

A STUDY OF THE PHYSICOCHEMICAL HETEROGENEITY OF
GAMMA GLOBULINS IN CATTLE EXPERIMENTALLY INFECTED
WITH EAST COAST FEVER (Theileria parva infection).

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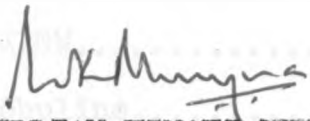
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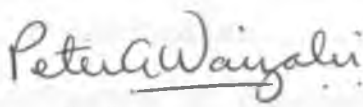
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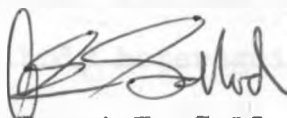

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SUMMARY

The formation of physically heterogeneous capillary agglutinating antibodies during experimental infection of cattle with Theileria parva was followed in anion-exchange chromatographic fractions. Early agglutinating antibody consisted exclusively of IgM by the 17th day post challenge with Theileria parva infected adult Rhipicephalus appendiculatus ticks. This initial antibody response due to the IgM was augmented by an immunoelectrophoretically fast IgG antibody at around the 28th day post challenge.

The bovine IgM component containing the antibody activity was characterized not only by its properties on the anion exchanger, DEAE-Sephadex A-50 but also by the methods of gel filtration on Sephadex G-200, immunoelectrophoretic analysis, sucrose density gradient ultracentrifugation, carbohydrate content and dissociation characteristics when treated with 2-mercaptoethanol. All of these procedures yielded results quite similar to those that have been obtained with human IgM (Kunkel, 1960) as well as those in the bovine (Butler and Maxwell, 1972).

Bovine electrophoretically fast IgG containing antibody activity was characterized using the same physicochemical and immunochemical methods applied to IgM. In addition, chromatographic and immunoelectrophoretic analyses of papain digestion fragments showed

the very close physicochemical relationship between fast and slow IgG in the bovine species. Fast and slow IgG were distinguished on the basis of differences in electrophoretic rates, chromatographic elution positions following fractionation on DEAE-Sephadex A-50 and antibody activity. Their papain digestion fragments were distinguished on the basis of differences in chromatographic elution positions and immunoelectrophoretic behaviour.

A STUDY OF THE PHYSICOCHEMICAL HETEROGENEITY OF GAMMA GLOBULINS IN CATTLE EXPERIMENTALLY INFECTED WITH EAST COAST FEVER (Theileria parva INFECTION).

INTRODUCTION

The term heterogeneity according to Fahey (1962) is used to describe the distribution of antibodies among immunoglobulin classes. This type of heterogeneity has been demonstrated repeatedly in many mammals including man as an initial production of immunoglobulin-M (IgM) antibodies followed by the synthesis of immunoglobulin-G (IgG) antibodies against the same antigenic stimulus.

Stemming from the multifaceted advances in the understanding of the nature of immune response have come up at least two concepts of immunoglobulin heterogeneity. In one usage of the term, heterogeneity has been applied at a "micro" level to describe variations in affinity for an antigenic determinant among antibody combining sites; whereas the use of the term at a "macro" level describes the physicochemical differences between antibody globulin populations themselves.

Murphy et al. (1965) have stressed that recognition of immunoglobulin classes by protein characterization is necessarily the foundation for understanding variations in antibody heterogeneity. Furthermore, it has been emphasized by Burnet (1963) that "no understanding of immunity is possible without an adequate scrutiny of the facts of antibody production and of the physicochemical

nature of the antibody itself".

This dissertation will be concerned with the concept of physicochemical macroheterogeneity of gamma globulin antibodies present in sera of cattle experimentally infected with East Coast Fever (Theileria parva infection). Classification of this heterogeneity based on (1) antigenic analysis, (2) immunoelectrophoretic analysis, (3) sucrose density gradient ultracentrifugation, (4) electrophoretic mobility and (5) carbohydrate content has led to the recognition of 7 S gamma globulin, gamma IgM and gamma IgA globulins (Murphy et al. 1965). This classification, having been derived from comprehensive study of human proteins is well founded, but classification of the immunoglobulins of other species by analogy rather than by physicochemical characterization has tended in some instances to overemphasize the similarities rather than the differences between species.

The aim of this study was to:-

(a) investigate the nature and levels of immunoglobulins in the bovine responding to Theileria parva infection with particular emphasis being placed upon the two globulin populations namely, IgG and IgM showing antibody activity.

(b) study the sequence of appearance and persistence of each molecular species of gamma globulin antibody as the disease progresses.

and (c) to investigate the ability of such humoral immunoglobulin response to suppress the progress of an ongoing disease (East Coast Fever).

Because of the very nature of East Coast Fever (Theileria parva infection), it was felt that substantial effects of immunoglobulins could be expected and that the changes found might help in obtaining a better understanding of its pathogenesis. Furthermore, such a study on the immunoglobulin response could be beneficial in the development of specific serodiagnostic techniques with which to demonstrate specific theilerial immunoglobulins.

In addition to consideration of the comparative properties of gamma globulins as they act in the immune response, there is current interest in the study of these proteins per se. Further understanding of the nature of these proteins will require relatively large quantities of purified material. Both IgG and IgM are present in quantities in bovine serum greater than in small laboratory animals or in human serum, which might in some regards make this species (the bovine) suitable for further study of these particular proteins.

LITERATURE REVIEW

(1) GAMMA GLOBULINS

Gamma globulins were first defined by Tiselius (1937) as the slowest migrating electrophoretic group of serum proteins in electrophoresis conducted at alkaline pH. In 1939, Tiselius and Kabat first demonstrated that the gamma globulin fraction was associated with the antibody activity. However, several subsequent experiments revealed that antibody activity was also associated with the proteins lying in the beta region of the electrophoretic pattern. Thus, Heremans (1959) put forward the "immunoglobulin concept". This was subsequently supported by Korngold (1961) and others. Fahey (1962) in contrast prefers not to use the prefix "immune" with regard to these serum proteins since it is not certain whether all are involved in the immune system; thus he still preferred the usage of "gamma globulins" to include all serum proteins formed in the lymphoid and plasma cells.

Within the gamma electrophoretic area, there are three components: IgG gamma globulin, gamma IgA globulin and gamma IgM globulin. These components are associated with antibody activities. From chemical, physical, biological and immunological standpoints they seem to exist as a system of closely related, though not identical proteins.

In this dissertation, both the terms "gamma globulins" and "immunoglobulins" will be used, (1) gamma

TABLE 1: SOME CHARACTERISTICS OF BOVINE IgG and IgM IMMUNOGLOBULINS

CLASS AND SUBCLASS	IgG Class			REFERENCE
	IgG ₁	IgG ₂	IgM	
Electrophoretic Mobility	Fast γ and B ₂	γ	B ₂	Butler (1969)
Sedimentation Coefficient (S _{20 w}).	6.5-7.0	6.5-7.0	19.5	Butler (1971)
Total Carbohydrate (%)	2.0-4.0	2.0-4.0	10-12	Butler (1971)
Hexose	1.05	0.99	-	Kickhofen et al. (1968)
Hexosamine	1.57	1.61	-	Kickhofen et al. (1968)
Molecular weight	163,000	150,000	900,000	Butler (1969)
Conc. Mg/ml serum	10.5	7.9	2.5	Mach and Pahud (1971)
Antibody activity	+ve	+ve	+ve	Butler (1971)

γ = gamma, B = beta.

globulin to emphasize the related character of the proteins themselves and (2) immunoglobulin to indicate the antibody activity of the protein. The terms gamma - 1A and gamma - 1M describe what appears to be rather definite homogeneous proteins but the complexity of the electrophoretic heterogeneity within the 7 S gamma globulin requires further classification. The very real distinction between electrophoretically fast and slow migrating 7 S gamma globulins is incorporated herein into the terms "fast 7 S gamma globulin" and "slow 7 S gamma globulin". These terms have been used by Silverstein et al. (1963) for analogous proteins present in sheep serum, but others have preferred to call them gamma - 1 and gamma - 2 globulins (Benacerraf et al. 1963). In this dissertation, the term "7 S gamma globulin" will be used where authors do not clearly show the mobility.

The immunoglobulin concept of Heremans (1964) was adopted by the World Health Organization Committee on nomenclature of human immunoglobulins (1964). The adopted concept was to designate proteins of animal origin with known antibody activity as well as other chemically related normal and pathological proteins (Cohen, 1965). For the human immunoglobulins, (Rowe, 1970), a complete set of terminology has been adopted and the names which were proposed by the Committee for

the different classes of immunoglobulins were IgG, IgM and IgA. However, names for new classes of immunoglobulins: IgD and IgE have since been added for man (Rowe, 1970).

In the bovine, three distinct classes of immunoglobulins have been described. They occur in serum and lacteal secretions and are designated IgA, IgM and IgG, the latter being divided into two subclasses, namely, IgG₁ and IgG₂ (Butler, 1969). The nomenclature follows the one adopted for human immunoglobulins and it is this same one which was proposed for the immunoglobulins of the domesticated Bovidae at a "Symposium on the Bovine Immune System" held on November 18-20 at the Interstate Inn, College Park, Maryland (Butler et al., 1970). Some properties and synonyms of the gamma globulins are shown in Table 1.

Chemical, physical and biological properties of gamma globulins and antibodies have been reviewed by Porter (1960) while the macroglobulins have been considered by Kunkel (1960). Fahey (1962) has reviewed the broad aspects of the heterogeneity of gamma globulins. Aalund (1968) has extensively reviewed the heterogeneity of ruminant immunoglobulins. Butler (1969) has reviewed the bovine immunoglobulins while Ogilvie (1970) has reviewed immunoglobulin responses in parasitic infections. East Coast Fever (E.C.F.) has been reviewed by Neitz (1959) and Wilde (1967).

The early studies on antibody heterogeneity started with the classical work of Tiselius (1937). This study defined the gamma globulins in free electrophoresis of whole serum. All the same, complexity of gamma globulins with antibody activity was puzzling to the early workers using free electrophoresis; but now the problem has been unravelled by using other physicochemical and immunochemical approaches to build upon the cornerstone erected by Tiselius.

Heidelberger and Pedersen (1937) found that when horses were challenged with pneumococcal antigens, the anti-pneumococcal antibodies had a much higher molecular weight than normal horse gamma globulin. Soon after the components of the immunoglobulin system were identified, their functional relationships between them were investigated. Stelos and Talmage (1956) and Talmage et al. (1956) described the separation by differential centrifugation of two antibodies in rabbit antisera to Forssman antigen and two antibodies to an isophile antigen of sheep erythrocytes which differed in haemolytic efficiency whereby antibodies with high molecular weight were very efficient haemolytically as compared with antibodies with low molecular weight.

Since a out 1958, the different fundamental aspects of immunoglobulin heterogeneity have been extensively studied by applying the more modern physicochemical and immunochemical tools. Several factors

such as (1) the chemical properties of the antigen, (2) the age of antigen recipient, (3) route and duration of immunization, (4) site of antibody formation, (5) genetic factors, and (6) the species injected have been found to influence the physicochemical properties of antibodies formed (Aalund, 1968). Those factors with influences bearing on the research to be reported in this thesis will be reviewed in detail in the following subsections of part two in the Literature review.

(2) THE 19 S (GAMMA - MACROGLOBULIN) VERSUS 7 S GAMMA - GLOBULIN HETEROGENEITY.

(a) Influence of chemical properties of antigen, antigen dosage and route of administration.

The implication of antigen properties and immunization schedule as factors influencing the sequence and amount of 7 S (IgG) and 19 S (IgM) antibody produced in response to an antigen was first demonstrated by Bauer and Stavitsky (1961) and Bauer et al. (1963). Rabbit antibodies formed during early response to single injection of several antigens were characterized. Antibodies produced during the primary and secondary responses to various proteins, T₂ bacteriophage and the "O" and "H" antigens of Salmonella organisms were studied. They found that, apart from the response to "O" antigen of Salmonella, an early antibody response consisted of the initial synthesis of 19 S gamma - Ig M globulin.

antibody. This finding by these workers has since been found to be a general pattern of the primary immune response in several mammalian as well as non-mammalian species (Aalund 1968). Bauer et al. (1963) proposed a hypothesis to explain the observations that different cells synthesized the two distinct molecular forms of the antibody though the significance and implications of the sequential synthesis of the two antibodies was unknown. Moreover, the concept of sequential synthesis of IgM and IgG antibodies may have to be re-evaluated in the light of the findings by Freeman and Stavitsky (1965) and Wei and Stavitsky (1967) of simultaneous appearance of IgM and IgG antibodies after the primary antigenic stimulation of rabbits.

Lospalluto et al. (1962) found that in humans, high molecular weight antibodies (19 S) but little or no 7 S (IgG) antibodies were formed following initial immunization with typhoid-paratyphoid vaccines. However, following booster doses antibodies to typhoid "H" antigen and paratyphoid vaccines slowly and incompletely shifted to the low molecular weight fraction (7 S), whereas typhoid "O" agglutinins remained in the high molecular weight fraction (19 S) of serum. Brown and Graves (1959) and Brown (1960) noted changes in specificity and electrophoretic mobility of precipitating antibodies present in the serum of cattle and guinea pigs immunized

with Foot and Mouth disease virus. antigen. Immuno-electrophoretic arcs developed with virus suspensions showed the 7 - day response was less specific (cross reaction with another strain of the virus) and had a mobility in the fast gamma range, while 14 and 21-day antisera were strain specific and migrated in the slow 7 S gamma globulin range. DEAE cellulose chromatography of sera from the same cattle and sera obtained from guinea pigs showed 7-day antibody mostly in fractions eluted with high molar phosphate (0.25 M) and 21 - day antibodies in breakthrough and low molar phosphate (0.02 M) fractions. From these findings it is clear that a 19 S to 7 S shift occurred between days seven and twenty one.

With regard to the antigen dosage and the route of administration as they affect the 19 S to 7 S shift, the work of Borel et al. (1964) is pertinent. These workers compared the anamnestic response to human erythrocyte antigens in rabbits immunized either intradermally (ID) or intraperitoneally (IP) with varying primary antigen doses. Six weeks following the secondary stimulation with a constant amount of antigen in all groups, no difference in 19 S and 7 S response could be attributed to primary dose variation. However at six months, difference due to the dosage used for the primary challenge were noted. Animals that received (IP) a large primary dose showed a ratio of 70% 19 S

antibody (2-Mercaptoethanol (2-ME) sensitive) and 30% 7 S antibody (2-ME resistant). Mean agglutination titer in this group was 1:1000. Those animals which received a small primary dose administered ID, a high titer was produced following the constant secondary injection. These antibodies came preferentially 2-ME sensitive at 2 months.

In East Coast Fever (Theileria parva infection) it has been established using electron microscopy (Jarrett and Brocklesby, 1966; and Mugeru and Munyua, 1973) that following the introduction of "infective particles" by ticks into cattle, the former sequentially develop into three phases, namely, macroschizonts, microschizonts and micromerozoites which are believed to invade the erythrocytes and become the piroplasms. Each one of these phases could have a dictating influence on the 19 S to 7 S gamma globulin shift. At present, it is not even known which stage of the parasite, if any, is primarily responsible for stimulating the protective response. Further, the nature of the immune mechanism and phase of the parasites life cycle acted upon by the immune reaction remain unknown (Jarrett et al. 1969). In their study of the kinetics of multiplication of Theileria parva, Jarrett et al. (1969) showed that the appearance of each phase of the parasite was dependent on time post infection and not on the dose of the parasite (antigen) the individual

bovine host was subjected to. Infections using 10 ticks, 100 ticks and 1000 ticks showed that in each instance the appearance of the parasite phase or phases was exponential.

Although heterogeneous, antibody globulin response has been obtained with relatively homogeneous (pure) protein antigens. The possibility remained that different determinant groups on the protein molecule gave rise to different antibody globulins. Bauer (1963) examined the molecular properties of antibodies formed in response to haptens coupled to protein carriers. His results showed that early appearing haemagglutination antibodies in the rabbit to sulfanilic acid - bovine gamma globulin and para-aminobenzoic acid-bovine gamma globulin were predominantly of the macroglobulin (19 S) type, but with continued challenge, shifted to the 7 S type. This evidence points to the fact that a heterogeneous antibody response may be elicited by a single antigenic determinant.

(b) The influence of the age of the antigen recipient upon antibody heterogeneity.

The influence of the age of the antigen recipient upon antibody heterogeneity has been extensively studied in several species by various workers. Jameson et al. (1942) showed by electrophoresis that newborn calf serum was relatively high in alpha globulin (36.8%),

low in betaglobulin (5.9%) and without any gamma globulin. Pierce (1955) using immunoelectrophoresis could distinguish both gamma - 1 and gamma - 2 globulins in sera of 10-day-old calves. By the 30th day after birth the amount of gamma - 2 globulin exceeded that of gamma-1 globulin. Merriman (1971) also applied immunoelectrophoresis and detected IgM, IgG₁ (fast) and IgG₂ (slow) globulins in both pre-colostral and post-colostral sera of Holstein calves. From his observations he suggested that the developing bovine foetus could be capable of independent immune response. Kniazeff et al. (1967) also demonstrated gamma globulins in sera of 4-6 month old bovine foetuses. These sera were capable of neutralizing certain bovine viruses. Therefore these workers concluded that transplacental transfer or possibly foetal synthesis of the gamma globulins was not infrequent in the bovine species as is held by most other investigators (Rice and Carriere, 1969; Stone and Gitter, 1969).

Barnett (1963) reported that higher resistance in calves to East Coast Fever was present only when these came from areas where the disease was enzootic and that this resistance was dependent on the immune status of the dams. Barnett (1957, 1963) fed immune calves with colostrum from susceptible dams and then exposed these calves (when one month of age) to

Theileria parva infected tick. The calves survived. When a similar treatment was followed, but supplying susceptible calves with colostrum from immune dams and then exposing them to infected ticks, the calves succumbed. The experiments indicated that the highest degree of resistance in calves was attained at about 4 months of age, very young calves being more susceptible. He inferred that the calf resistance was not linked with the maternal antibodies in the colostrum. However, at a meeting of investigators on the Immunology of East Coast Fever held in Nairobi on December 9 - 10, 1974 under the auspices of FAO/UNDP, it was pointed out that "infective particles" of Theileria parva harvested by in vitro feeding technique (Purnell and Joyner, 1967) from ticks, the infectivity of the particles could be preserved in foetal calf serum (FCS) but lost in normal, adult bovine serum (NBS). This raised the question: What would the composition of the component responsible for the inactivation effect in NBS or the protective effect in FCS be?

Bellanti et al. (1963) showed that when newborn rabbits were intensively immunized with large amounts of Salmonella paratyphi flagella antigens, higher titer agglutinins were produced by the 7th to 10th day of life. The earliest antibody was IgM globulin; slow IgG gamma globulin did not appear until the 4th - 5th week

of life. In contrast, adult rabbits produced macroglobulin (IgM) antibodies for only 3 - 5 days before the IgG gamma globulins appeared. The neonatal macroglobulin was similar in all respects to the adult macroglobulin antibodies. The immune response of the newborn and adult rabbit appear to differ mainly in the interval that precedes development of the capacity to form IgG antibodies. Uhr et al. (1962a) found that antibody response to bacteriophage ϕ X 174 in newborn premature children consisted of 19 S antibody which changed to IgG antibody within 6 weeks after immunization.

Silverstein et al. (1963a, 1963b) studied the influence of the age of antigen recipient on the capacity to respond to antigenic stimulus. They stimulated immature lambs in utero with various antigens and studied the serum gamma globulins and antibody responses after the foetuses were delivered by hysterotomy. Sera from foetuses and their dams were assayed. They compared the gamma globulin and antibody in normal and antigenically stimulated fetal lambs with that in adult sheep. Immunoelectrophoresis of the adult sheep serum produced arcs from the fast and slow IgG gamma globulins in the shape of a "gull wing" figure. Another arc was identical to the IgM globulin of human serum by its shape and position. Most fetal lambs given an antigenic stimulus responded with the production of

increasing amounts of IgM globulin which occasionally reached levels as nearly high as in normal adult sheep. Although normal foetal lambs possessed only trace amounts of IgG gamma globulin in their sera, most stimulated lambs responded with the production of varying amount of fast IgG gamma globulin. Exceptions were foetuses that received antigen without adjuvant and animals that were sacrificed six days post stimulus. Slow IgG gamma globulin was produced by fetal lambs only after prolonged stimulus, and even then production of only small amounts was noted in foetuses nearing birth and in those having the greatest amounts of fast IgG gamma globulin.

Thorbecke (1964), working with guinea pigs found that IgM globulin was synthesized by the spleen in fetuses and by both lymph nodes and spleen in newborns. Slow IgG gamma globulin formation began two weeks after birth while synthesis of fast IgG gamma globulin was first evident in guinea pigs which were at least one month old.

(c) The kinetics of development
of IgM and IgG antibodies.

Several kinetic studies of the development of the IgM to IgG antibody response have formed the basis for concepts of the role of antigen in regulating the relative rate, duration and molecular species ratio of

antibody formation. Uhr and Finkelstein (1963) and Uhr et al. (1962) observed the kinetics of the primary and secondary responses of guinea pigs to bacteriophage ϕ X 174. Their results showed that the relative rate of 19 S antibody formation was maximal and dose independent above a threshold dose of antigen; below this dose, slower relative rates were obtained. IgM antibody synthesis was relatively short-lived. In contrast, primary IgG antibody could be detected as early as seven days post immunisation. It was synthesized for many months and led to a preparation for a secondary IgG antibody response ("immunological memory"). It was shown that the relative rate of 7 S antibody formation is antigen dose dependent.

Svehag and Mandel (1964a, 1964b) detected IgM antibody within 8 - 12 hours and IgG antibody within 36 - 48 hours following a single intravenous poliovirus injection into rabbits. The rate of synthesis and peak titers of the two antibody types were dose dependent. Single small antigen doses induced only IgM antibody, the synthesis of which ceased abruptly on day 4 or 5 post immunization. The minimum antigen dose required for the induction of IgG antibody formation was necessary for induction of an enduring response. This minimum amount of antigen was 50 times or more greater than that needed for IgM antibody response. The kinetics of secondary IgM and IgG antibody responses were similar to the respective primary responses.

It was found that the presence of IgG antibody did not alter the secondary IgM responses, and that immunological memory in IgM antibody formation was demonstrable only within 2 - 3 days following discontinuation of synthesis. Svehag and Mandel (1964a, 1964b) were of the opinion that the kinetics of the formation of both IgM and IgG were similar (a) for infectious and non-infectious poliovirus antigens; (b) when poliovirus was administered by different routes; (c) for the serologically unrelated poliovirus and Coxsackie B-4 Virus; (d) in one-day-old or adult rabbit (e) in antibody responses to poliovirus in rabbit, guinea pig and man. These workers concluded that all the differences observed in the formation of IgM and IgG could best be explained on the assumption that the two antibodies are produced by different cells. This is in agreement with suggestions made previously by Askonas et al. (1956) that different types of gamma globulins are synthesized by different cells which are unevenly distributed in the tissues making gamma globulin. Nossal et al. (1964) also found that both IgM and IgG antibodies were formed by cells of the plasma cell family with IgM producers consisting of blast and immature plasma cells while IgG producers mainly consisted of mature plasma cells.

In their study of the kinetics of the antibody response to Anaplasma marginale infection, Murphy et al. (1966) found that the early complement-fixing antibody

consisted exclusively of γ M (19 S) globulin but within 4 to 5 days this was augmented by an electrophoretically fast γ G globulin (6.2 S) eluted from DEAE - Sephadex. Slow IgG complement fixing antibodies were never observed. At the peak of parasitemia, 8 and 92 per cent of the total amount of complement fixing antibody activity resided in the fast IgG and IgM classes, respectively. Approximately 30 days after the haemolytic crisis the distribution had changed to 25 and 75 per cent fast IgG and IgM complement fixing antibody activity, respectively, and this ratio remained fairly constant for several months and was noted in other animals in the postacute phase of naturally acquired anaplasmosis.

(d) The influence of chemical and physical factors upon 19 S and 7 S antibody heterogeneity.

There is considerable evidence that splenectomy impairs antibody formation in many species, especially in the early part of the primary response (Taliaferro and Taliaferro, 1950). This infers that the spleen is involved in the synthesis of macroglobulin (IgM) antibodies, although there is no conclusive evidence that it produces macroglobulin at a rate out of proportion to other antibody-producing tissues. Davidsohn et al. (1964) reported that the normal shift of rabbit anti-mouse erythrocyte antibodies from IgM to IgG gamma globulin was completely inhibited, regardless of the

number of antigen injections, by splenectomy. In splenectomized calves infected with Anaplasma marginale, Jones et al. (1968) observed a delay in the appearance of complement fixing (CF) antibodies. These early CF antibodies were predominately in the IgM fraction of the immunoglobulins, although later in the disease IgG antibodies appeared (Murphy et al. 1966).

In their investigation of the influence of splenectomy on the kinetics of the immunoglobulin response to Anaplasma marginale infection in calves, Klaus and Jones (1968) demonstrated by the quantitative radial diffusion test that splenectomy depressed IgM levels but had no effect on IgG levels. However, Blinkoff (1964) found no difference in proportions or kinetics of 19 S and 7 S antibody production following splenectomy in specific pathogen free mice. The same shift of 19 S to 7 S antibody synthesis was inhibited by the immunosuppressant 6 - mercaptopurine (Sahiar and Schwartz, 1964). The 19 S antibody response in the treated animals was greatly prolonged, with some rabbits forming 19 S antibodies for almost 50 days after antigenic stimulus whereas untreated ones completed the shift to exclusive 7 S antibody synthesis by 18 to 21 days. Another antigen injection produced typical anamnestic response in normal and treated rabbits which was marked by the synthesis of large amounts of 7 S antibody. Methotrexate (Blinkoff, 1964) likewise did

not change the time of onset and titer production of 19 S antibody following antigen administration in mice. However, the appearance of 7 S antibody was delayed 5 - 10 days and its titer was reduced 2 - 4 fold.

(e) Examples of 19 S - 7 S antibody heterogeneity in infectious diseases.

At least two physicochemically distinct groups of antibodies to the purified protein derivative of tuberculin were found in human serum by Turcotte et al. (1964). The haemagglutinating (HA) titer of the sera of patients with active tuberculosis was primarily due to antibodies in the 7 S gamma globulin fraction, whereas the haemagglutinins produced by health tuberculin skin test positive individuals were macroglobulins. It appears that the antigenic challenge to the host during active tuberculosis is distinct enough from that of other less formidable sources of antigen to result in this markedly different antibody distribution. Williams and Wemyss (1961) studied the immunoelectrophoretic patterns of serum proteins of specific pathogen free mice undergoing acute infection. These mice had very low gamma globulin levels normally, but within 2 - 3 weeks following infection with Mycobacterium tuberculosis var. bovis, large amounts of slow 7 S gamma globulin and a trace of gamma-IM globulin were present in sera as shown by electrophoresis. Staphylococcus aureus infection elicited some gamma-IM and large amounts of slow 7 S gamma globulin synthesis,

as assayed by incorporation of C^{14} label into these proteins by spleen and lymph node tissue cultures of infected mice followed by autoradiography of immunoelectrophoretic patterns.

Abele et al. (1964) noted certain alterations in immunoelectrophoretic patterns in the sera of human subjects infected with Plasmodium vivax whereas no significant changes were seen in individuals infected with Salmonella typhi. There was a consistent, marked increase in the IgM globulin which appeared simultaneously with the first appearance of anti-malarial antibodies as demonstrated serologically. This increase persisted in some cases for as long as 60 days. Sephadex G-200 gel filtration of immune sera indicated that the increase in the IgM globulin was partially due to the presence of anti-malarial antibodies in this component. Abele and his co-workers' findings were later confirmed by Tobie et al. (1966) who employed the single radial diffusion technique to study the serum immunoglobulin levels in relationship to antibody production in human subjects infected with malaria.

Chandler et al. (1969) studied the immunoglobulin and antibody responses in human subjects suffering acute and chronic histoplasmosis. They noted that sequential immunoglobulin response in acute histoplasmosis was typical, with an early IgM rise followed by an increase in IgA and IgG gamma globulin levels. Patients with

the chronic form of the disease had markedly elevated IgA and relatively normal IgM and IgG gamma globulins. The authors observed that Latex-agglutinating antibody activity was located in the IgG component. Complement-fixing activity was found primarily in IgG and, less often, in IgM. Luckins (1972) studied immunoglobulin levels in Zebu cattle (Bos indicus) exposed to trypanosomiasis. He found an increase in both IgM and IgG levels as compared with pre-infection levels. Increases of 2 - 9 times above pre-infection levels of IgM were detectable two weeks after cattle were introduced into the enzootic area. These changes persisted for several months. However, IgG levels showed only a 2 - fold increase during the same period.

(3) HETEROGENEITY WITHIN THE 7 S GAMMA GLOBULINS
(FAST AGAINST SLOW 7 S GAMMA GLOBULINS).

The heterogeneity that has so far been reviewed has centered on the relationships between immunoglobulins of different molecular sizes viz. the 19 S and 7 S gamma globulins. However, several reports have recently been published with regard to the heterogeneity between the "fast" and "slow" components of the 7 S gamma globulin. The two components were previously thought to differ only in their electrophoretic mobility.

In so far as the study of the two components is concerned, much work has been done in studies of heterogeneous properties of guinea pig 7 S gamma globulins.

Benacerraf et al. (1963) in the course of immunization of guinea pigs with single protein antigens or hapten conjugates emulsified in complete Freund's adjuvants obtained two types of precipitating antibodies. Both had sedimentation coefficients of 7 S but with different electrophoretic mobilities; the "slow" migrating antibody appearing earlier while the "fast" appeared later. When the antigens were administered in the absence of complete Freund's adjuvant, the guinea pigs produced primarily 7 S gamma-I globulins. These two types of gamma globulins had both common as well as distinct antigenic determinants. They were designated "7 S gamma-I" (fast 7 S gamma) and "7 S gamma-2" (slow 7 S gamma).

Ovary, Benacerraf and Bloch (1963) showed that fast 7 S gamma-I antibodies mediated passive, systemic or cutaneous anaphylaxis whereas slow 7 S gamma-2 antibodies did not do so. Guinea pig fast 7 S gamma-I which carries receptors for fixation to skin produces homologous skin sensitization while guinea pig slow 7 S gamma-2 produces heterologous sensitization. Noting that the antibody fraction which sensitizes for wheal and flare reactions in human serum migrates ahead of the main gamma globulin fraction in electrophoresis, Ovary and his associates suggested that it is possible that in all mammalian species, gamma globulin components of fast mobility have analogous function. In the same series of studies on guinea pig antibodies, Bloch et al. (1963a) found guinea

pigs hyperimmunized with Escherichia coli in complete Freund's adjuvant produced large amounts of slow 7 S gamma globulins, but with only one exception, did not produce fast 7 S gamma antibodies. These workers could not account for the large amount of slow 7 S gamma globulin contained in these sera and they suggested that a property of the antigen as being decisive in determining the type and amount of antibody produced. Bloch et al. (1963b) state that "although electrophoretic fractionation of guinea pig serum readily permits separation of the two major types of biological activities, which in turn may be identified as properties of two distinct types of antibodies.....similar studies in man and other species will be required to explore whether antibodies directed against a single antigenic specificity but subserving different biological activities can be found in species other than the guinea pigs".

Murphy et al. (1965) demonstrated both the slow and fast gamma globulins in bovine suffering from Anaplasmosis. Fast gamma globulin contained antibodies that fixed complement following reaction with Anaplasma marginale antigen, whereas the slow gamma globulin component could not fix complement. This was both an indication of specialized function and a demonstration of difference among species since complement fixation in guinea pig serum has been associated with the slow gamma globulins

and not with the fast gamma globulin as has been demonstrated by Bloch et al. (1963b). Similarity in molecular weight and a major degree of common antigenicity of the bovine fast and slow gamma globulins, and fragments from papain digestion, indicated a close relationship between these two gamma globulin groups (Murphy et al. 1965). The authors also demonstrated distinct spur formation of fast over slow gamma globulin in the immunoelectrophoretic analysis with whole bovine serum. In sheep, Aalund et al. (1965) demonstrated similar findings as presented by Murphy et al. (1965).

(4) ANTIGENIC RELATIONSHIP AMONG THE GAMMA GLOBULINS

If antigenic identity rather than electrophoretic mobility is to be the determining factor as to whether a particular antibody species is fast 7 S gamma globulin or gamma-IA globulins, then actual antigenic and structural comparison between fast and slow 7 S gamma globulins and between fast gamma globulin and IgA will decide this issue. The earliest evidence of antigenic differences between components of 7 S gamma globulin spectrum came from the demonstration of immunoelectrophoretic arc splitting. Goodman (1960) using fowl antihuman serum found an immunoelectrophoretic arc in human serum parallel to the usual 7 S gamma globulin line. Augustin and Hayward (1960) discovered in human sera three arcs in place of the usual single

7 S gamma globulin arc by means of a rabbit antiserum for a pathological macroglobulin (Waldenström's Macroglobulin). They also noted that human 7 S gamma globulin had an increasing tendency toward spontaneous splitting as sera was stored for progressively longer times. They saw a split in the 7 S gamma globulin line in sera that had been stored for 5 - 8 years at 4°C. In another paper Augustin and Hayward (1961) showed that reagins were 7 S gamma globulins with special limited range of mobilities and as such, they would be expected to share the lability peculiar to 7 S gamma globulins, particularly in dilute solutions, and no other special lability need be evolved to explain their behaviour. They stated that, "it remains to be seen what subspecies of 7 S gamma globulins reagins belong to, to give them a greater tendency to adhere to tissues than other proteins and whether they have fewer or more or possibly some determinants different to those of normal 7 S gamma globulins". However, the nature of reagins was clarified by the work of Fireman et al. (1963) when working with sera from ragweed pollen sensitive individuals. They demonstrated that the skin-sensitizing antibody activity was associated with gel filtration protein fraction where IgA was located but not in the protein fraction containing either the 7 S gamma globulin or the macroglobulin.

In the work of Edelman et al. (1960) intact 7 S gamma globulin producing double, split or spur precipitation lines in immunoelectrophoresis were compared with papain digestion fragments to demonstrate separate antigenic determinants on gamma globulin molecules. They obtained several horse and rabbit anti-normal human gamma globulins which showed either double or split arcs and also several rabbit anti-gamma-IgA myeloma protein sera and rabbit-anti-pathological macroglobulin serum which showed the spatial separation of the two arcs better than anti-normal globulins. Comprehensive studies failed to demonstrate that a separate type of 7 S gamma globulin was responsible for the second arc. These workers demonstrated the relationship between the double precipitation arcs of gamma globulin with the papain digestion products, F and S in several ways. In this work, the arc closest to the antibody trench has been called the "outer precipitin arc" and that nearest the antigen source the "inner precipitin arc". The Ouchterlony's immunodiffusion technique served to relate the inner precipitin arc of undigested gamma globulin with chromatographically isolated 3 fragments and the outer precipitin arc with the F fragments. When intact gamma globulin producing a double precipitation arc was run side by side on the same immunoelectrophoretic slide with papain split 7 S gamma globulin with the center trench interrupted, fusion of the S fragment arc with

the inner arc and fusion of the F fragment with outer arc was dramatically demonstrated. Absorption experiments confirmed these findings whereby the inner arc was selectively removed by absorption of the antiserum with isolated S fragments and the outer arc was reduced to a thin residual arc by absorption of the antiserum with F fragment. These workers stated that "these antigen relationships make it highly improbable that double lines are completely non-specific phenomena resulting from the physicochemical conditions of precipitation In many cases the doubling seems most obvious at equivalence..... In many instances separation at the ends of arcs could be found on careful observation of the immunoelectrophoretic patterns. These observations suggest that the doubling phenomenon was a common one for gamma globulin but in most instances the lines were so close together that they appeared to be single". Two hypotheses were suggested to explain these findings: the first favoured by these authors, suggests that multiple different antigenic determinants are present on each gamma globulin molecule, all molecules having similar antigenic structure; the second suggests that certain groups of molecules possess single antigenic determinants different from those of their neighbours. However, the important question raised here is whether single precipitation lines are produced by an antigen with multiple determinants and double arcs by antigenically different molecules, or

whether multiple precipitin zones may be formed by one antigen with several determinants which seems to be the case with these gamma globulins.

Fahey and Askonas (1962) compared the electrophoretic heterogeneity and the distribution of antigenic determinants of normal gamma globulins and of gamma type myeloma mouse proteins. After digestion by papain following the method of Porter (1959), the S fragments of the two gamma globulin groups differed markedly in their electrophoretic mobility. The S fragments of slowly migrating globulins migrated more slowly than those from rapidly migrating ones. These workers concluded that it is most likely that the S peptide chains contributed importantly to the electrophoretic properties of the intact molecules in this species. The discrete S and F components seen on starch gel electrophoresis and immunoelectrophoresis of papain digested gamma-IgA myeloma globulins were similar to those obtained with 7 S gamma type proteins except for a slight increase in the mobility of each fragment. Fahey and his co-worker concluded that comparison of the S and F fragments from gamma-IgA and 7 S gamma globulins reveals that the antigenic features shared by the various globulins derived from plasma cells (7 S gamma and gamma-IgM myeloma proteins, the range of normal 7 S gamma globulins) are largely the properties of the S fragments, whereas the distinctive antigenic differences between the normal and myelomatous 7 S gamma and gamma- myeloma proteins are properties

which appear in the F fragments of the molecules (Askonas and Fahey 1962; Fahey, 1963).

Edelman et al. (1960) subjected human 7 S gamma globulin to zone electrophoresis on starch. They isolated from the leading and trailing portion of the protein peak two gamma globulin components with different mobilities. Following papain treatment, these gamma globulins yielded identical F. fragments but the S component of the slow 7 S gamma globulin had a markedly lower mobility than that of the 7 S globulin. Edelman and his associates state that "these results suggest that the amino acid side chains responsible for antigenic structure differ from those responsible for mobility differences in sub-fractions of electrophoretically separated gamma globulin and that the mobility differences of the pair of gamma globulins appeared to be primarily related to the differences in the S component".

Franklin and Stanworth (1961) in a study of human proteins reported that the S fragment of 7 S gamma globulin was responsible for the antigenic determinants shared with the gammaIgA globulin and macroglobulin.

Richards and Marrack (1963) separated the fast 7 S gamma and slow 7 S gamma globulins of ovine serum on DEAE cellulose chromatography and subjected each of the molecular species to papain hydrolysis. They found that the fragment with the lowest electrophoretic mobility (S fragment) was identical in these two proteins.

(5) GEL FILTRATION CHROMATOGRAPHY

Gel filtration is a chromatographic procedure utilizing the molecular sieve properties of gels (Flodin, 1962). The Sephadex (Pharmacia, Uppsala, Sweden) preparations have proved extremely useful as molecular sieves. The principle of the separation process on Sephadex columns is that large solutes which cannot enter the gel pores emerge first at the void volume (the volume of liquid surrounding the gel in a column) while small solutes which penetrate the gel pores freely are retarded and emerge at a volume $V_0 + V_i$, where V_i is the volume of liquid inside the gel particles (Kwapinski, 1972).

Flodin and Killander (1962) fractionated human serum by gel filtration in the dextran gel Sephadex G-200. Analytical ultracentrifugation of the gel filtration peaks showed an inverse relationship between sedimentation coefficient ($S_{20,w}$) and elution rate. The molecular sieving effect of Sephadex is explained by the fact that the liquid imbibed by the gel particles is differentially available as solvent to solute particles of different sizes. The degree of availability depends upon porosity of the granules, thus the distribution of solute inside and out of the granules is determined not by differences in solubility but by the volume available inside the gel particles and the total volume of the column. Thus ions and small molecules can diffuse in all the fluid imbibed by the gel and on the other hand it has been found that very large molecules do not enter the gel at all, presu-

mably due to the steric hindrance of the gel network. Of those molecules intermediary in size that do penetrate the gel network. Of those molecules filter faster and are eluted faster than smaller ones. The exclusion value for Sephadex G-200 is 200,000 Molecular weight for dextrans and approximately the same value for proteins.

Using paper electrophoresis, immunoelectrophoresis and double diffusion in agar, Flodin and Killander (1962) identified in the Sephadex G-200 peak one the serum alpha-2 macroglobulin, alpha-beta lipoproteins and the 19 S gamma globulin. In the second peak, they found 7 S molecules and antibodies while serum albumin and transferrin were present in the third peak.

Hogman and Killander (1962) and Killander and Hogman (1963) fractionated blood group antisera on Sephadex G-200 columns and were able to separate the 7 S and 19 S types. The 7 S type was incomplete and demonstrable only by the indirect antiglobulin technique, while 19 S antibody behaved as a saline agglutinin.

The shortcomings of any particular serum fractionation method led Gelotte et al. (1962) to investigate combination of two or more fractionation techniques including gel filtration, zone electrophoresis and anion exchange chromatography. Of particular interest was their success in separating gamma-M by a combination of gel filtration of whole serum on Sephadex G-200 followed by preparative electrophoresis of Sephadex G-200 peak one.

When a pathological serum with a high content of IgM globulin was fractionated by this combination of techniques, two protein peaks were obtained; one having in immunoelectrophoresis a precipitin arc corresponding to IgM the other having an arc corresponding to alpha-2M globulin.

(6) ANION EXCHANGE CHROMATOGRAPHY

Anion exchange chromatography was first developed by Peterson and Sober in 1956 when they prepared the first ion exchange adsorbents from cellulose with properties suitable for column chromatography of proteins.

Sober and Peterson (1958) pointed out that instability of protein molecule has imposed some special conditions such as (1) limited choice of solvents and eluants and (2) restricted pH range. The range of conditions in which adsorption and desorption occur is small, the former being enhanced at low salt concentration while the latter is favoured by increasing salt and/or a change in pH. The major effect of changing pH is that of changing the number of charges on the protein and occasionally on the adsorbent. On the other hand, the effect of salt must be one of promoting dissociation of electrostatic linkages established between protein and adsorbent. The narrow range of conditions under which finite adsorption equilibrium could be established as well as being able to achieve the desired spread of protein components, has led to an increased employment of gradient elution in chromatography. This technique provides the required range of eluting power; and it was

found in experiments with human serum that maximum resolution was obtained with a gradient "rising fairly rapidly for a brief initial period, then across the center of the chromatogram and, finally, swinging upward towards the end to sharpen the tail" (Sober and Peterson, 1958).

Sober et al. (1956) developed gradients of pH and salt concentration for elution of serum proteins from anion exchange cellulose and characterized the protein distribution in the eluant fractions by electrophoretic techniques. The study of these workers indicated the great potential usefulness of anion-exchange cellulose chromatography for the study of proteins, but considerable time and work was required to carry out a single determination. Thus Fahey et al. (1958) and Fahey and Horbett (1959) introduced a modification of the technique described by Sober et al. (1956) to reduce the time and work involved and to decrease the initial loss of euglobulins.

Fahey (1962) further reported on the distribution of serum protein components fractionated on columns of diethylaminoethyl (DEAE) cellulose. Proteins of gamma-globulin mobility were shown to be widely distributed throughout the entire serum chromatogram effluent. However, the bulk of each group of gamma-globulins (7S-gamma, gamma-IA and gamma-IM) were found in different parts of the chromatogram. Most of the 7S-gamma globulins were located in the 5-15% effluent region although small amounts of these proteins were distributed throughout the chroma-

togram. The gamma IgA globulins were detected in the 40-65% effluent region while IgM globulins were present in the 55-83% effluent region.

MATERIALS AND METHODS

(1) THE PARASITE

The organism used was the strain known as Theileria parva (Muguga) which has been maintained in the laboratory by alternative passages in Rhipicephalus appendiculatus ticks and Kenya "high-grade" cattle since 1951 (Brocklesby et al. (1961).

Infection was established by attaching Theileria parva (Muguga) infected adult Rhipicephalus appendiculatus ticks to the ears of steers using a 10 tick challenge.

Rectal temperatures were recorded twice daily from all animals after tick attachment. Blood smears were also made daily. Lymph node biopsy smears were made from the parotid and prescapular lymph nodes commencing when these became clinically enlarged. All smears were Giemsa stained and were examined for the presence of Theileria parva parasites.

(2) EXPERIMENTAL ANIMALS

These were "high grade" steers of Bos taurus type aged between 9 and 12 months. They were of various exotic dairy breeds obtained from different farms in Kenya where vigorous tick control was practised. In order to ascertain that these animals had no previous exposure to East Coast Fever (Theileria parva infection) at the time of purchase, their sera were tested by capillary agglutination test as described by Schaeffler (1963). Animals showing a positive reaction were excluded from this study. All animals were

housed in tick-proof stalls surrounded by a moat of approximately 6 inches in width. They were maintained under standard conditions of husbandry and periodically checked for internal and external parasites. A total of fourteen steers were used in these studies. In order to investigate the influence of splenectomy on the kinetics of the immunoglobulin response to Theileria parva infection, three steers (NOs 221, 226 & 227) were initially splenectomized and infected 28 days after surgery.

(3) SURGICAL PROCEDURE

Steers were starved for 24 hours before splenectomy. The site of incision in the left para-lumba fossa was shaved. Animals were premedicated with a tranquilizer, acetylpromazine hydrochloride (2mg/ml) injectable solution. They were anaesthetized with a 20% solution of chloral hydrate given to effect. As a safety precaution complete anaesthesia was avoided and a local anaesthetic was used to infiltrate the incision area. The steers were secured to a metal operating table and the surgical area thoroughly cleaned with water and soap and then dressed with surgical spirit to minimize contamination. A 4 to 6 inch incision about 1½ to 2 inches posterior and parallel to the last rib in the left para-lumbar fossa was made through the skin and subcutis, abdominal muscles and peritoneum.

The spleen was then loosened by manually breaking down (blind dissection) the connective tissue and fascia holding it in place. Caution was necessary to avoid tearing the splenic artery and vein which enter at the hilus on the dorsal third near the anterior border. The spleen was exteriorized through the incision, care being taken to avoid rupturing the splenic capsule or breaking the splenic blood vessels. The vessels were ligated with a non-absorbable "Vetafil Bengen" synthetic surgical suture (0.60 mm). The splenic blood vessels were then severed and the spleen removed.

The peritoneum and M. transversus abdominis were closed with a simple continuous suture using medium chromic catgut No. 2. Internal abdominal (obliquus abdominis internus) muscle was also closed with a simple continuous suture using medium chromic catgut No. 2. The external abdominal (obliquus abdominis externus) muscle was also with simple interrupted suture using medium chromic catgut No. 2. Finally the subcutaneous tissue and the skin were closed with a horizontal interrupted mattress suture using "Vetafil Bengen" synthetic non-absorbable suture (0.60 mm).

Post surgical care consisted of 20 ml Terramycin injectable solution (50mg/ml) given intramuscularly to prevent bacterial infection. The animals were kept under antibiotic cover for 3 days. They were placed in dry and well-bedded stalls. Skin sutures were

removed 3 weeks post surgery and infection with Theileria parva was carried out one week later.

(4) COLLECTION OF BLOOD SAMPLES

Blood samples were collected three times a week. Technical details pertaining to collection of blood samples for haematological analysis and for determinations on serum were performed according to the methods of Munyua (1971). Details concerning differential leucocyte counts and total serum protein (TSP) determination were also performed according to the methods of Munyua (1971).

Portions of TSP samples were used for immunoglobulin studies. Samples that were not immediately used after collection were stored at -20°C .

Determination of packed cell volume (PCV), erythrocyte count (RBC), and white blood cell (WBC) count were performed by means of automatic electronic Coulter Counter (Coulter Electronics, Coulter Model ZB Inc. 590 West, 20th Street, Hialeah, Florida 33010). Haemoglobin concentration (Hb) was estimated through a haemoglobinometer attached to the main instrument.

(5) GEL FILTRATION CHROMATOGRAPHY

Bovine sera were fractionated by gel filtration in the cross-linked dextran gel Sephadex G-200 (particle size of 40-120 μ ; Pharmacia Fine Chemicals Inc. Piscataway, N.J. 08854) following the method described by Flodin and Killander (1962), Gelotte et al. (1962), Hogman and Killander (1962), Killander and Hogman (1963)

and Aalund et al. (1965). Minor modifications were introduced into their methodology. The gel was allowed to swell with continuous stirring for at least 24 hours in excess TRIS-HCl buffer (0.05 M tris (hydroxymethyl) aminomethane pH 8.0 + 0.15 M NaCl). Merthiolate or sodium azide were added to the buffer solution at concentrations of 0.01% and 0.1%, respectively to prevent bacterial growth.

A clean column (Pharmacia Fine Chemicals AB S-751 25 Uppsala 1, Sweden), 2.5 cm by 100 cm was mounted in a vertical position and fitted with a flow adaptor at the bottom. The top of the column was fitted to the gel reservoir. The gel was then added into the column through the open top of the reservoir. In order to dislodge air bubbles from the surface of the flow adaptor, a glass tube was inserted down through the top of the column and suction applied as required.

The effluent tube was then opened and eluant drained from the column until all the gel had settled from the reservoir and into the column. Before the serum sample was applied into the column, the buffer was allowed to run through the gel bed over night to let it stabilize.

Five to ten ml. of serum sample was applied to the column in each fractionation experiment. The gel filtrations were performed at room temperature using upward flow elution. The elution flow rate was 20 ml/hr and was controlled by the regulation of the hydrostatic pressure. The effluent was collected in 5 ml. fractions

using an Automatic LKB 7000 UltraRac fraction collector (LKB-Produkter AB, S-161 25 Bromma 1, Sweden). The protein content of the effluent was recorded as UV transmission at 280 m μ in an LKB 8300A Uvicord II Absorptiometer (LKB-Produkter AB S-161 25 Bromma 1, SWEDEN). Protein appeared in the eluent in three main peaks as has been shown with human serum by Gelotte and his co-workers (1962). The distribution of protein in these peaks was analysed by immunoelectrophoresis (IEP).

(6) IMMUNOELECTROPHORESIS (IEP) AND DOUBLE DIFFUSION

PRECIPITIN TESTS

Microimmunoelectrophoresis was performed on microscope slides according to the method of Scheidegger (1955) but with some modifications. "Ionagar" No. 2 (1%) (Oxoid Ltd. Southwark Bridge R^d Lond., SE 1) was brushed onto 25-by 76-mm microscope slides and dried overnight. The pre-coating helped to anchor the main agar layer. Two to three ml. of 1% "Ionagar" in barbital (Veronal) buffer of ionic strength 0.05M pH 8.6 and with 0.01% Merthiolate added as a preservative was poured on each slide. The agar-covered slides were placed on a slide frame and standard well and trench templates stamped out with the Shadon gel punch set (Shadon Scientific Co. Ltd. London, N.W. 10) as follows:- double well, single trench for comparison of antigens; and single well, double trench for comparison of antisera. The gel plug of the antigen wells was sucked off with a Pasteur pipette

connected to a Vacuum water pump. The Shadon electrophoresis chamber (Shadon Scientific Co. Ltd. Lond N.W. 10) used could accommodate up to eight slides in one row at each time of running. Serum samples, gel filtration fractions, DEAE Sephadex chromatographic fractions, papain digestion fragments and any other antigens required for analysis in the studies were applied to wells in a semi-quantitative method using the dimensions of the well to determine volumes.

After the wells were filled with the appropriate antigen using plain capillary tubes, the slides were placed in the electrophoresis chamber containing barbital (Veronal) buffer. Contact between the slide and the electrode vessels was established by using approximately 1 cm wide filter paper strips. About 5 mm of both ends of slide was covered with the paper strips soaked in barbital (Veronal) buffer while the other end of the papers was immersed in the electrode vessels. Electrophoresis chamber was covered and the d.c. power supply equipment (Buchler Instruments, Fort Lee, N.J; U.S.A.) was turned on. Electrophoresis was allowed to proceed at a constant voltage of 150 Volts. With this arrangement, approximately 2 mA were going through each slide. After 60 minutes, the power was turned off and the slides removed from the electrophoresis chamber. The trenches were excavated to remove the gel strip and appropriate antisera added, thereby allowing double diffusion step to start. Subsequent immunodiffusion was done in an

insulated moist chamber at room temperature overnight. Most precipitin arcs were well developed after 18-20 hours, but in some cases longer periods of diffusion were necessary.

Following double diffusion, slides were rinsed for 24 hrs with several changes of phosphate buffered saline (PBS) 0.15 M, pH 7.2 and then rinsed briefly in distilled water. They were dried in contact with moist filter paper at room temperature and then stained for 10 minutes with 0.1% Amido Black 10B (BDH Chemicals Ltd., England) in acetate-glycerol following the method described by Crowle (1961). They were cleared in 5% acetic acid, rinsed in distilled water, dried and then photographed.

The Ouchterlony technique (1958) of double diffusion in gel following a micro-format was used for comparison of two antigens with respect to one antiserum (Crowle, 1961). Three ml. of the agar solution (1% Iona-gar No. 2 in PBS 0.15 M, pH 7.2) used for IEP as described above was poured on each microscope slide and placed on a horizontal levelling table for the agar to set. Patterns to be punched in the agar layer had been outlined on a piece of paper placed under the slide, and the wells were stamped out. The agar gel was removed from wells by suction using a Pasteur pipette connected to a vacuum water pump (Dimensions of the wells: Holes diameter: 3 mm, distance between holes: 7 mm). The

desired reagents were applied to the wells, and the slides were subsequently handled as described for the immunoelectrophoresis slides.

(7) ANION EXCHANGE CHROMATOGRAPHY

Anion exchange chromatography was performed according to the method described by Fahey et al. (1958) and Sober and Peterson (1958) with some modifications. Diethylaminoethyl (DEAE) - Sephadex A-50 (Pharmacia Fine Chemicals, Uppsala, Sweden) was used as the anion exchanger rather than DEAE - Cellulose because of its higher adsorbent capacity to reduce proportionally the amount of eluent used by Fahey et al. (1958), which proportionally increased the protein concentration in the effluent.

Elution of proteins was accomplished by a continuous gradient in phosphate molarity from 0.005 to 0.50 M and a decrease in pH from 8.0 to 4.5 in the buffer entering the column. This was achieved as illustrated in Figure 1. The apparatus consisted of a mixing chamber, a one-litre Erlenmeyer flask which at the start of the gradient contained 300 ml. of 0.005 M phosphate buffer pH 8.0. The flask at the start of the gradient was connected by a siphon to a second one-litre Erlenmeyer flask containing 300 ml. of 0.5 M phosphate buffer, pH 8.0. Mixing was accomplished with a magnetic stirrer.

Three gms of DEAE - Sephadex A-50 medium (particle size of 100 - 200 mesh; Pharmacia, Uppsala, Sweden) was allowed to swell for 40 minutes in 160 ml. phosphate

Figure 1: Diagram of the apparatus used in the anion exchange chromatography. At the beginning of the chromatographic procedure, Erlenmeyer flask No. I contained at the start of the gradient 300 ml. 0.5 M PO_4 , pH 8.0 while flask NO. II contained 300 ml. 0.005 M PO_4 , pH 8.0. Mixing was accomplished with a magnetic stirrer.

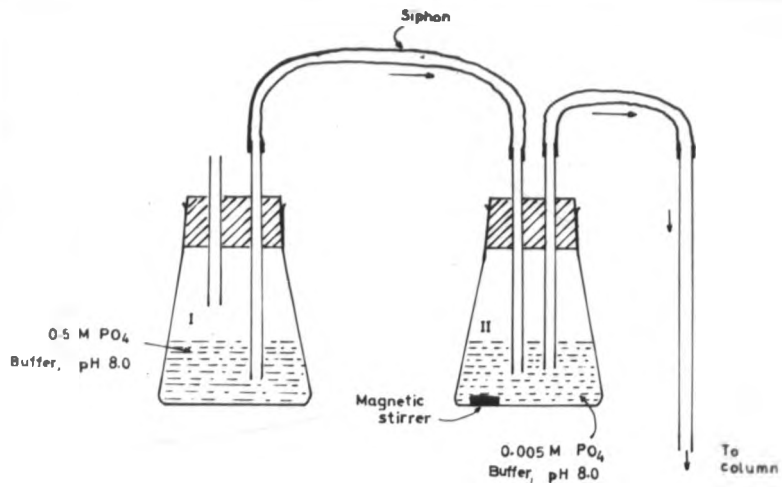


Fig. 1: Diagram of the Apparatus used in the Anion Exchange Chromatography.

buffer (0.005 M, pH 8.0) while being stirred. Swelling and stirring was repeated three times after which it was packed in a 2.5 cm by 40 cm glass column. Merthiolate or sodium azide at concentrations of 0.01% and 0.1%, respectively, were added to the buffer solution as a preservative. The column was packed by gravity.

Each serum sample was dialyzed against 0.005 M phosphate buffer, pH 8.0 for 24 hrs at 4°C and a 2-ml aliquot was applied to the column. The effluent was collected in 5 ml. fractions in an Automatic LKB 7000 UtraRac fraction collector (LKB-Produkter AB, S-161 25 Bromma 1, Sweden). Protein concentration of the effluent was recorded as UV absorption at 280 mμ in an LKB 8300A Uvicord II Absorptiometer (LKB Produkter AB S-161 25 Bromma 1, Sweden).

(8) ANTISERUM PRODUCTION

The following antisera were produced in rabbits:=-

- (1) Rabbit anti-whole bovine serum
- (2) Rabbit anti-bovine macroglobulin, namely IgM
(Protein eluted as Peak 1 from gel filtration of whole bovine serum on Sephadex G-200).
- (3) Rabbit anti-bovine IgG gamma globulin
(Protein eluted as Peak 1 in DEAE-Sephadex A-50 chromatography of whole bovine serum).

Immunization Procedure

Rabbits were immunized intramuscularly in the thigh at weekly intervals. Antigen preparations were emulsified in an equal volume of Freund's complete

adjuvant (Difco Laboratories, Detroit 1, Michigan).

Rabbits were bled from the ear eight days after the last injection. Their ears were initially shaven with a blade and xylene applied to allow dilation of the veins. Antisera were collected after centrifugation. Their specificity and quality were checked by immunoelectrophoresis and then stored at -20°C .

(9) 2-MERCAPTOETHANOL (2-ME) SENSITIVITY OF BOVINE
SERUM IMMUNOGLOBULINS

Deutsch and Morton (1957) and Kunkel (1960) reported that sulfhydryl compounds such as 2-ME dissociates human macroglobulins into smaller units. This treatment reduces their sedimentation constants from 19 S to 6.5 S - 8.0 S and also abolishes all antibody activity of the macroglobulin. The breakdown products of the dissociated macroglobulin can be recombined if the 2-ME is removed by dialysis. However, conformation of the reaggregated macromolecules differs from that of the native globulin. The recombination can be prevented by addition of sulfhydryl blocking agents such as iodoacetamide to the phosphate buffer in which the dialysis of the 2-ME and macroglobulin mixture is carried out (Deutsch and Morton, 1957).

Kunkel (1960) further demonstrated that when IgG gamma globulins were treated as described above, there was no loss of their antigen combining capacity. Further, their gross physical properties were not affected.

The following experiment was therefore performed to study the sensitivity of bovine IgM globulin and IgG gamma globulin to 2-ME.

A fraction of Sephadex G-200 Peak I which was rich in IgM component and a fraction from DEAE-Sephadex Peak I containing IgG gamma globulin were separately incubated in equal volumes of 0.15M PO_4 buffer pH 7.2 for 20 minutes at 4°C. Three ml. of iodoacetamide (BDH Chemicals Ltd., England) 0.34 M were then added to bring the final concentration of both reagents to 0.02 M. Following the incubation period, immunoelectrophoresis was performed using homologous antisera. Some of the materials were dialysed against 0.14 M NaCl and subsequently tested for antibody activity.

(10) DETERMINATION OF TOTAL PROTEIN CONCENTRATION

The protein content of the IgG and IgM immunoglobulin solutions was determined by nitrogen analysis using the micro-Kjeldahl technique described by Chibnall, Rees and Williams (1943) with slight modifications. The determination was performed at the Nutrition Laboratory of the Department of Animal Production.

The sample (2 ml) to be analysed was transferred into a Kjeldahl digestion flask. One tablet of Kjeldahl catalyst made of selenium (one tablet contained 1 gm anhydrous sodium sulphate and one equivalent of 0.05 g of selenium) was added. This was followed by the addition of 10 ml. concentrated sulphuric acid. The digestion mixture was now boiled and the boiling continued for 8 hrs.

The mixture was allowed to cool before 5 ml of distilled water was added to the digest which was then transferred to the distillation apparatus. The digestion flask was rinsed thoroughly with distilled water. Twenty ml. of 50% sodium hydroxide solution was subsequently added to the digest and the distillation started and continued until approximately 20 - 30 ml. of fluid had been collected in an Erlenmeyer flask containing approximately 10 ml. 10 mNHCl. Then 5 - 6 drops of methyl red methylene blue indicator solution (Chibnall et al. 1943) was added to the distillate before titration.

Each sample was analyzed in duplicate and the difference between the two figures in per cent of their mean was used as an estimate of the accuracy of the analysis the correction factor 6.25 being used for protein estimation.

(11) PAPAIN DIGESTION OF BOVINE GAMMA GLOBULINS

The enzymatic degradation technique of Porter (1959) which splits rabbit gamma globulin (Porter, 1959) and human gamma globulins (Edelman, et al. 1960; Hsiao and Putnam, 1961) into three fragments of approximately equal size, was used for digestion of bovine gamma globulins.

A pool of Peak 1 fractions from six of the DEAE-Sephadex whole serum chromatograms of animals Nos. 58 and 64 was concentrated by dialysis in Visking tubing against polyethylene glycol 4000 (Carbowax) BDH Chemicals Ltd., England). Likewise an aliquot from a pool

of Peak II protein containing about 150 mg of globulin was also prepared for digestion. As with Porter's method (1959), each sample of 150 mg of gamma globulin together with 1.5 mg papain (BDH Chemicals Ltd; England) were dissolved in 10 ml of 0.1 M potassium phosphate buffer pH 7.0 containing 0.1 M cysteine and 0.002 M ethylenediaminetetraacetic acid (EDTA) as activators. The digestion mixture was then incubated at 37°C for 16 hrs. The sample was then dialysed against distilled water overnight at 4°C. Porter (1959) showed that this procedure which removed both cysteine and EDTA and facilitated oxidation, inactivated the enzyme. The globulin fragments in the papain digest were then dialysed against 0.005 M phosphate buffer, pH 8.0. They were fractionated by chromatography on DEAE-Sephadex using a gradient elution as previously described with the exception that the fraction size was smaller (3 ml. fractions were collected instead of 5 ml fractions).

(12) SUCROSE DENSITY GRADIENT ULTRACENTRIFUGATION

The sucrose density gradient ultracentrifugation (SDGU) was performed according to the techniques of Edelman, Kunkel and Franklin (1958) and Martin and Ames (1961).

The sucrose density gradient was prepared by placing 40% w/v sucrose at the bottom and 10% w/v sucrose at the top in plastic cellulose nitrate centrifuge tubes (Beckman Instruments, Inc., Spinco Division, Palo Alto,

California, U.S.A.) of 5 ml capacity. The gradients were stored for 24 hours at 4°C before use. Serum samples to be studied were diluted 1:2 with saline solution and 0.50 ml aliquots containing 7.0 to 14.0 mg/ml protein layered on the gradient; care being taken to avoid bubble formation. A sharp interface between the sucrose and serum sample was always observed and it remained distinct during the period of time required to load the centrifuge tubes into the rotor buckets. Droplet formation was minimized by slight stirring of the serum sample to the sucrose below it, and by commencing ultracentrifugation immediately thereafter. (Edelman et al. 1958). The total volume used in these experiments was 4.50 ml. (4.00 ml. gradient plus 0.5 ml. sample).

The gradients were ultracentrifuged in a SW-39L swinging bucket rotor at 124,135g. for 18 hours at 4°C in a Spinco model L preparative ultracentrifuge (Beckman Instruments, Inc., Spinco Division, Palo Alto, California, U.S.A.).

After centrifugation, the density gradient fractions were isolated by puncturing with a needle a small hole in the bottom of each centrifuge tube. Three serial drop fractions were then collected from the bottom of each centrifuge tube, the flow of drops being controlled by negative pressure. They were diluted with 3 ml. of saline solution and the protein concentration of each fraction was analysed at 280 mμ using a DU-2 spectrophotometer

(Beckman Instruments, GmbH, München 45 Germany). Final identification of the proteins was made by IEP.

(13) QUANTITATIVE IMMUNOGLOBULIN DETERMINATION

The single radial immunodiffusion (SRD) was performed by the method devised by Mancini, Carbonara and Heremans (1965) as modified by Fahey and McKelvey (1965) with minor modifications for the determination of serum concentrations of IgG and IgM.

Anti-IgM and Anti-IgG antisera were each used at a 1:14 dilution, with phosphate buffered saline as diluent prior to mixing with an equal volume of 1% molten Ionagar. The 10 cm by 10 cm glass plates contained 40 wells, each 3 mm. in diameter. Each plate contained one row of doubling dilutions of a standard serum assayed for IgM and IgG against preparations of these immunoglobulins. The protein content of the latter was determined by the micro-Kjeldahl technique as described previously. All unknown sera were assayed in duplicate. Plates were incubated for 16 hrs in moist chamber at room temperature, dried and then stained in 0.1% Amido Black 10B following the procedure described under IEP. Precipitin ring diameters were then measured by Vernier Calipers. A graph was prepared for each plate comparing the precipitate ring diameter to the logarithm of the protein concentration. The protein concentration in a test sample was determined by comparing its ring diameter and the graphic curve obtained with the standard protein.

(14) CARBOHYDRATE ANALYSIS

It is now clearly established that many proteins contain tightly bound carbohydrate (Meyer, 1945). In particular all of the gamma globulins and specific antibodies which have been obtained in highly purified form have been demonstrated to contain small amounts of carbohydrate (Smith et al. 1952) and may be classified therefore as glycoproteins (Meyer, 1945). Most of the studies on carbohydrate content in gamma globulins have been performed with human and mouse globulins, since normal as well as pathological globulins (myeloma globulins) are available in both species (Oettgen, et al. 1965). In the bovine, Kickhofen et al. (1968) have presented data for normal carbohydrate content in γ G type immunoglobulins.

Being one of the fundamental aspects in the study of gamma globulin heterogeneity (Aalund, 1968) it was the purpose of the investigation reported hereunder to study the carbohydrate content of IgG and IgM in the bovine suffering from East Coast Fever. Only hexosamine and hexose were analysed and all analyses were performed in duplicate and compared with a standard curve in each determination.

(a) HEXOSE ANALYSIS

Total hexose was assayed by the method described by Mokrasch (1954). Fractions, 1.0 ml each from Peak I and Peak II of Sephadex G-200 column chromatography and containing IgM and IgG respectively were separately used as test samples. Prior to analysis for hexose all protein

preparations (IgM and IgG) were precipitated twice in 18% Na_2SO_4 - solution (Oettgen et al. 1965). As a rule, one volume of protein solution was mixed with nine volumes of a 20% Na_2SO_4 - solution, and the mixture was kept for 1 hour at 37°C in a water bath. After centrifugation, the precipitate was reconstituted in one volume of distilled water, centrifuged for 20 minutes at $2,500 \times g$ and the supernatant reprecipitated by adding nine volumes of 20% Na_2SO_4 - solution. The protein precipitate was reconstituted in one volume of distilled water, centrifuged for 20 minutes at $2,500 \times g$ and the supernatant then subjected to hexose analysis. It was found that this procedure eliminated most of hexose contamination.

For the analysis, 2.0 ml of anthrone reagent (BDH, Chemicals Ltd. England) prepared by dissolving 0.4 gm anthrone in 200 ml of concentrated sulphuric acid, was pipetted into the bottom of pyrex test tubes in a water bath at 10° to 15°C . Then 1.0 ml of either IgG or IgM sample was carefully layered above the reagent. Water blanks and standard solutions of hexose (glucose) were treated similarly. Each tube was vigorously shaken while immersed in the cold water bath until the contents were thoroughly mixed. The mixture was then heated in a water bath at 90°C for 16 minutes, cooled and the absorbance measured at 620 mu in a Beckman DU-2 Spectrophotometer.

(b) HEXOSAMINE ANALYSIS

Total hexosamine in either IgG or IgM samples was assayed by the method of Elson and Morgan (1933) as modified by Belcher et al. (1954) and Rondle and Morgan (1955). However, minor modifications were introduced. Samples to be analysed for hexosamine content were obtained as described under hexose analysis and so was the procedure for the elimination of contamination.

The test samples were separately pipetted into Pyrex test tubes. The tubes were placed in a boiling water bath at 100°C and 2N hydrochloric acid added to hydrolyze proteins. This was allowed to proceed for 2 hours. Then 1.0 ml of acetylacetone solution was added. This solution had previously been prepared by dissolving 1.0 ml of acetylacetone (BDH, Chemicals Ltd. England) in 50 ml of normal Na₂CO₃. The contents in each tube were mixed by gentle rotation. The tubes were stoppered and placed in a boiling water bath for 20 minutes. Then they were removed, cooled to room temperature and 3.0 ml of absolute ethanol added. The contents were again mixed by gentle rotation. Then 1.0 ml of Ehrlich's reagent was added with stirring. The reagent was prepared by dissolving 0.8 mg of p-dimethylaminobenzaldehyde (BDH, Chemicals, Ltd. England) in 30 ml of absolute ethanol and 30 ml of concentrated hydrochloric acid. The tubes were stoppered and the

contents mixed thoroughly but gently. They were then placed in a water bath at 75°C for 15 minutes to accelerate liberation of carbon dioxide. Then they were cooled to room temperature and the absorbance measured at 540 mμ in a Beckman DU-2 Spectrophotometer. Water blanks and standard solutions of hexosamine (glucosamine hydrochloride, BDH, Chemicals Ltd. England) were treated similarly.

(15) CAPILLARY TUBE AGGLUTINATION SEROLOGY

The preparation of the piroplasma antigen and the operation of test were performed according to the procedure of Schaeffler (1963) and Ross and Lohr (1972). However, apart from carrying out the test for the selection of experimental animals and testing for antibody response in the chromatographic eluates, no titrations were done during the study.

RESULTS

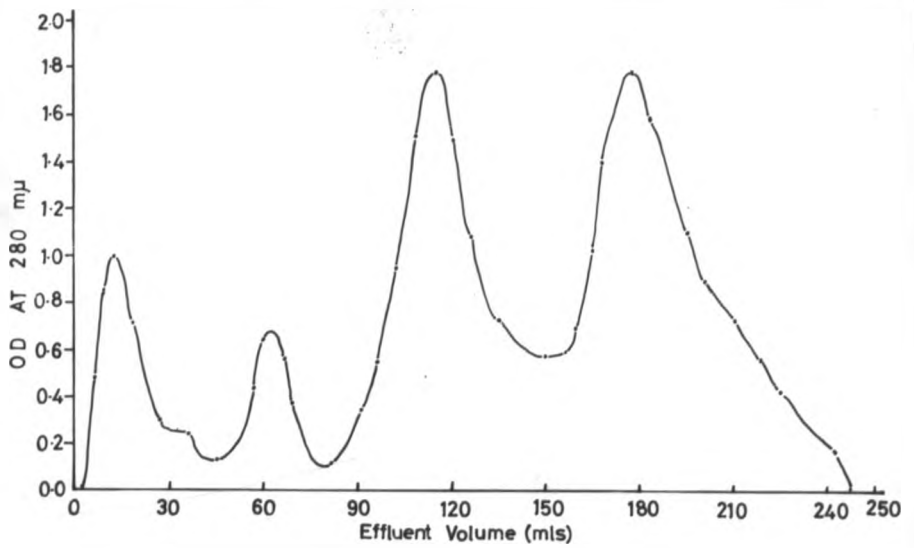
All the experimental animals (14 steers) including those that were splenectomized became demonstrably infected with typical East Coast Fever. The clinical manifestations of the disease were as reported previously by Neitz (1959) and Munyua (1971). In addition to the morbidity rate of 100% all of the steers died thus exhibiting a 100% mortality rate.

(1) ANION EXCHANGE CHROMATOGRAPHY

Serum samples for chromatographic analysis were selected to represent several of the phases in the course of Theileria parva infection in the experimental animals. These phases were (a) the time of incubation period (period from the day that Theileria parva infected ticks were placed on the animal to the day that temperature rose sharply to more than 39.5°C, both days inclusive), (b) times when macroschizonts and microschizonts could be demonstrated in lymph node smears, (c) time of piroplasm appearance and (d) various later phases such as time of death. During the course of this study, a total of fifty different whole bovine serum samples were subjected to anion-exchange chromatography on DEAE-Sephadex A-50 columns. The elution profiles (Fig 2) were analogous to those obtained with human serum (Fahey et. al., 1958). In the majority of sera analysed, proteins were resolved into four major peaks.

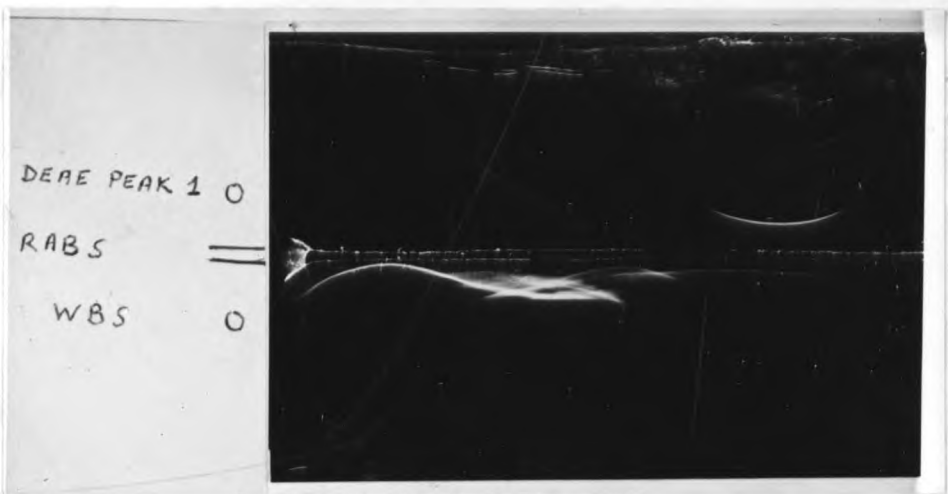
Peak I (front peak) was usually sharp and of a higher magnitude than Peak II. The front peak, also called the

Figure 2: DEAE-Sephadex chromatogram of whole bovine serum from steer No. 74. Elution gradient progressed from 0.005M PO_4 , pH 8.0 to 0.5 M PO_4 , pH 4.5 commencing at 300 ml. eluate volume. Column, 2.5 cm x 40 cm. (Peak 1 is on the left: see text)..



DEAE SEPHADEX CHROMATOGRAM OF WHOLE BOVINE SERUM FROM STEER No 74.

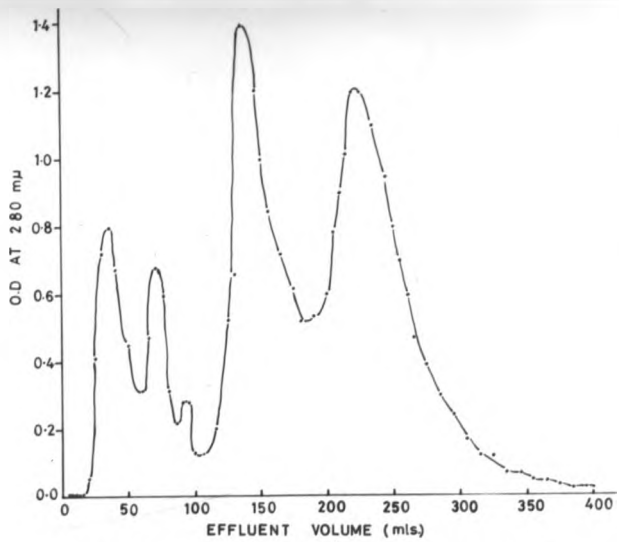
Figure 3: Immuno-electrophoretic analysis of Peak I (Slow IgG) from DEAE-Sephadex chromatography of bovine serum. Top well: Concentrated pool of fractions from Peak I in the DEAE-Sephadex chromatogram. Bottom well: WBS= Whole bovine serum. Trench: RABS= rabbit antiserum. Anode is to the left.



"breakthrough peak" was comprised of proteins with little electronegative charge. Thus, they were not subject to ion-exchange under the experimental conditions employed in this study. They were eluted within the void volume of the chromatographic column. Immuno-electrophoretic analysis of serial chromatographic fractions in peak I revealed exclusively the presence of molecules migrating in the typical gamma region. The precipitin arcs, especially those formed by fractions in the ascending slope of the peak, were located most cathodally in the immunoelectropherogram (Fig. 3). Thus, the breakthrough peak contained the slowest IgG (IgG-2).

Peak II was eluted from the DEAE-Sephadex column as the molarity of the eluate increased (starting buffer 0.5M PO_4 , pH 8.0). In most chromatograms, this peak appeared to be of lower magnitude in height compared with peak I. Its shape also differed considerably from that of peak I. In some chromatograms it was partially subdivided into two halves forming a "twin-peak" (Fig. 4). Formation of "twin-peaks" was usually observed in sera obtained when schizonts could be demonstrated in Giemsa-Stained lymph node smears. However, such peaks were not observed in chromatograms of sera collected at periods when the intensity of the infection was at its height. In most cases, this was at 16 to 25 days post-infection. Immuno-electrophoretic examination of the chromatographic fractions in the ascending part of peak II indicated the presence of precipitin arcs in the typical gamma

Figure 4: DEAE-Sephadex chromatogram of whole bovine serum from steer No. 64 demonstrating the partial subdivision of Peak II into two halves to form a "twin-peak". Elution gradient progressed from 0.005M PO_4 , pH 8.0 (300 ml) to 0.5M PO_4 , pH 4.5 (300 ml).



DEAE SEPHADEX COLUMN CHROMATOGRAM OF WHOLE BOVINE SERUM FROM STEER No. 64

Figure 5: Immuno-electrophoretic analysis of Peak II from DEAE-Sephadex chromatography of serum from bovine No. 58. Top. well: concentrated pool of fractions from the protein peak II in the DEAE-Sephadex chromatogram. Bottom well: WBS = Whole bovine serum. Trench: RABS = rabbit antiserum. Anode is to the left.



region. Compared with gamma globulins observed in the sub-fractions from peak I, those from peak II migrated most anodically (Fig. 5). Therefore, peak II contained mostly the fast IgG (IgG-1). No concrete interpretation could be offered for the composition of the "twin-peak." In the ovine where such peaks have been identified in DEAE-Sephadex chromatograms, Aalund et al. (1965) demonstrated the presence of transferrin and alpha-macroglobulins (alpha-2M). In sera taken from cattle in post-acute phases of Anaplasma marginale infection, the bulk of transferrin was identified in the descending part of peak II (Murphy et al., 1965). Identification of transferrin was accomplished by its colour as well as by its immunoelectrophoretic behaviour when compared with human transferrin. The bovine transferrin was not as well separated as ovine transferrin from the bulk of fast IgG (Murphy et al., (1965). It could be that the "twin-peaks" observed in the bovine chromatogram during this study also contained transferrin as has been described by Aalund et al. (1965). However, its concentration could have been so low as not to be demonstrable in immunoelectrophoretic patterns.

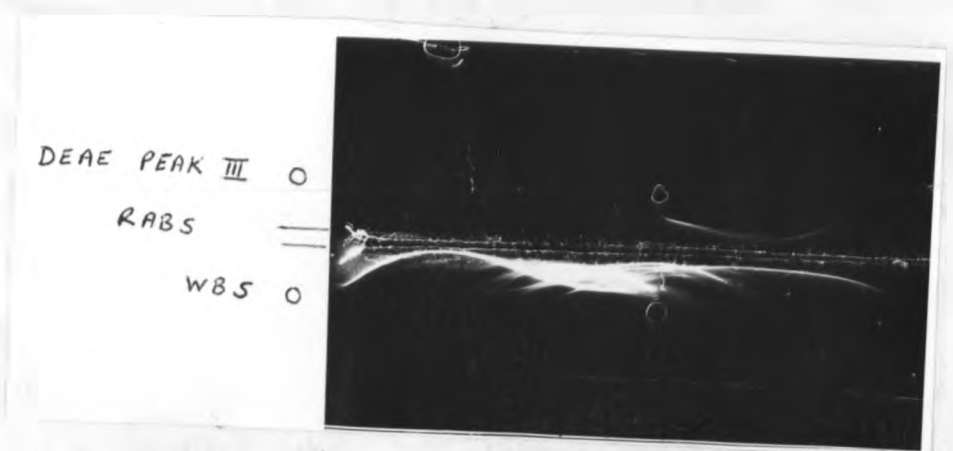
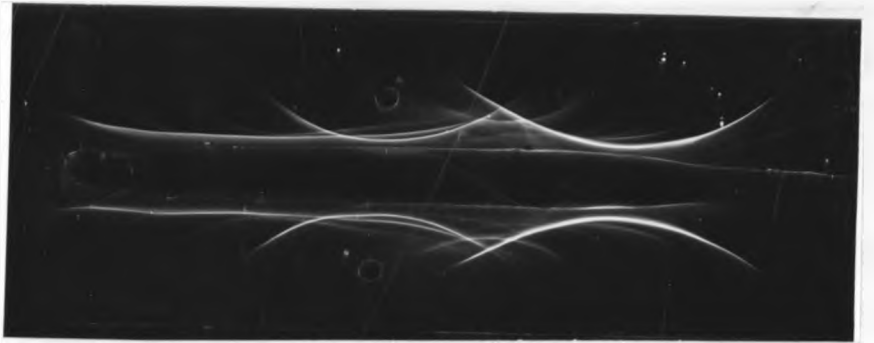
Peak III was sharp and usually higher in magnitude than the other peaks. A precipitin arc resembling the human IgM arc in width and distribution was seen in the immunoelectropherogram of a concentrated pool of fractions from the valley between peaks II and III; together with

TABLE 2: Course of infections in five steers infected with Theileria parva (Muguga).

Animal Number	Status of Spleen	Incubation period	Days to first Detectable Schizont	Days to first Detectable Piroplasm	Days to first Detectable Agglutinating Antibody	Days to Death
58	Intact	14	17	17	17	21
64	Intact	14	16	23	17	24
74	Intact	18	19	24	20	29
221	Splenectomized.	16	18	22	17	25
226	Splenectomized.	13	16	21	17	22

Figure 6: Immuno-electropherogram of serum from bovine NO. 224 showing the heavy precipitin arc of albumin, the major protein component of DEAE-Sephadex Peak IV. Wells: Whole bovine serum. Trench: rabbit antiovine serum. Anode is to the right.

Figure 7: Immuno-electrophoretic analysis of Peak III from DEAE-Sephadex chromatography of serum from bovine NO. 221. Top well: concentrated pool of fractions from the protein Peak III in the DEAE-Sephadex chromatogram. Bottom well: WBS=Whole bovine serum. Trench: RABS = rabbit antiovine serum. Anode is to the left.



those fractions from the ascending slope of peak III.

Peak IV consisted mostly of albumin. Other protein components were only present in amounts that gave trace reactions in immunoelectrophoresis. Precipitin arc of albumin was always thick in appearance as compared to the other arcs in immunoelectropherograms and was located most anodically (Fig. 6).

(2) AGGLUTINATING ANTIBODY ACTIVITY IN CHROMATOGRAPHIC FRACTIONS

As stated in the review of literature the sequence of production of antibodies capable of agglutinating Theileria parva antigen were made. This was done by applying the capillary tube agglutination test (CAT) using the antigen prepared by sonicating erythrocytes from cattle infected with Theileria parva (Schaeffler, 1963; Ross and Lohr, 1972). Five infected steers were used (Table 2), three non-splenectomized and two splenectomized. For each animal, peak I was tested together with at least two other fractions, one from peak II and two from peak III. Before the test was performed, effluent fractions from each particular peak were initially concentrated extensively using polyethylene glycol (PEG) 4000.

The agglutinating antibody response in each particular peak was recorded as either positive, trace positive or negative. This was because the limited sensitivity of capillary tube agglutination procedure made it necessary to place more emphasis on the qualitative presence of

antibody activity in particular peaks rather than upon the degree of reactivity. Antibody activity was first detected in four of the steers (Nos. 64, 58, 221 and 226) on the 17th day post infection. Antibody activity in animal number 74 was demonstrated on day 20 post infection. In all cases, agglutinating antibody activity was exclusively detected in peak III. It was also at this period of infection that macroschizonts could be demonstrated in Giemsa-stained lymph node smears. The agglutinating antibody activity then increased rapidly and was positive on the 23rd day post-infection. This was especially marked in animals 64 and 74 where piroplasms began to appear in the blood smears at this period in the disease. Among the animals tested, only animal number 74 exhibited trace agglutinating activity in peak II at day 28 post infection. This animal had the longest course of the disease among the five steers whose antibody activity was tested. It had a reaction period (period from the day of temperature rise to more than 39.5°C to day of death, both days inclusive) of thirteen days. At no stage of the disease did peak I show any agglutinating activity in any of the steers tested.

From these results, it appeared that during the course of East Coast Fever, the agglutinating antibody activity was initially localized in peak III (the IgM position) of the DEAE-Sephadex chromatograms followed sequentially by antibody activity in peak II (fast IgG position). It follows that antibody distribution during

the infectious process was initially associated with IgM. Antibodies synthesized were later associated with the fast IgG.

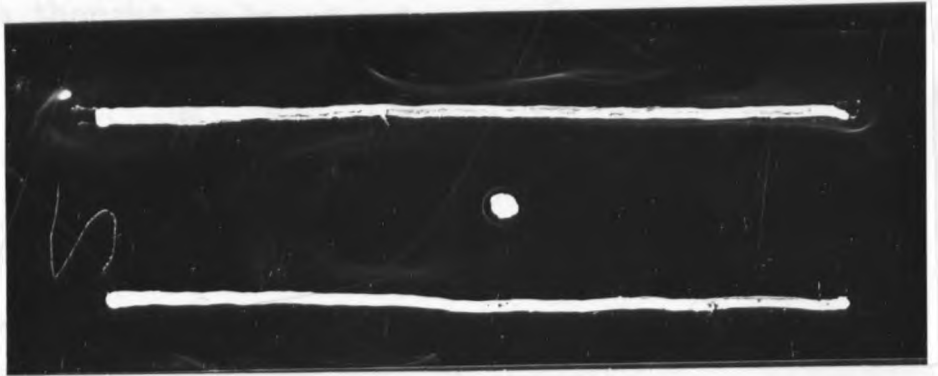
(3) CHARACTERIZATION OF IgM FROM PEAK III.

(a) Immuno-electrophoretic analysis

It has been demonstrated by Fahey and Horbett (1959) that gamma globulin with the highest anionic binding capacity in human serum is IgM, the macroglobulin. As indicated in the review of literature, the evidence available on all of the species studied to date indicates that IgM globulin is localized in the late effluent fractions of DEAE-Sephadex chromatography. For this reason, in these studies efforts were made to relate the agglutinating antibody activity found in cattle experimentally infected with East Coast Fever, that localized in peak III of the DEAE-Sephadex chromatograms to the IgM globulin of the bovine serum. In this work, known immunochemical and physicochemical properties of IgM antibodies served as a guide for comparison with IgM globulin isolated from the steers.

The first investigation was planned to detect whether a protein component with the immunochemical properties of IgM globulin could selectively be localized in peak III. For this purpose, effluent fractions from peak III taken from the area of maximum antibody activity were concentrated via dialysis against PEG and then analysed by IEP. A typical precipitin arc similar in appearance to the well defined IgM arc of human serum was demonstrated with its

Figure 8: Immunolectropherogram comparing human and bovine sera. Upper trench: rabbit antibovine serum. Lower trench: horse anti-human normal serum. The well contained equal volumes of normal human serum and bovine serum.



relative position close to the sample well (Fig. 7). The slow rate of its formation due to the slow diffusion rate formed another basis for its characterization. This typical precipitin arc was not seen in other similarly treated fractions of peak III. However, in some other immunoelectrophoretic preparations of DEAE-Sephadex peak III fractions, other cathodal proteins were seen. These probably represented the rapidly migrating 7 S gamma globulins.

Although analogy to human immunoelectrophoretic pattern does not necessarily constitute conclusive evidence in the identification of bovine proteins, such comparison was thought to be of value in these studies. It was offered as one link in the chain of evidence leading to the identification of the bovine gamma globulin precipitin arcs. Equal volumes of normal human serum and bovine serum were mixed and applied to immunoelectrophoretic wells. Following electrophoresis, rabbit anti-bovine serum was applied to one trench and horse anti-human normal serum* was applied to the other trench. Controls to demonstrate that the anti-bovine serum would not develop human protein precipitin arcs and vice versa were included. In the immunoelectropherogram (Fig. 8) only one alpha protein cross-reacted with the two anti-sera. However, similarities between human and bovine proteins were well demonstrated. The mobility of each albumin was identical. Human IgM globulin formed an

* Horse anti-human normal serum was kindly supplied by Professor V. Houba, WHO Lab., Faculty of Medicine, University of Nairobi, Kenya.

Figure 9: Immunoelectrophoresis of pooled fractions from peak I of whole serum gel filtration on Sephadex G-200 reduced with 0.1M 2-Mercaptoethanol (2-ME) and untreated whole bovine serum. Top well: Whole bovine serum Bottom well: Sephadex G-200 Peak I + 0.1M 2-ME. No appearance of IgM precipitin arc after reduction with 2-ME.



arc practically similar to the arc in bovine serum being characterized as IgM.

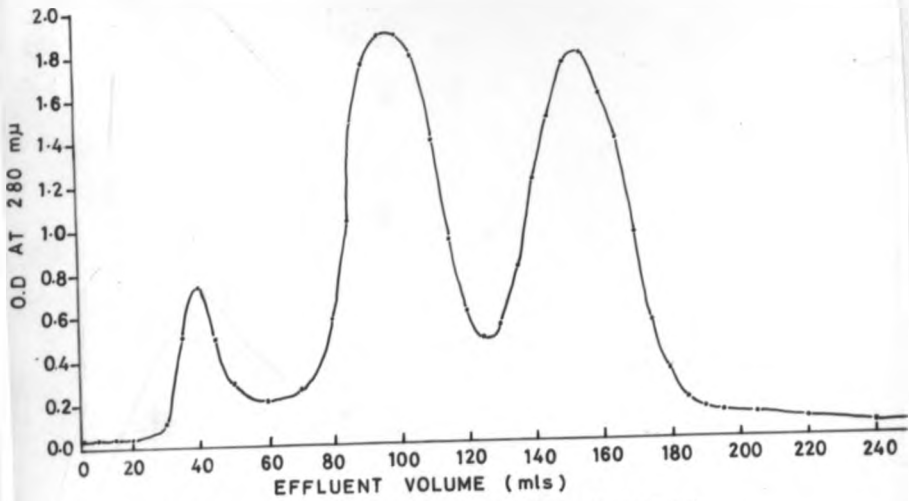
(b) 2-Mercaptoethanol sensitivity of bovine immunoglobulins

Reduction with 2-ME has been shown to split human macroglobulins (IgM) into smaller units (6.5-8.0S) by breaking disulfide bonds; and also abolishes all antibody activity (Deutsch and Morton, 1957; Kunkel, 1960). Similar treatment of IgG immunoglobulins does not destroy their antigen binding capacity or affect their gross physical properties (Kunkel, 1960). After reduction of the proteins in the first peak of the Sephadex G-200 chromatogram (rich in IgM component) with 2-ME, the IgM of this material (Fig. 9). This is in accord with similar findings with human IgM globulin and serves further to identify the immunoelectrophoretic arcs of bovine serum in terms of their human counterparts.

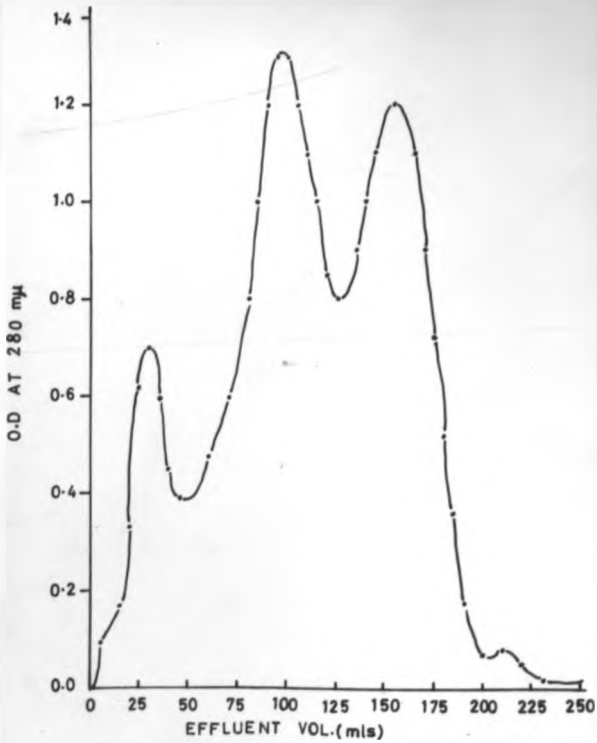
It had been reported by Wiedermann et al. (1963, 1964) that 2-ME treatment of human 7 S gamma globulin antibodies abolished their ability to bind complement when reacting with antigen. In another study, Amiraian and Ferris (1964) reported that treatment of 7 S and 19 S rat anti-sheep haemolysins with 2-ME destroyed the haemolytic activity of the 19 S. In the present studies, the capillary agglutinating ability of both IgM and fast IgG globulin antibodies were abolished following incubation with 2-ME. Therefore it was not possible to use the

Figure 10: Sephadex G-200 chromatogram of serum from bovine NO. 64 eluted in TRIS-HCl buffer (0.0M Tris (hydroxymethyl) aminomethane + 0.15M NaCl, pH 8.0). Column, 2.5 cm x 100 cm.

Figure II: Sephadex G-200 chromatogram of serum from bovine NO. 58. Legend as for Fig. 10.



SEPHADEX G-200 CHROMATOGRAM OF WHOLE BOVINE SERUM STEER No. 64.



SEPHADEX G-200 CHROMATOGRAM OF SERUM FROM STEER No. 58.

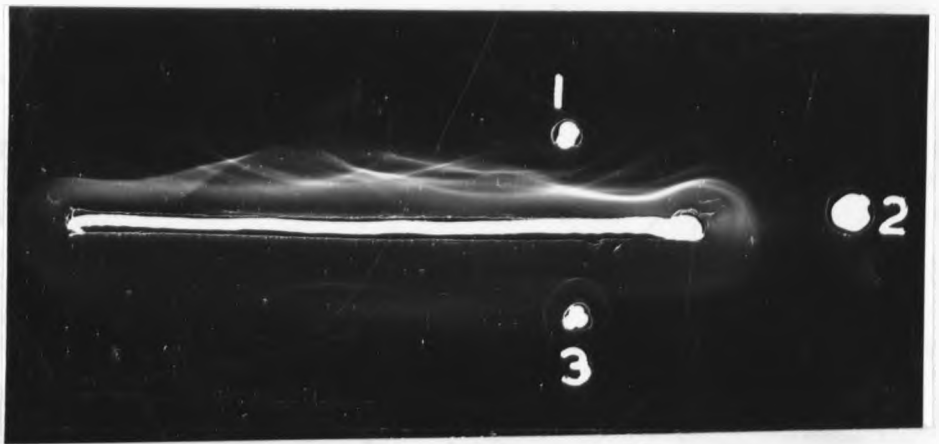
difference in 2-ME sensitivity as a presumptive test to distinguish these two classes of agglutinating antibodies.

(c) Gel filtration chromatography and IEP.

Gel filtration using Sephadex G-200 provided the definitive proof of the macroglobulin nature of peak III. Since the loosely-linked dextran of Sephadex G-200 excludes those molecules of molecular weight greater than 200,000 from the bound water of the gel matrix, the macroglobulins of serum are eluted as an exclusion peak while the other proteins are delayed proportionally to the amount of the bound water phase that is available to them. Flodin (1962) states that 18% of the water occluded by the gel is available for the diffusion of human albumin delaying its elution from the column enough to form peak III.

Fractionation of bovine sera on Sephadex G-200 yielded three major protein peaks (Fig. 10 and 11) comparable in size and position to those obtained with human (Killander and Hogman, 1963) and ovine (Aalund et al. 1965) serum. According to Aalund et al. 1965), approximately 8% of the total amount of the serum proteins in normal ovine serum were present in peak I while the remainder were equally divided between peak II and III. This is fairly comparable with the findings in these studies. However, peak II tended to be slightly higher in magnitude as compared with peak III in most of the chromatograms.

Figure 12: Immuno-electrophoresis in combination with double diffusion in agar gel for the analysis of specific immunoglobulin associated with antibody activity. Well 1: Whole bovine serum. Wells 2 and 3: Sephadex G-200 Peak 1. Trench: rabbit antibovine serum.



Immuno-electrophoretic analysis of peak I demonstrated the single cathodal precipitin arc of IgM while peak II and the valley between peak I and II showed a predominance of IgG gamma globulins. Peak III contained serum albumin. The capillary agglutinating antibodies were found to be distributed proportionally between peak I and II of Sephadex G-200 gel filtrations just as they were distributed between peaks II and III from DEAE-Sephadex A-50 chromatography.

In order to associate the antibody activity with the appropriate immunoglobulin(s), additional experimental approach was necessary. It was demonstrated that the cathodal immuno-electrophoretic arc from Peak I protein obtained by gel filtration of serum on Sephadex G-200 coalesced only with arc of whole serum immuno-electrophoretic pattern that was analogous to the human IgM in shape and position (Fig. 12). Fusion occurred between the electrophoresed IgM of Sephadex G-200 peak I, the IgM of G-200 peak I applied to a double diffusion well for arc extension, and the IgM arc of whole serum. In the whole serum pattern, IgM is seen as a flat arc in the gamma-I region which is closest to the antigen source and furthest from the antiserum trench. This investigation helped to dispell uncertainty concerning the common identity of the immuno-electrophoretic arc from the partially isolated protein and the analogous arc developed from whole serum.

(d) Immunelectrophoretic Analysis Throughout E.C.F.

Another investigation involving IEP of serum samples throughout the acute phase of E.C.F. further implicated macroglobulin (IgM) in the early response of the host to the invading Theileria parva parasites. A series of serum samples from animal Nos. 7231, 851 and 74 were applied to the wells of immunelectrophoretic slides in volumes as nearly equal as possible, using the dimensions of the well to limit the volume. During these studies, it has been shown that accuracy of sample volume can as well be controlled this way as by using micropipettes. Following electrophoresis, antiserum was applied to the trenches, again using the dimensions of the trench to limit the volume. These slides were stained with Amido Black 10 B and used as negatives in an enlarger allowing photographic reversal and superimposition for accurate comparison of arcs from different samples. Although most arcs appeared identical throughout the series indicating the success achieved in controlling volumes, differences in the IgM arc were noted.

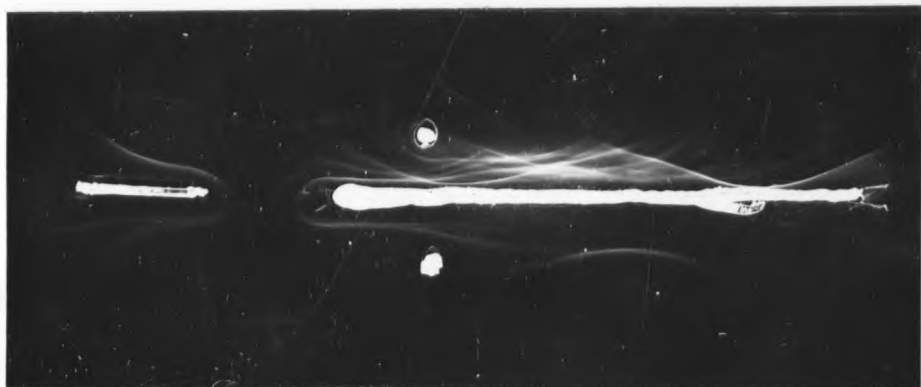
The increase in IgM protein precipitin arcs noted around day 17 postinfection roughly approximated the development of IgM agglutinating antibodies.

(4) CHARACTERIZATION OF IgG GAMMA GLOBULIN

(a) Chromatography and IEP

In instances where serum of man (Fahey and Horbett, 1959), sheep (Harrison and Mage, 1967) goat (Gray et al.,

Figure 13: Immunolectropherogram with antiserum trench interrupted to demonstrate the continuity of the precipitin arc of DEAE-Sephadex Peak I or Peak II with that of 7 S gamma globulin of the reference serum. Top well: Whole bovine serum. Bottom well: DEAE-Sephadex Peak 1 or Peak II. Trench: rabbit anti-bovine serum Anode is to the right.



1967) or hamster (Coe, 1968) have been chromatographed on DEAE-Cellulose, the 7 S gamma globulin in each case were first to appear in effluent fractions. In these studies, chromatography of bovine serum on DEAE-Sephadex A-50 accompanied by immunoelectrophoretic analysis of the fractions revealed that the same spectrum of IgG mobilities as had been described by these workers was also the case for bovine gamma globulins. When fractions from peak I and II in the DEAE-Sephadex chromatograms were examined by IEP, anti-peak I serum developed a single precipitin arc which was in the position of electrophoretically slow-and fast-migrating gamma globulins respectively.

The continuity of the immunoelectrophoretic arc of isolated peak I protein with that of the 7 S gamma globulin precipitin arc of the reference serum was demonstrated by means of the technique where the antiserum trench was interrupted (Fig. 13). This provided evidence of the identity of the isolated protein. The same modification of immunoelectrophoretic procedure using peak II protein resulted in similar coalescence of precipitin arcs. The identification of the DEAE-Sephadex peak I protein required no further experimentation. There is only one serum protein component, the slow 7 S gamma globulin; that migrates as far toward the cathode at pH 8.6 as this protein.

Figure 14: Ouchterlony double diffusion in gel analysis of Peak I and Peak II obtained from DEAE-Sephadex chromatography of bovine serum. Well 1: rabbit antiovine serum. Wells 2 and 5: concentrated pools of DEAE-Sephadex Peak I Wells 4 and 7: Concentrated pools of DEAE-Sephadex Peak II. Antiserum gave single precipitin lines with either of the peaks showing identity.

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Figure 15: Ouchterlony double diffusion in gel analysis of Peak 1 and Peak II obtained from DEAE-Sephadex chromatography of bovine serum. Rabbit antibovine serum (Well 1) gave single precipitin line when reacted with Peak II (Well, 5) but when reacted with Peak 1 (Well, 2) a second precipitin line appeared.

Fractions making up Peak 1 and Peak II were obtained from a different DEAE-Sephadex chromatogram from those in Fig. 15.

7 6 5
2 1 4
3

(b) Antigenic analysis by the Ouchterlony double diffusion in gel technique

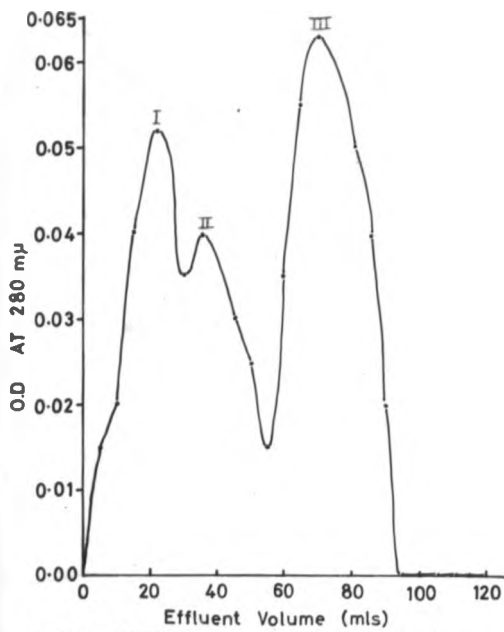
Ouchterlony double diffusion analysis further provided information regarding the antigenic similarities between peak I and peak II globulins.

Several peak I and peak II globulin preparations of the DEAE-Sephadex chromatograms were subjected to double diffusion in gel analysis using different rabbit anti-bovine sera. No trace of arc intersection was detected when peak I globulin was compared with peak II (Fig. 14). The antisera employed detected no antigenic difference between peak I and peak II proteins. Very weak second precipitin arcs due to unidentified trace contaminants were observed in several of the comparisons (Fig. 15). These second precipitin arcs could be due to trace contamination with transferrin in peak II. However, such doubling of precipitin arcs has been found by Edelman et al. (1960) to relate to distinct antigenic determinants on the S and F papain fragments of gamma globulin. In some preparations, peak II (fast IgG) showed spurs of partial antigenic identity over peak I (slow IgG) and the spurs were confluencing.

(c) Papain digestion, Chromatography and Immunoelectrophoretic analysis of fragments.

Analysis of papain digestion fragments was used in an attempt to further characterize the antigenic relationship between DEAE-Sephadex peak I and peak II gamma globulins.

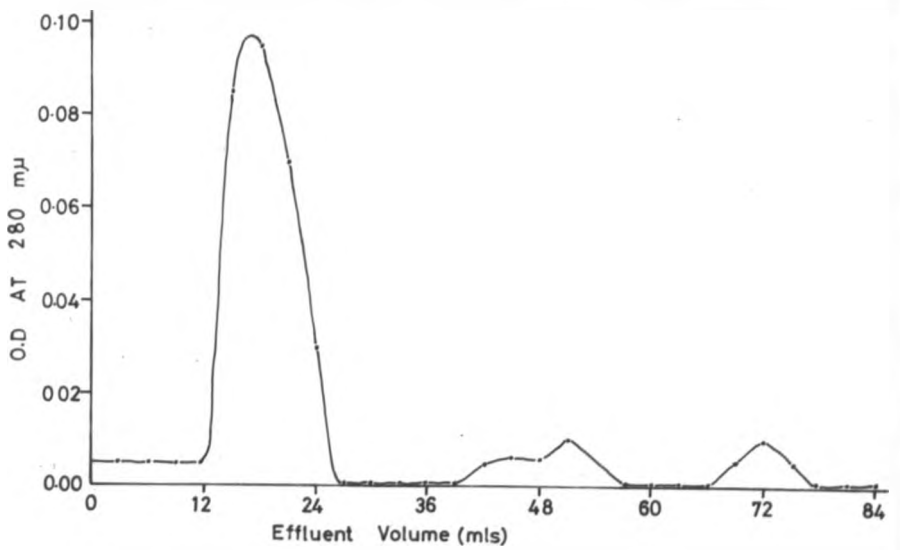
Figure 16: DEAE-Sephadex chromatogram of papain digest of bovine IgG. Elution gradient progressed from 0.005M PO₄, pH 8.0 to 0.5M PO₄, pH 8.0 commencing at 300 ml eluate volume. Column, 2.5 cm x 40 cm.



DEAE SEPHADEX COLUMN CHROMATOGRAM OF
PAPAIN DIGEST OF γ G OF WHOLE BOVINE SERUM
FROM STEER No. 64

A pool of whole IgG was digested with papain and cysteine for 16 hours at 37°C according to the method of Porter (1959). The digestion products were then dialysed against 0.005 M PO₄ buffer, pH 8.0 to remove diffusible peptides. The products of the papain digestion were then chromatographed on DEAE-Sephadex following the procedure described elsewhere in this dissertation. Three components were resolved and were designated according to Porter (1959) as Fractions I, II and III in order of chromatographic elution (Fig. 16). However, these subunits have also been referred to as Fab and Fc fragments to represent fractions I and II; and fraction III respectively. On the other hand, Edelman et al. (1960) designated fractions I and II and fraction III as S (slow) and F (fast) respectively on the basis of their electrophoretic mobility. The recommended notations in 1964 by the W.H.O. Committee on the nomenclature of human immunoglobulins is Fab(s) and Fc(F) to represent fractions I and II; and fraction III respectively. This terminology will be used in the text. Similarly, treatment as described above for whole IgG globulin was applied to Peak I and Peak II protein of the DEAE-Sephadex chromatograms. In each case, the resulting chromatogram was however complex but similar to the elution profiles that have been obtained with human (Olins and Edelman, 1962) and mice (Fahey and Askonas, 1962)

Figure 17a: Chromatography of papain digest of DEAE-Sephadex Peak 1 (slow IgG) on DEAE-Sephadex. Elution gradient progressed from 0.005M PO_4 , pH 8.0 to 0.5M PO_4 pH 8.0 commencing at 300 ml eluate volume. Column, 2.5 cm x 40 cm. Fab(S) fragment was eluted in the breakthrough peak and Fc(F) fragment in subsequent fractions.



DEAE SEPHADEX CHROMATOGRAM OF PAPAIN DIGEST OF γG_1 FROM STEER No. 64.

yG-globulins. Such a chromatogram is illustrated by Fig. 18 following papain degradation of peak I globulin (IgG₁ or slow IgG).

Immuno-electrophoretically identified Fab(s) fragments from both peak I and peak II globulins (slow and fast) appeared in the breakthrough peaks of their respective DEAE-Sephadex chromatograms (Fig. 17a), and this peak was considerably higher in magnitude in each case. Two smaller peaks were eluted at 40 - 70% of effluent (the expected Fc(F) fragment region) of each chromatogram. Each Fc(F) fragment peak appeared distinct in elution position. When papain digestion fragments of human gamma globulin were chromatographed on DEAE-Cellulose by Edelman et al. (1960), similar separation of Fab(s) and Fc(F) components as reported in these studies was obtained and the bipartite elution curve of Fc(F) fragment was demonstrated.

Immuno-electrophoretic analysis revealed that in each of the peaks, some gamma globulin remained which had not been digested and therefore was eluted according to its usual anionic binding capacity. The undigested gamma globulin component from the peak I (slow IgG) digestion was found in the breakthrough peak while that from the peak II (fast IgG) digestion was localized in the Fc(F) fragment fractions. The presence of undigested gamma globulin encountered in these studies could perhaps be justified since Hsaio and Putnam (1961) demonstrated that conditions of papain digestion set out by

Figure 17b: Immuno-electrophoresis (line drawing) of intact bovine IgG and IgG hydrolysed with papain and cysteine for 16 hours at 37°C. Top well: Intact IgG. Bottom well: Papain treated IgG. Trench: rabbit anti-bovine IgG. Anode is to the right.

NORMAL γ -GLOBULINS

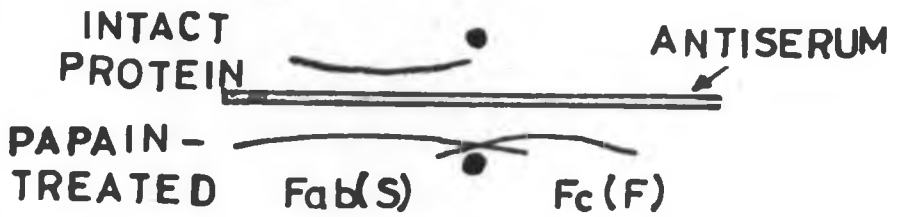
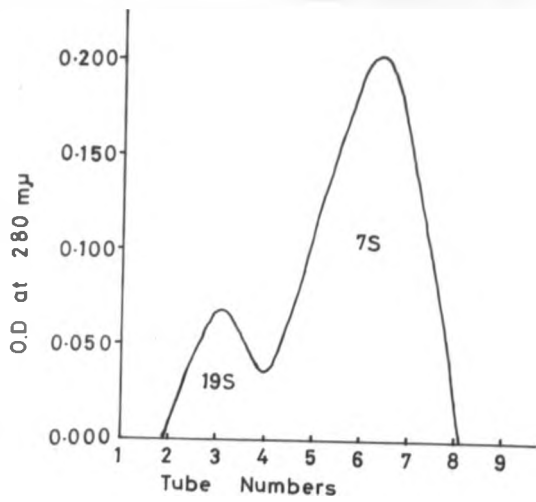


Figure 18: Representative pattern of bovine IgM and IgG sedimentation rates obtained by sucrose density gradient ultracentrifugation. Serum sample was from steer No. 74 and protein concentration was measured at 280 mu.



Determination of Sedimentation Rate of IgM and IgG by Sucrose Density Gradient Ultracentrifugation of Serum from Steer No. 74.

Porter do not produce complete degradation to 3.5 S (Fab, Fc) fragments of gamma globulins of species other than man and rabbit.

Immuno-electrophoretic comparison of the Fab(S) fragments from both peak I and II (slow IgG and fast IgG) demonstrated identical mobilities while the Fc(F) fragments were found to be immuno-electrophoretically heterogeneous. The Fc(F) fragments moved toward the anode while the Fab(S) migrated toward the cathode (Fig. 17b). These differences have been ascribed to be due to antigenic as well as relative electrophoretic mobility between the two groups of papain digestion products.

(5) SUCROSE DENSITY GRADIENT ULTRACENTRIFUGATION.

The pattern obtained by sucrose density gradient ultracentrifugation (SDGU) of serum from bovine with East Coast Fever is shown in Fig. 18. The figure shown here represented serum from steer number 74 bled on day 20th postinfection when agglutinating antibody activity was first detected in peak III of the DEAE-Sephadex chromatogram. Results of the SDGU indicated that the heavy density gradient fractions of the serum sedimented in the bottom of the centrifuge tubes while the lighter fractions were found mainly in the middle fractions. Since a considerable amount of evidence is now available that in many species, macroglobulin (19 S) antibodies are produced early in immunization with many antigens (Bauer and Stavitsky, 1961; Uhr and Finkelstein,

Figure 19: Appearance of ring diffusion in an IgM antibody-agar plate after 24 hours. Six dilutions of a standard serum of known IgM concentration were placed in the remainder of the antigen wells.

REPRESENTATIVE AGAR RING DIFFUSION TEST

Bovine IgM - globulin - 24 hour

Reference
Serum

TEST
SAMPLES

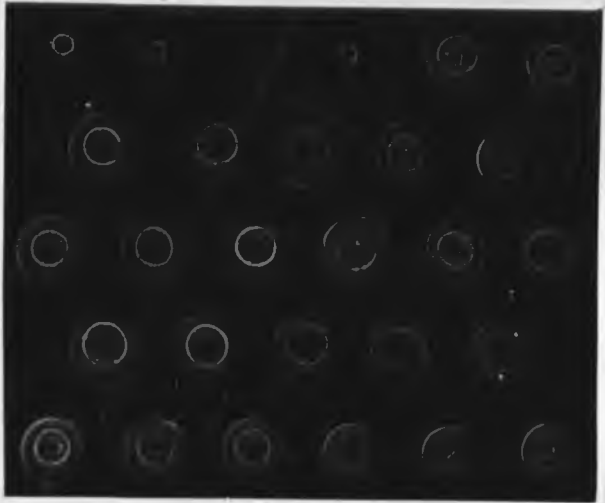


Figure 20: Diffusion rings obtained in antibody-agar plates with serial dilutions of IgG. Photographed at 24 hours.

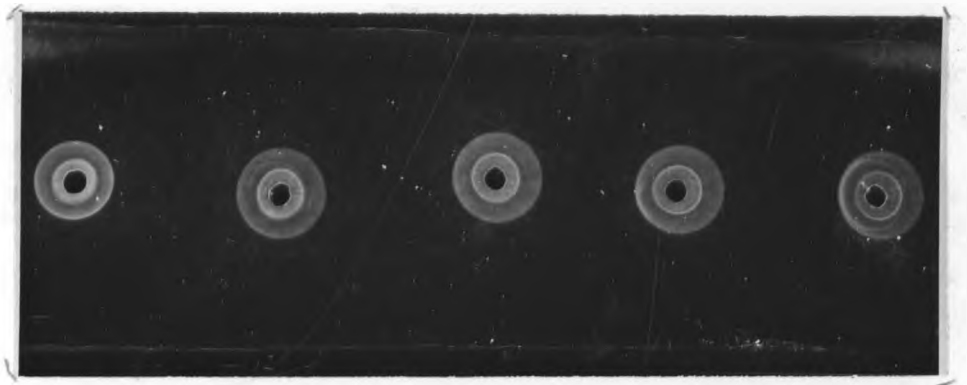


Figure 21a: A graph showing the relationship between bovine serum IgG concentration and the precipitin ring diameter. IgG concentration is given in micrograms/milliliter on logarithmic scale (ordinate). Ring diameters are in millimeters (abscissa).

Relation of Precipitate Ring Diameters to
Concentration of Antigen (IgG Globulin)

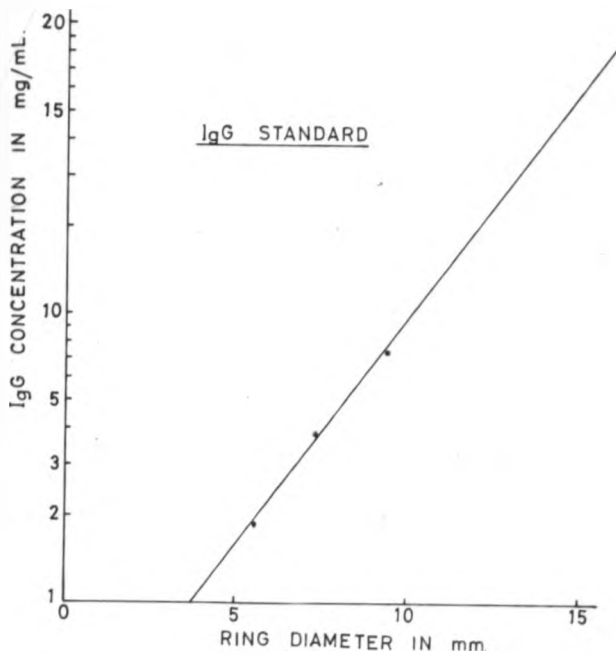
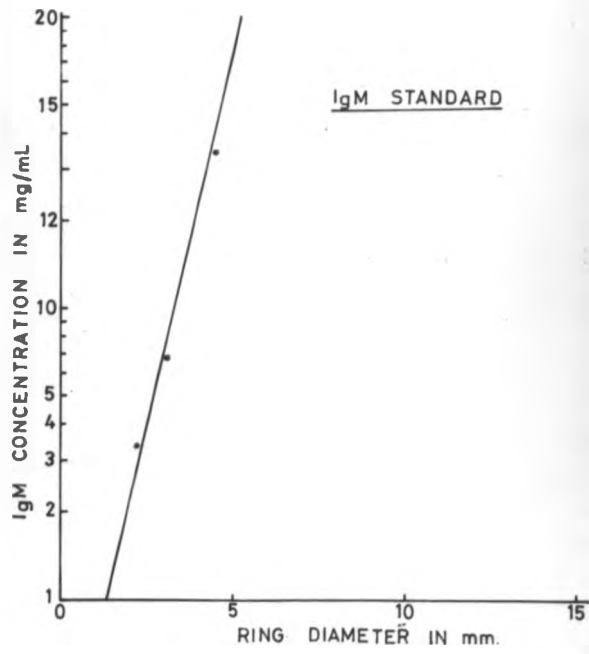


Figure 21b: A graph showing the relationship between bovine serum IgM concentration and the precipitin ring diameter. IgM concentration is in micrograms/milliliter on logarithmic scale (ordinate). Ring diameters are in millimeters (abscissa).

Relation of Precipitate Ring Diameters to
Concentration of Antigen (IgM Globulin)



1963) and that a transient and early rise in the macroglobulin antibody is followed later by a more sustained rise in the level of IgG antibodies, then the immunoglobulin distribution of anti-Theileria parva antibodies described here would fit quite well with these observations. Immunoelectrophoresis confirmed that the fractions with the highest sedimentation rate were macroglobulins while those that were collected from the middle fractions of the density gradient migrated in the 7 S gamma region.

(6) SERUM IMMUNOGLOBULIN CONCENTRATION AS DETERMINED BY THE SINGLE RADIAL IMMUNODIFFUSION.

An agar plate was prepared incorporating antibody throughout the agar. The test sample was put into antigen wells, and on diffusion into the agar, formed rings of antigen-antibody precipitate around the wells. Serial dilutions of a standard serum of known IgM and IgG concentration was included on each plate as seen in Figure 19 while Figure 20 demonstrates a representative precipitate pattern obtained with serial dilutions of IgG (7sy-globulin). Precipitin ring diameters were measured and a graph was prepared for each plate comparing the precipitate ring diameter to the logarithm of the protein concentration. With both IgG and IgM standard antigen solutions, the diameter of the precipitin ring was found to be related directly to the logarithmic concentration of antigen (Fig. 21a and b). The protein concentration

in a test sample was determined by comparing its ring diameter and the graphic curve obtained with the standard antigen.

The results from SRD assay for the concentration of serum IgG and IgM in cattle infected with East Coast Fever are given in Table 3. In these studies, pre-infection period is taken to represent sera obtained from the experimental animals between day zero and the day temperature rose to 39.5°C. Post-infection period represents sera obtained between day temperature rose to 39.5°C to day of death.

TABLE 3:

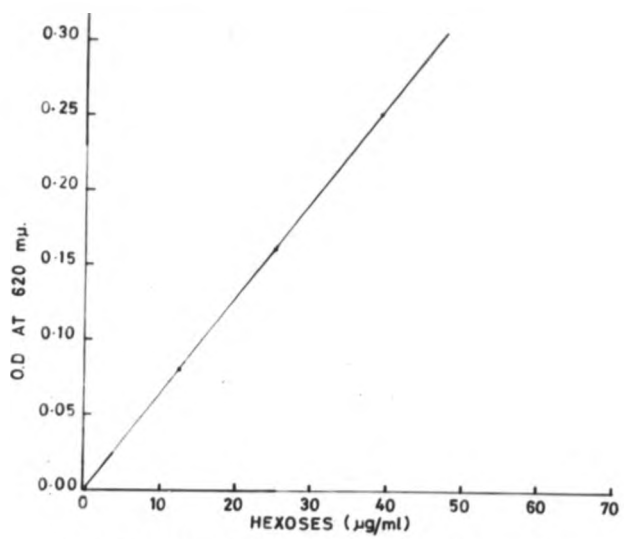
CONCENTRATIONS OF SERUM IgG and IgM IN CATTLE EXPERIMENTALLY INFECTED WITH EAST COAST FEVER (*Theileria parva* infection).

Serum	No of Samples	IgG (mg/ml)		IgM (µg/ml)	
		Mean ± SD	Range	Mean ± SD	Range
Pre-infection	10	12.4 ± 0.3	12.6-14.0	3.22±1.42	1.2-5.2
Post-infection	10	28.08± 3.9	23.1-36.0	20.1±3.3	8.5-31.0

SD = Standard deviation.

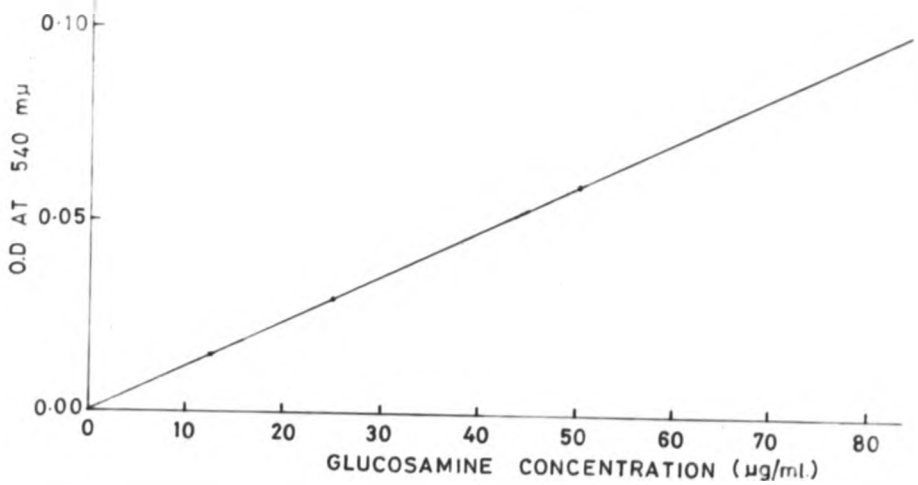
During the pre-infective period, the IgM levels were in the range 1.2 to 5.2 mg/ml while in the post-infective period which was the actual period of the clinical disease, the levels were between 8.5 to 31.0 mg/ml.

Figure 22: Standard curve for the determination of hexose concentration in bovine serum IgG and IgM. Hexose concentration was measured at 620 m μ and is given in micrograms/milliliter (abscissa).



STANDARD CURVE FOR THE DETERMINATION OF HEXOSES CONCENTRATION IN BOVINE IgG AND IgM.

Figure 23: Standard curve for the determination of Glucosamine concentration in bovine serum IgG and IgM. Glucosamine concentration was measured at 540 m μ and is given in micrograms/milliliter (abscissa).



STANDARD CURVE FOR THE DETERMINATION OF GLUCOSAMINE CONCENTRATION IN BOVINE IgG AND IgM.

The mean concentration of serum IgM in the animals when reacting from the clinical disease was significantly much higher than when disease manifestations had not set in ($P < 0.001$). Serum IgG levels showed a fairly narrow range both in the pre-infective period (12.6 - 14.0 mg/ml) and during post-infective period (23.1 to 36.0 mg/ml). However, the mean concentration of IgG was significantly higher in the post-infective period than in the pre-infective one ($P < 0.01$).

(7) CARBOHYDRATE ANALYSIS IN THE BOVINE IgG AND IgM.

The hexose and hexosamine content of the bovine immunoglobulins, IgG and IgM were analyzed chemically. In each case a standard curve was set up as illustrated in Figures 22 and 23 respectively. The amount of either hexose or hexosamine was then calculated from the calibration curve and the results are given in Table 4.

TABLE 4.

CARBOHYDRATE CONTENT OF THE BOVINE IgG AND IgM
(% of Protein)

<u>Immunoglobulin Class</u>	<u>Hexoses</u>	<u>Hexosamine</u>	<u>Hexosamine: Hexose Ratio.</u>
IgG	1.8	1.3	0.72
IgM	4.0	3.4	0.85

The results of the analyses indicated that IgG preparations had comparatively lower hexose and hexosamine values than the IgM component. However, the hexosamine/hexose ratio of γ -G globulin (IgG) (0.72) appeared to have relatively little difference from that of the macroglobulin (IgM) which was 0.85.

DISCUSSION

(1) ANION-EXCHANGE CHROMATOGRAPHY OF SERUM FROM BOVINE EXPERIMENTALLY INFECTED WITH EAST COAST FEVER (*Theileria parva* infection).

(a) Antibody heterogeneity in relation to the course of the disease and the apparent level of the parasite antigen(s).

The noteworthy similarity of the antibody response following infection with *Theileria parva* in the fourteen animals studied reflects in one way upon the nature of the mechanism responsible for such constant manifestation. This relationship between initial synthesis of IgM and IgG must be indicative of a rather basic protective mechanism, if the immune system is protective in a teleological sense, since it occurs in many independent antibody responses to different antigens in different species (Aalund, 1968).

In this work, capillary agglutinating antibody activity appeared to reside exclusively in IgM globulin during the early stage (17th day post infection) of antibody synthesis. Macroschizonts could be demonstrated in the Giemsa-stained lymph node smears at that time. However, during this early stage of the detection of antibody activity there were relatively few parasites. If the estimated severity of the infection is taken as an indication of the antigenic burden presented to the reticulo-endothelial system (RES) at a given time, then the marginally detectable level of the parasites at this time might mean that early IgM globulin antibody

synthesis was evoked by a small amount of antigen. This is in conformity with the observations made by Duffus and Wagner (1974) on the development of Theileria parva schizont and piroplasm which suggested that parasitism of only a few cells was necessary for induction of an antibody response.

It has been shown that the amount of antigen in immunologically competent cells may serve as a major control mechanism for several immunological functions (Uhr and Finkelstein, 1963). Thus, Uhr and Finkelstein (1963) demonstrated in guinea pigs that antigen can regulate the relative rate of antibody formation and the duration of the synthesis of rapidly sedimenting antibody.

By about the 23rd day post-infection, the agglutinating antibody activity had rapidly increased at the time when intraerythrocytic piroplasms had started to be demonstrated in Giemsa-stained blood smears. However, it was not until about the 28th day post-infection that the synthesis of fast IgG gamma globulin antibody was coincident with severe parasitemia (Schizont and piroplasm) and thus was probably characterized by the production of large amounts of antigen from the microorganisms. It has been demonstrated by Hill and Matson (1970) that red cells in cattle infected with Theileria lawrencei are very fragile around day 23 of infection and become destroyed in the hemolytic process; thus RES removal of

affected erythrocytes presents antigen to cells capable of humoral immune response.

Ross and Lohr (1972) in their study on the production of serum antibodies against Theileria parva using capillary tube agglutination test found that antibodies were first detected 13 - 23 days after infection and reached a peak on the 30th day. They established infections by attaching Theileria parva infected adult Rhipicephalus appendiculatus ticks, a similar method as applied in these studies. Although they did not report on the type of antibody molecules produced during the course of the disease, their results roughly corroborate the observations reported in this dissertation with reference to the time of detection of the anti-Theileria parva antibodies when it is taken for consideration that ticks will only transmit Theileria parva to the host an average of 5 days after attachment (Wilde et. al., 1968).

Some interesting observations which were not encountered during the present studies were made by Spooner et. al. (1973) during their investigation of the serum globulin changes in the sera of cattle suffering from severe East Coast Fever. They found a brief fall in IgG concentration 14 days post-infection. However, there was increased levels by day 18. They observed that the fall coincided with the peak of the clinical reaction when the Theileria parva macroschizont parasitosis and

the febrile response of the infected cattle were most marked. Likewise, IgM protein fell in concentration after day 14, i.e. coinciding with the peak of the clinical reaction. Unlike IgG, the IgM levels showed a very rapid increase after the disappearance of the macroschizonts followed by a gradual decline. In such a situation of very severe East Coast Fever, it would then appear that there is a suppression of immunoglobulin synthesis which would not be surprising considering the marked leukopenia associated with the massive parasitosis of lymphoid cells that develop in severe infections.

That a difference in the levels of parasitemia achieved can radically alter the humoral immune response has been reported for Plasmodium infection (Cox and Turner, 1970a). However, if as others have demonstrated, antigen dosage is an important factor in determining the sequence and rate of different molecular species of antibody molecules, then it may be inferred that the shift from exclusive IgM synthesis initially followed by the synthesis of fast IgG is either (i) an antigen independent function that would take place regardless of the peak antigen load provided enough was present to trip the "all or none" switch or (ii) that the progression is antigen dependent and a function of the very small amounts of antigen present in the first few days of response (of IgM antibody) as well as the subsequent exponentially

increasing amounts (IgM + IgG antibody). Although Uhr and Finkelstein (1963) found that IgM antibody response in guinea pigs challenged with bacteriophage ϕ X 174 is maximal and antigen dose independent above a "threshold" dose, while the intensity and rate of development of IgG antibody is antigen dose dependent, the question remains unanswered in the bovine species in its response to Theileria parva antigens, be they schizontal or piroplasm antigens.

In studies on Babesia microti infection in mice, Cox and Turner (1970b) using fluorescent antibody (IFA) test were able to detect both IgM and IgG but were unable to demonstrate a sequential production of IgM and IgG, the latter occurring early in the antibody response and always to a much higher level than the IgM antibodies. Similar result have been described by Cox and Turner (1970) for Plasmodium infection in mice. However, since it is well documented that IgM antibodies are more efficient at complement fixation (CF) and agglutination than IgG (Grey, 1964; Robbins et al., 1965) while the latter class of immunoglobulin is known to be a more efficient precipitin (Pike, 1967), the acceptance of the usual sequential production of IgM and IgG during an immune response should be re-evaluated as has been suggested by Freeman and Stavitsky (1965). Nevertheless, the sequential production of IgM and IgG in the present

studies was well demonstrated in terms of agglutination activity during the course of the disease.

It has been observed by Abele et.al., (1965) that the different methods of assay for malarial antibodies could lead to variable results when studying specific immunoglobulins. As shown in this dissertation, the immunoglobulin response as detected by capillary agglutination test demonstrated a normal sequential production of IgM and IgG. Murphy, Osebold and Aalund (1966) applying both complement fixation and capillary agglutination tests published results with Anaplasma marginale infection in cattle, where there was an early increase in IgM followed by an increase in IgG activity. In contrast, Rose, Amerault and Roby (1974) applying the modified card agglutination test reported the simultaneous appearance of 19 S and 7 S antibodies in bovine anaplasmosis. When Wagner et. al. (1974) applied several serological tests to examine for specific immunoglobulin activity in response to isolated Theileria parva antigens, they observed that the early antibodies reactive in the complement fixation and indirect haemagglutination tests were of the IgM class whereas the indirect fluorescent antibody test only detected 7 S Ig antibodies.

Although only limited interpretations can be drawn from the results of agglutination antibody studies due to the rather insensitivity of the serologic assay (Murphy et. al., (1965), it appeared that no better correlation could be obtained between detection of this

activity and the manifest signs of immune protective activity. This is not to say that there are or are not other antibody populations unknown during the clinical disease which are protective in nature. Furthermore, cell-mediated rather than humoral immunity was suggested by Hulliger et. al. (1965) as a possible mechanism of immunity to Theileria parva infection. The immune clearance of parasitized erythrocytes and leukocytes and the immune suppression of the replication of Theileria parva organisms or passage from cell to cell must act in concert with immunological mechanism to alter a course bent on progression to a critical leukopenia and anaemic state. The total response must allow the animal to reach a more favourable equilibrium of co-existence with the invading parasites if resolution is to be the ultimate result.

Muhammed, Lauerman and Johnson (1975) studied the capability of humoral antibodies to influence the course of East Coast Fever. They inoculated serum and gamma globulins from vaccinated (donor cattle) to cattle (principals) exposed to Theileria parva. The principals were treated with normal serum, with serum containing high antibody titer against Theileria parva or with globulins prepared from normal and from immune serums. They established infections by subcutaneously inoculating the principal cattle and controls with standardized suspension of the tick Rhipicephalus appendiculatus containing

Theileria parva infective particles (Cunningham et al. 1973). Comparisons were then made between treatments with normal serum and globulin and treatments with immune serum and globulin, using observations of time lapse after exposure to febrile response (39.5°C), to appearance of Theileria parva macroschizonts, and to death. They observed that neither the establishment of infection nor the clinical and hematologic changes in the principals, were influenced by their treatment with immune serum or concentrated globulins; otherwise all died of East Coast Fever. This demonstrated in one way that humoral antibodies did not protect cattle against fatal East Coast Fever, thus indicating that the resistance against the disease could probably require the involvement of both humoral and cell-mediated immune mechanism.

Conclusive evidence of cell-mediated immune (CMI) response in Theileria parva infection was demonstrated by Muhammed et al. (1974). They applied leukocyte migration inhibition (LMI) test as in vitro evidence of CMI response. In the LMI test, peripheral blood leukocytes were used. These investigators demonstrated that cattle inoculated with Theileria parva produce CMI response. However, the response was limited to animals inoculated with live parasites which is similar to the findings in anaplasmosis by Carson et al. (1975). Only 76% of cattle that were exposed to live Theileria parva antigens showed CMI response. This finding somehow resembles

that obtained by Nyindo (1975) who studied CMI in Alsatian dogs infected with Ehrlichia canis and found that only 58% of infected animals responded in the test. A possible analogy between infections induced by both parasites is that they both invade the lymphatic system. Under the circumstances it is possible that a considerable in vivo drainage of lymphokines may be taking place, thereby depriving sensitized lymphocytes of their full in vitro performance.

(b) Other influence upon the physicochemical heterogeneity of the antibody response.

In addition to discussing that antigen dosage may influence the physicochemical heterogeneity of the antibody response, other workers have shown the influence of

- (1) the age of the antigen recipient,
- (2) the site of antibody formation,
- (3) the species injected,
- (4) the chemical properties of the antigen,
- (5) the route of antigen administration

and (6) the genetic factors (Aalund, 1968). The relationship of these factors to the antibody response of interest in these studies justifies the following discussion.

The age or immunological maturity of the infected animals although it may be of importance in terms of the clinical and haematological aspects of the disease, had no influence upon the properties of the antibody molecules

synthesized by the steers used in the study. Similarly, splenectomy did not appear to alter the kinetics or the distribution of the antibody response. The status of the spleen had only some influence upon the clinical aspects of the disease as was indicated by steer No. 226 (Table 2).

The localization of Theileria parva piroplasmas in erythrocytes which may help the distribution of the antigens to all parts of the reticuloendothelial system (RES) may be important in minimizing the part played by the spleen as an antibody synthesizing organ. This might explain the failure to detect differences in antibody response between intact and splenectomized steers. In this same regard, the influence of the route of antigen administration cannot be compared with results obtained by others with non-viable antigens. However, a sequential production of IgM and IgG antibodies has been demonstrated by Duffus and Wagner (1974) following subcutaneous inoculation of a stabilate containing infective particles of Theileria parva (Cunningham et al., 1973) although the antigenic effect of the tick material in the stabilate is unknown. With regard to the influence of the way Theileria parva antigens are presented to the RES of the host, little may be said since replication of the parasites within the cells during the incubation period of the disease and other early events have not been studied in detail.

Although the genetic factors also influence the physicochemical properties of the molecular form of the antibody response (Fahey, 1962), this aspect of investigation was outside the scope of this project.

The limited host range of Theileria parva prevented infection of other species. Therefore, in this as in most other experimental models, comparison of physicochemical character of the antibody response with that in other species has not depended upon comparable antigenic challenge with species as the only varying parameter. Nevertheless, species is of prime importance in determining the nature of heterogeneous antibody response. Some examples of similar primary antibody responses in other species follow: In rabbit sequential synthesis of two types of antibodies IgM and IgG have been observed after primary injection of sheep erythrocytes (Stelos and Talmage, 1956), bacteriophage (Bauer and Stavitsky, 1961), bovine serum albumin (Benedict, Brown and Ayengar, 1962), bacterial flagellae (Bellanti, Eitzmann, Robbins and Smith, 1963) and poliovirus (Svehag and Mandel, 1964a); while in guinea pigs similar response were observed following injection of bacteriophage ϕ X 174 (Uhr, Finkelstein and Baumann, 1962b).

The only exceptions to the above described sequences are the antibody responses in rabbits to the O-antigen of Salmonella typhosa, to sheep and human erythrocytes

and to sulfanilazo-hapten (Bauer et al. 1963), wherein primary, secondary and tertiary injection of the O-antigen resulted in the synthesis of macroglobulin antibody only, while a second or third injection of human erythrocytes resulted in the synthesis of 7 S gamma globulin antibody after the primary injection had elicited only IgM antibody. Injection of bacteriophage ϕ X 174 in guinea pigs by Uhr and Finkelstein (1963) resulted in the synthesis of rapidly sedimenting 19 S antibodies and later slowly sedimenting 7 S molecules. Blinkoff (1964) has shown that the same sequence of antibody synthesis occurs in mice. He demonstrated that in mice antibodies capable of immobilizing flagella of Salmonella were initially of 19 S type and later 7 S molecules. It would appear from these and many other observations in various species that the bovine antibody response to Theileria parva infection does not reflect any factor unique to this species.

The fact that some antigens induce 7 S gamma globulin and others 19 S gamma globulin antibodies indicates that chemical properties play an important role in the antibody response. For example in the rabbit, Bellanti et al. (1963) demonstrated that Salmonella somatic lipopolysaccharide O-antigen induced only a 19 S antibody response whereas the flagella H-antigen induced early 19 S and later 7 S antibody response.

The exclusive production of IgM antibodies in the rabbit immunized with somatic antigens of Salmonella enteritidis and Escherichia coli has also been documented by Weidanz et al. (1964). Since it is now widely accepted that lipopolysaccharide is an antigen which selectively stimulates thymus independent (B) cells (Gery et al., 1971; Manning et al., 1972), and it has been demonstrated by Dasgupta (1960) that cellular material of Theileria parva organisms contain polysaccharide component, then this could be antigenic and activate the B cells during the course of East Coast Fever. Once activated, B cells divide and differentiate into high-rate antibody secreting cells. Britton and Moller (1968) and Miranda (1972) showed that IgM was the prevalent class of antibody to be secreted by activated B cells. However, the production of both IgM and IgG antibodies depends on the specific co-operation of thymus - dependent (T) and thymus independent (B) cells (Claman et al., 1966) although the IgM is the first class of antibody that circulates in peripheral blood at higher concentrations (Vaerman and Heremans, 1970). From this, it can then be postulated that IgM and IgG were produced in response to Theileria parva parasites although IgM class was the one which could initially be detected serologically. However, whether the significant factor is the chemistry of the antigen directly or the manner in which its chemi-

stry affects its presentation to the immune system remains to be determined. In many cases, IgM inducing antigens are attached to large molecules, while antigens which induce synthesis of both IgM and IgG antibodies are complex molecules but often soluble, especially the larger proteins. In the light of these observations, the double agglutinating antibody response reported in this dissertation is what might be expected if Theileria parva organism stimulates the synthesis of IgM antibodies and the soluble antigenic products of its growth stimulate a heterogeneous antibody response. However, the serological assay employed would not have detected differences in specificity.

(2) BOVINE IgM

(a) Its characterization

Inspite of the fact that gamma globulins of different mammalian species are antigenically distinct and that they cross react only slightly or not at all, there are some gross physicochemical properties of striking similarity between species. The immunoelectrophoretic patterns of sera of all of the animals that have been studied contain only minor variations of which the bovine species is no exception. The near identity of its immunoelectrophoretic pattern with the one described for human serum (Kunkel, 1960) confirmed this point. However, in terms of functional relationship between

particular proteins in different species, comparable position and shape of immunoelectrophoretic precipitin arcs have shown to be too shallow a criterion to rely upon. Therefore in conjunction with defining the position and shape of immunoelectrophoretic arc of bovine IgM globulin, it was shown that (i) this protein migrated in the gamma region in electrophoresis (ii) it behaved as a macroglobulin when submitted to gel filtration in Sephadex G-200 (Fig. 10), (iii) it sedimented as a macroglobulin in the sucrose density gradient ultracentrifugation (Fig. 18), (iv) following treatment with 2 - ME, it was unable to produce its characteristic immunoelectrophoretic precipitin arc (Fig. 9) and (v) it had the highest carbohydrate content (Table 4). All of these criteria contribute to the description of a bovine protein which meets the specifications of IgM globulin. Moreover, agglutinating antibody activity was definitely associated with it.

(b) IgM heterogeneity

The current knowledge of heterogeneity of IgM as compared with that of IgG is limited. The existence of electrophoretic heterogeneity of this globulin in all the mammalian species that have so far been studied is indicated by the length of its immunoelectrophoretic precipitin arc. By applying some electrophoresis in Pevikon, Murphy et al. (1964) demonstrated that IgM

extend in a spectrum of electrophoretic mobility from the slowest γ -region through the B-region. However, the extent of electrophoretic heterogeneity was less than that of the IgG gamma globulins. They found no electrophoretic sub-groups of particularly significant concentration, as described for IgG. Furthermore, the CF antibody activity was found to be associated with this bovine macroglobulin.

The electrophoretic mobility of proteins depends primarily upon the net charge of their molecules. This is consonant with results obtained by anion exchange chromatography of serum on DEAE-Sephadex A-50. Following fractionation of the bovine serum, a progressive increase in the electrophoretic migration of the protein in successive chromatographic fractions was demonstrated until the end of the major albumin peak was reached.

The chemical basis of electrophoretic heterogeneity does not seem to be the carbohydrate content of the gamma globulin molecules, whereas the different amounts of sialic acid in the molecule may partly account for the differences in electrophoretic mobilities between immunoglobulin classes (Aalund, 1968). It was demonstrated by Schultze (1962) that the electrophoretic mobility of glycoproteins (e.g. IgM in this case) additionally depends on the localization of bound sialic acid in the molecule. Murphy et al. (1964) contend

that if this residue is split off physiologically, it may have an effect upon the mobility of this protein.

(3) BOVINE FAST IgG GAMMA GLOBULIN

(a) Its characterization

The gamma globulin eluted from the DEAE-Sephadex in peak II of the chromatogram and contained some agglutinating antibody activity in the sera of the experimental steers reacting to infection with Theileria parva produced an immunoelectrophoretic precipitin arc with mobility very similar to IgM and gamma IgA globulin (Fahey, 1962b; Fireman et al. 1963). Proof that the agglutinating antibody globulin molecules belonged to the fast IgG class was based on the merging of the precipitin arc formed by the chromatographically isolated globulin (viz. DEAE-Sephadex Peak II) with the long gamma globulin arc of whole bovine serum in a modified immunoelectrophoretic technique, where the antiserum trench was interrupted (Fig. 13). Further proof of the association of fast IgG globulin with the antibody activity was confirmed by antigenic comparison of the DEAE-Sephadex Peak I protein, the slow IgG with the DEAE-Sephadex Peak II protein. Papain digestion of either fast or slow IgG yielded the Fab(S) and Fc(F) fragments. It was noted that the whole globulins as well as their papain digestion fragments were antigenically similar. It should be pointed out that a marked difference was noted between fast IgG and slow IgG in their resistance to papain

hydrolysis. Fast IgG was more sensitive to papain than slow IgG even in the absence of cysteine. This agrees with the results obtained by Micusan and Borduas (1975) when they hydrolysed the two IgG subclasses isolated from goat serum. However, the presence of undigested gamma globulin encountered during these studies could perhaps be justified since Hsaio and Putnam (1961) demonstrated that conditions of papain digestion set out by Porter do not produce complete degradation to 3.5 S (Fab, Fc) fragments of gamma globulins of species other than man and rabbit. The Fab(S) fragments obtained from both IgG subclasses could not be distinguished both chromatographically and immunoelectrophoretically. However, Murphy et al. (1965) showed that the common antigenic determinants of both fast and slow IgG reside in the Fab(S) fragment.

(b) Immunoglobulin-G (IgG) heterogeneity

The bovine IgG has been divided into two subclasses on the basis of antigenic, physicochemical and biological criteria (Nansen, 1970) and the designations of the two subclasses refer to their electrophoretic mobilities, the slow IgG being the most cathodically and fast IgG the most anodically moving. One very conspicuous feature is the antigenic heterogeneity demonstrated within the IgG class (Pierce and Feinstein, 1965; Kickhofen et al. 1968). This phenomenon was observed in the immunoelectropherogram in Figure 6. The cathodic

precipitin spur of fast IgG was a distinct and constant finding. However, occasionally an anodic spur of slow IgG over fast IgG was observed. The existence of both spurs was indicative of discrete antigenicity and the confluencing major precipitates indicated shared antigenicity of the two IgG subclasses.

It should be stressed here that the division of bovine IgG into slow and fast electrophoretic subclasses is not an original idea. It is in accord with studies in several species such as the guinea pig (Benacerraf et al., 1963), mice (Fahey et al., 1964) and sheep (Silverstein et al., 1963; Aalund et al., 1965). In the guinea pig, Benacerraf et al. (1963) and Bloch et al. (1963) showed distinctions between slow and fast IgG which were correlated with the nature of the antigenic stimulus, age of the animals and the stage in the immune response. Antigenic differences were noted between the two IgG subclasses. In the present studies on the serum from bovine responding to Theileria parva antigens, fast IgG (DEAE-Sephadex Peak II) was found to contain agglutinating activity whereas similar activity was not present in the slow IgG (DEAE-Sephadex Peak I). This was of interest since in the guinea pig, the slow IgG antibodies are associated with fixation of complement and not the fast IgG as has been demonstrated by Benacerraf et al. (1963). In the bovine reacting from Anaplasma marginale, Murphy et al. (1965)

showed that complement fixation was associated with the fast IgG antibodies and not slow IgG subclass.

The common antigenicity of the bovine fast and slow IgG and fragments from papain digestion indicated a close relationship between these two subclasses of IgG.

The serum concentrations of fast and slow IgG were not analyzed in the steers employed in these studies. However, serum DEAE-Sephadex chromatograms appeared to be indicative of higher concentrations of slow IgG (IgG₂) than of fast IgG (IgG₁). However, applying the quantitative method for the determination of these two subclasses of IgG in serum of cattle responding to Trypanosoma congolense infection, Kobayashi and Tizard (1976) found higher serum levels of fast IgG (IgG₁) than of slow IgG (IgG₂). According to Fahey and Robinson (1963), these differences could be explained on the basis of different synthetic rates, but a common catabolic control mechanism for the IgG subclasses.

From these studies, it was observed that there was considerable heterogeneity in the immunoglobulin response to Theileria parva with initial appearance of IgM antibodies which were later augmented with the appearance of IgG antibodies. The appearance of IgG component was apparently coincident with the appearance

of Theileria parva piroplasms and it was at this stage of infection that parasitosis was markedly increased. Considering the antigenic mosaic of the rather complex organism, Theileria parva presented to the bovine host, it is difficult to elaborate upon the skew of the agglutinating antibody activity noted in these studies as a specific effect of a particular antigenic stimulus.

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