, 15 and 30 uM 4-methyl umbelliferone in 0.25 M citrate buffer, pH 4.6, and dilutions of a standardised mastitic milk. Uninoculated MUFAG substrate was also included as a control. Incubation was for 30 min. at 4^{o}C. The NAGase reaction was terminated by addition of 200 ul of 0.2 M glycine NaOH buffer (pH 10.7) to both test and standard wells. Emitted fluorescence was read by a fluorometer:- wells were scored as positive or negative in relation to the controls. A positive in either of the duplicate wells scored the individual sample as positive. A cut-off point of >400 000 cells/ml was taken for MCC: milk with MCC levels above this were regarded as mastitic. The majority of MCCs of > 400 000 had detectable NAGase activity (6).

4.3 Results.

4.31 MAb "5A10" Specificity.

The only significant activity noted from the ELISA readings were with all the *M. bovis* isolates from various sources, and the *M. agalactiae* type culture. No other cross-reactions were observed.

Table 8. Serological cross-reaction of MAb 5"A10" with M. bovis and M. agalactiae.

	M.agalactiae	M. bovis sc38
B5A10		
500	0.4	1.0
1000	0.3	0.9

The literature cites the cross-reactivity of bovine serum with mycoplasma antigens of bovine, caprine, avian, and environmental sources, with the strongest reaction between M. bovis and M. agalactiae (17, 36, 77, 80).

4.32 Sensitivity Assay.

Table 9. MAb "5A10" sensitivity assay by serial dilution of 48 hour inoculated broths.

	M. bovis strain	
	sc38	130
Broth diln.		
10	0.3	0.4
10^2	0.090	0.102
10^3	0.097	0.09

Conjugate-control absorption: 0.100

Test is sensitive for broth dilution of 1/10, representing a sensitivity of 10^8 cfu/ml.

Table 10. Detailed ELISA results for experimental milks.

Table 10a. Cow no. 1

Days p.i.	Milk Clots	ELISA *M. bovis*	NAGase	Reisolation (log_{10})
1		-	+	7
2	+	-	+	7
3		+	+	7
7	++	+	+	7
10	++	+	+	7
14	++	+	+	7
17	++	+	+	7
21		+	+	7
24	++	-	-	7
28	++	-	+	7
31	++	-	+	7
35	++	-	+	7
38	++	-	+	7
No of NAGase +ve *M. bovis* isolations				12
No of *M. bovis* ELISA +ve isolations				6
M. bovis ELISA % sensitivity				50.0

Table 10b. Cow no. 2

Days p.i.	Milk Clots	ELISA *M. bovis*	NAGase	Reisolation (log_{10})
1		-	+	4
2		-	+	6
3		-	+	7
7	++	+	+	7
10	++	+	+	7
14	++	+	+	7
17	++	+	+	7
21	++	+	+	7
24	++	+	-	7
28	++	+	+	7
31	++	+	+	7
35	++	+	+	7
38	++	+	+	7

No of NAGase +ve *M. bovis* isolations	11
No of *M. bovis* ELISA +ve isolations	10
M. bovis ELISA % sensitivity	90.9

Table 10c. Cow no. 3.

Days p.i.	Milk Clots	ELISA *M. bovis*	NAGase	Reisolation (log_{10})
1		-	-	4
2		-	+	6
3		-	+	7
7	+	+	+	7
10	++	+	+	7
14		-	+	3
17		-	-	2
21	++	-	-	3
24		-	+	-
28		-	-	-
31		-	-	-
35		-	+	-
38		-	+	-

No of NAGase +ve *M. bovis* isolations	8
No of *M. bovis* ELISA +ve isolations	2
M. bovis ELISA % sensitivity	25

Table 10d. Cow no. 7

Days p.i.	Milk Clots	ELISA *M. bovis*	NAGase	Reisolation (log_{10})
1		-	+	-
2		-	-	-
3		-	-	-
7		-	-	-
10		-	-	-
14		-	-	-
17		-	-	-
21		-	-	-
24		-	-	-
28		-	-	-
31		-	-	-
35		-	-	-
38		-	-	-

No of NAGase +ve *M. bovis* isolations	0
No of *M. bovis* ELISA +ve isolations	0
M. bovis ELISA % sensitivity	

Table 10e. Cow no. 8

Days p.i.	Milk Clots	ELISA *M. bovis*	NAGase	Reisolation (log_{10})
1		-	-	3
2		-	+	5
3		-	+	7
7	+	+	+	7
10	+	+	+	7
14	+	+	+	7
17	+	+	+	7
21	+	+	+	7
24	+	+	+	7
28	+	+	+	7
31	+	+	+	7
35	+	+	-	7
38	+	+	+	7

No of NAGase +ve *M. bovis* isolations	8
No of *M. bovis* ELISA +ve isolations	7
M. bovis ELISA % sensitivity	87.5

Table 10f. Cow no. 9

Days p.i.	Milk Clots	ELISA *M. bovis*	NAGase	Reisolation (log_{10})
1		-	+	-
2		-	+	3
3		-	+	4
7	+	+	+	7
10	+	+	+	7
14	+	-	+	6
17		-	+	7
21		-	-	3
24		-	+	7
28		-	+	1
31		-	-	-
35		-	-	-
38		-	-	-

No of NAGase +ve *M. bovis* isolations	8
No of *M. bovis* ELISA +ve isolations	2
M. bovis ELISA % sensitivity	25

4.33 Results of ELISA examination of milk samples.

Table 11. Summary of ELISA results for experimental milks.

No of NAGase +ve *M. bovis* isolations	47
No of *M. bovis* ELISA +ve isolations	27
M. bovis ELISA % sensitivity	57.5

These results indicate a sensitivity of approximately 10^7 organisms/ml:- higher than with broth culture.

In general, the ELISA results detected antigen where it had been demonstrated by standard cultural techniques. In all cases, the ELISA detected antigen in milks where it had been isolated at a concentration of 10^7 organisms per ml. No isolations were made from milks where the reisolation was less than that level. The detailed results are presented in Tables 10 and 11.

4.34 N-acetylglucosamide.

These results would also tend to suggest that the sensitivity of the ELISA test is c. 10^7 c.f.u./ml.

4.4 Discussion

The construction of the capture ELISA initially was impaired by the poor titre achieved from *in vitro* production of large volumes of monoclonal antibody as sac fluid. The sac fluid produced from MAb 5A10 required a titre of 1/50, whereas the

production of ascites fluid from the same line achieved a titre of 1/1000 for the same test format.

The NAGase ELISA was carried out on the German experimental milks in the V.R.L., Belfast after the end of the project. The cultural examination for M. bovis was performed in East Germany, and is thus the source of the data on reisolation. The results of NAGase and cultural examination carried out by others are included here and aid the interpretation of results using the M. bovis ELISA developed in this study. It is interesting to note that the test sensitivity appeared to be an order of magnitude higher basing the measurement on testing clinically affected milks from acute cases, compared to estimations from serial broth dilutions. The fact that the M. bovis ELISA did not pick up any positive-culture milks earlier than day 3 or after d38 is also of interest with a view to the use of the test in the case of a herd epizootic.

Further work to improve the sensitivity of the test would obviously be desirable. Investigation of hybridoma reserve lines held in frozen storage might produce a monoclonal which would produce a more sensitive capture ELISA. On the basis of the previous experience with the development of a capture ELISA to detect M. californicum, pre-processing the milk sample might prove worthwhile. In this context, centrifuge collection of the milk cells, and inoculation into broth might prove beneficial to the overall sensitivity. Obviously the detracts from the potential of using the test for screening subclinical chronic carriers in a herd.

Chapter 5.

General Discussion.

5.1 Present Study

The objective of the study was achieved by the development of an antigen capture ELISA test for the rapid detection of *M. bovis* in milk, with a sensitivity of approximately 10^8 cfu/ml of broth culture. The test, as currently used, has good specificity, with cross reaction being detected only to *M. agalactiae* (80). As the latter is not an agent associated with bovine mastitis, this cross-reactivity does not invalidate this ELISA as a rapid diagnostic test. Indeed, it opens the possibility of testing sheep and goats for *M. agalactiae.* Although this test has been developed to assay for the presence of antigen in milk samples, the MAb technique could be used to isolate the antigenic epitope from the original antigen fractionation, thus allowing for the construction of an ELISA to test for serum antibodies directed against *M. bovis* (17, 23, 33, 36, 77, 83).

During development of the monoclonals, it became apparent one of the 6 cloned hybridomas was directed against media constituents. The origin of the antigenic stimulation by broth constituents may have arisen from their incorporation in the mycoplasmal membrane prepared in Chapter 2 (80). This is a recognised problem as cited in the discussion of Chapter 3, having been manifest in previous work by the incorporation of porcine serum constituents

(10). The early screening of hybridomas in Chapter 3 sought to avoid this problem by directly testing for activity against porcine serum. In addition, the immunising antigens were cultured in broths supplemented with murine serum. The serum necessary to grow the cultures for antigen preparation was of equine origin (79).

5.2 Areas for Further Investigation.

Assessment of the test sensitivity was by comparison of cultural isolation and NAGase with the mastitis ELISA, as documented in Chapter 4. The results compared favourably with experimental results using 48-hour broths, indicating a sensitivity level of about 10^7 cfu/ml. This might be improved by the selection of other lines for cloning, which would necessitate screening more of the original stage-1 clones. The decision to embark on this course was made when the selected clones were not showing satisfactory sensitivity in the early stages of test formulation. The sensitivity of the test may have been affected adversely by the presence of homologous antibody (whey IgG & IgA) in the clinical milks used for field testing, especially given the chronic nature of the infection in some of the samples. Literature cites ELISA detected antibodies against M.bovis at titres up to 1:25 000 in milk from cows with mastitis. An indirect haemagglutination test (IHT) detected titres up to 1:8192 in milk from the same cows (44).

The literature cites the use of culture-amplified EIA procedure as a standard method of enhancing the sensitivity of the ELISA

test developed. Compared to standard cultural techniques, improvements in sensitivity from 65 to 85% have been recorded (55). Another technique which might prove beneficial would be ultrasonication of such 18-hour broth cultures. This might increase the number of epitopes available to the monoclonal antibody, and thus the signal produced in the ELISA format.

The theory of making more epitopes available for capture might be exploited by capturing continuously growing culture. This might be achieved by mixing the milk sample 1:1 with broth and incubating the mixture overnight in the ELISA plate wells which had previously been coated for the capture format. The potential of this adaptation exploits the fact that M. bovis is known to be one of the most rapidly growing of the mycoplasmas, thus creating a larger amount of antigen in overnight capture testing.

Since a monoclonal antibody has successfully been developed against an epitope of M. bovis, it would be possible to use it in a minigel MAb immunoblot assay rather than that of an EIA (55). Following ultrasonication of the test sample, the immunoblot technique would show the detection of the target 30 kD epitope. The actual sensitivity of detection of this protein would be the subject of further investigation. An additional advantage of the immunoblot assay is its ability to detect antigen already complexed with antibody. M. bovis antigen in the form of immune complexes may not be detected by conventional immunoassays or by culture. In addition, this assay is not affected by Protein A (SpA) binding of monoclonal antibody by Staphylococcus spp. found in the test specimen (milk) (50). The system has been capable of

achieving a sensitivity equivalent to 200 ccu/ml by standard culture techniques. Many hybridomas were frozen down during the development of the test, and are available for further study. Perhaps one of these would give a higher sensitivity, or unique specificity for M. bovis if further time was available. The possibility of incorporating it with MAb "5A10" in an immunoblot technique would have potential, especially were it to be directed against an an epitope other than the 30 kD target: this might increase the sensitivity to the point of using the test for detection of individual carrier cows (82).

5.3 Conclusion.

Given this level of sensitivity, it appears the test is suitable as a diagnostic tool for herd testing following the identification of the index case in an M. bovis mastitis outbreak. In its present form, the test would seem not to possess the level of sensitivity necessary to reliably detect subclinical carriers, in the case of screening individuals prior to entry to a clean herd. Further evaluation of the role of milk antibody in chronically infected or convalescent cows would be necessary to determine if there was an antibody interference component to the level of sensitivity achieved with this sort of clinical milk sample.

Appendix

PAGE Gel staining method.

1. 500 ml distilled water
   50.0 g trichloroacetic acid
   17.5 g 5-sulphosalicylic acid
   150.0 ml methanol

   Immerse gel for 1 hour.

2. 500 ml distilled water
   150.0 ml methanol
   60.0 g trichloroacetic acid

   Immerse gel for 30 minutes.

3. 500 ml distilled water
   50 ml ethanol
   25 ml acetic acid

   Immerse gel for 30 minutes.

4. 600 ml distilled water
   150.0 ml methanol
   60.0 g trichloroacetic acid
   19.2 g developer (BIO-RAD)

   Immerse gel for 30 minutes.

5. 180 ml deionised water
   20.0 ml oxidiser concentrate (BIO-RAD)

   Swirl over gel for 10 minutes.

6. Wash in changes of deionised water for 30 minutes.

7. 180 ml deionised water
   20.0 ml silver reagent (BIO-RAD)

   Agitate over gel for 30 minutes.

8. Quickly was off silver reagent with 200 ml deionised water.

9. Add 200 ml developer (BIO-RAD). Swirl until solution turns yellow/brown, then pour off and repeat twice at 5 minute intervals.

10. Stop the reaction by covering the gel for 5 minutes with 5% acetic acid solution.

11. Store the gel in 40% ethanol.

PAGE Gels

Gel buffer, pH 8.9

TRIS 18.15 g
SDS 0.40 g
H_2O to 100 ml.

Spacer Gel, pH 6.7

TRIS 5.9 g
SDS 0.4 g
H_2O to 100 ml.

Tank Buffer

TRIS 3.16 g
Glycine 2.0 g
SDS 1.0 g
H_2O to 500 ml.

Boiling Mixture

Spacer gel buffer	1.0 ml
25 % SDS in H2O	0.8 ml
B-mercaptoethanol	0.5 ml
glycerol	1.0 ml

Stacking gel

Acrylamide	1.0 ml
Spacer gel	1.5 ml
H2O	3.5 ml
"AP"	0.065 ml
"Temed"	0.004 ml

12.5 % Resolving Gel.

Acrylamide	10.0 ml
Gel buffer	6.0 ml
H2O	4.4 ml
"AP"	0.200 ml
"Temed"	0.020 ml
Glycine	3.60 ml
Total volume	24.0 ml
Bromophenol blue	0.060 ml

0.060 ml fraction + 0.0010 ml bromophenol blue solubiliser were loaded into the wells of the stacking gel. Fractions for PAGE were run in HEPES buffer, pH 7.20 + 0.10 % SDS.

Western Blotting

1. Prepare 7.5 % polyacrylamide gel and prepare sample:boiling mixture 2:1. Boil 3 minutes, cool and add to gel. Run at 30 mA.

2. Western Blot overnight as follows:
Soak "Scotch Brite pads", filter paper and nitrocellulose in blot buffer.

3. Assemble in order:
"Scotch Brite";
x2 filter paper;
gel;
nitrocellulose;
x2 filter paper;
"Scotch Brite".

4. Put into blot apparatus with the nitrocellulose TOWARDS the ANODE.

5. Wash nitrocellulose in distilled water and air dry. Mark on the nitrocellulose the outline of the gel (corners, top and which side was next the polyacrylamide gel).

6. When dry, cut the nitrocellulose paper into strips. Put into individual Petri dishes and block using 2.5 ml per dish of PTNE + 5 % horse serum. Incubate at 37°C for 1 hour.

7. Add sera to dishes. ($^{1}/_{50}$ sera = add 0.050 ml neat sera to 2.5 ml PTNE).

8. Incubate for 2 hours at 37°C.

9. Wash strips several times with PTNE.

10. Add 2.5 ml conjugate. Incubate 1 hour at 37°C.

11. Wash with several changes of PTNE.

12. Add substrate and leave for 5 minutes before rinsing in distilled water. Air dry strips.

PTNE

PBS	**100.0 ml**
NaCl	**2.0 g**
EDTA	**1.0 ml**
10 % "Tween 80"	**5.0 ml**
Horse serum	**5.0 ml**

Blot Substrate

0.020 M TRIS (0.242 g TRIS/100 ml distilled water, pH 7.2)
50.0 mg DAB (3,3'-diaminobenzidine tetrahydrochloride)
0.033 ml hydrogen peroxide.

Blot buffer

25 mM TRIS (9.105 g/4l)
112 mM glycine (57.656 g/4l)
20 % methanol (200.0 ml/4l)

7.5 % Acrylamide gel

Acrylamide	9.0	ml
Gel buffer	9.0	ml
Water	18.0	ml
"AP"	0.225	ml
"Temed"	0.015	ml

0.1 M Phosphate buffer, pH 8.0 -
0.1 M Na2HPO4 + 0.1 M NaH2PO4
0.1 M Citrate buffer, pH 4.0 -
0.1 M trisodium citrate + 0.1 M citric acid

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